**Thèse de Doctorat de l'Université de Strasbourg**

En Aspects Moléculaires et Cellulaires de la Biologie

Mention Neurosciences

Présentée par **Paul Chu Sin Chung** pour l'obtention du grade de

Docteur de l'Université de Strasbourg

# Conditional gene knockout approach to investigate Delta Opioid Receptor functions in the forebrain

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A l'Institut de Génétique et de Biologie Moléculaire et Cellulaire

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*« Et il n'est rien de plus beau que l'instant qui précède le voyage, l'instant où l'horizon de demain vient nous rendre visite et nous dire ses promesses »*

*Milan Kundera*

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# Abbreviations

| | |
|---|---|
| aa: | amino acids |
| AC: | adenylate cyclase |
| ACTH: | adrenocorticotropin |
| ADL: | adolor |
| AMG: | amygdala |
| Arc: | arcuate nucleus of the hypothalamus |
| ATP: | adenosine triphosphate |
| BLA: | basolateral nucleus of the amygdala |
| BNST: | bed nucleus of the stria terminalis |
| cAMP: | cyclic adenosine monophosphate |
| cDNA: | complementary desoxyribonucleic acid |
| cKO: | conditional knockout |
| CoA: | cortical nucleus of the amygdala |
| CPA: | conditioned place aversion |
| CPP: | conditioned place preference |
| CPu: | caudate putamen nucleus |
| CRF: | corticotropin-releasing factor |
| CTB: | cholera toxin subunit B |
| DOR: | delta opioid receptor |
| DPDPE: | [D-Cys2, L-Pen5]-and [D-Cys2, D-Pen5]enkephalin |
| DR: | dorsal raphe nucleus |
| EA: | extended amygdala |
| eGFP: | enhanced green fluorescent protein |
| EPM: | elevated plus-maze |
| ERK: | extracellular signal regulated kinase |
| FST: | forced swim test |
| GABA: | $\gamma$-aminobutyric acid |
| GAD: | generalized anxiety disorder |
| GAD: | glutamic acid decarboxylase |
| GFP: | green fluorescent protein |
| GIRK: | G-protein inwardly rectifying potassium conductance |
| GP: | globus pallidus |
| GPCR: | G protein coupled receptor |
| GRKs: | G protein-coupled receptor kinases |
| GTP: | guanosine triphosphate |
| Hb: | habenula |
| HDB: | horizontal nucleus of the diagonal band |
| HPA: | hypothalamic-pituitary-adrenal axis |
| Hyp: | hypothalamus |
| InsCx: | insular cortex |
| ITCs: | intercalated cell masses |

| | |
|---|---|
| KO: | knockout |
| KOR: | kappa opioid receptor |
| LC: | locus coeruleus |
| LD: | light/dark box |
| LH: | lateral hypothalamus |
| MAPK: | mitogen-activated protein kinase |
| MeA: | medial nucleus of the amygdala |
| MOR: | mu opioid receptor |
| NAc: | nucleus accumbens |
| NOR: | novel object recognition |
| NSF: | novelty suppressed feeding |
| NTS: | nucleus of the solitary tract |
| OCD: | obsessive-compulsive disorder |
| OF: | open field |
| OFC: | orbitofrontal cortex |
| PAG: | periacqueductal gray |
| PDYN: | prodynorphin |
| PENK: | proenkephalin |
| PFC: | prefrontal cortex |
| PKA: | protein kinase A |
| PKC: | protein kinase C |
| POA: | preoptic area |
| POMC: | proopiomelanocortin |
| PTSD: | post-traumatic stress disorder |
| SN: | substantia nigra |
| ST: | stria terminalis |
| Th: | thalamus |
| TM: | transmembrane domain |
| TST: | tail suspension test |
| Tu: | olfactory tubercule |
| VAFP: | ventral amygdalofugal pathway |
| VP: | ventral pallidum |
| VTA: | ventral tegmental area |
| WT: | wild-type |

# General Introduction

**A.**

**B.**

**C.**

**D.**

Figure 1:
**(A)** Picture of opium poppy seed pods.

**(B)** Picture of Opium poppy (*Papaver somniferum*) with (left) mature fruit and seed and (right) detail of flower. *(J. Fujishima--B.W. Halstead, World Life Research Institute)*

**(C)** Latex trickling from incisions on a green immature capsule.

**(D)** Drawing of *Papaver somniferum* pods, flowers, seeds and plant.

# I. The endogenous opioid system

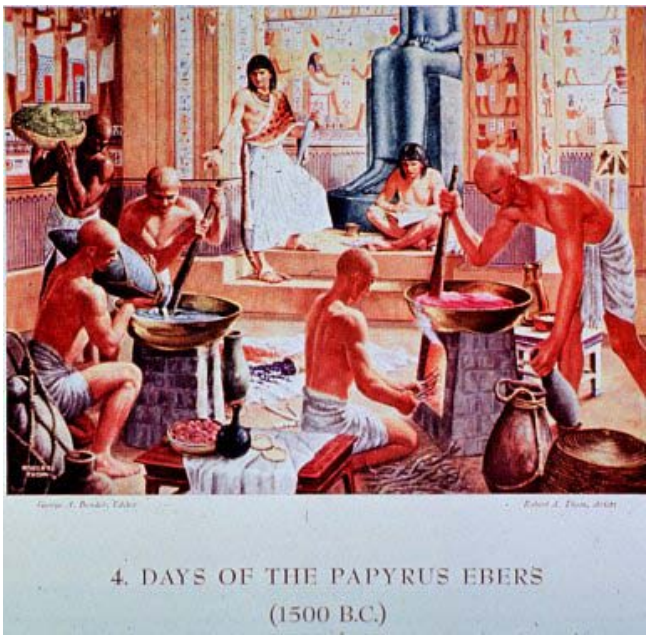
The term “Opioid” corresponds to endogenous peptides while “Opiates” more classically refers to exogeneous molecules. The opioid receptors recognize both opioids and opiates. The discovery and consumption of opiates date from several centuries. Since the use of opiates for clinical and recreational purposes expanded, their chemical action aroused interest which helped for the more recent discovery of the opioid system.

## A. History: From opium to the discovery of endogenous opioid receptors

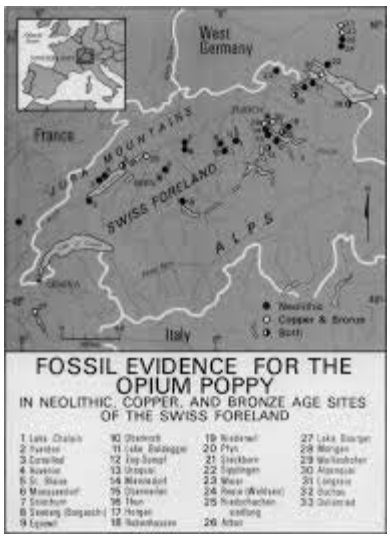
The opium poppy is considered as the oldest opiate used and from which all other opiates derive. Opium is extracted from the opium poppy (*Papaver somniferum*) following a simple incision on the green capsules (seed pods) (Figure 1). The pods may be incised three or four times with intervals of two to three days. The “poppy tears” are collected the following day as dried brown latex. The dried latex contains several alkaloids responsible for the pursuit effects of opium such as the morphine, codeine, noscapine, papaverine and thebaine.

Opium is used since several centuries for his sedative, analgesic and euphoric effects. The geographic origin of the opium poppy is not well-known. The opium poppy does not really exist as a wild form, and then opium cultivation likely followed the man migrations at the latest ages. Archeological evidence suggest that the opium poppy has been domesticated in Asia Minor, by the Sumerians (4000 B.C.). Lately, the opium poppy cultivation spread to the Persian region and Egypt. At this time, the first written proofs of opium consumption, mainly for medical purposes, were reported (1500 B.C., *Ebers Papyrus*). The opium poppy cultivation reached the eastern part of Asia between 400 and 1200 C.E., in India and China, likely from the Arab traders. The opium use remained rare until the 17$^{th}$ century. At this time, the opium addiction began to provoke serious troubles and started to be recognized. China prohibited opium consumption in 1729. However, the opium use increased at this period, partially encouraged by the British and the East India Company. After the two opium

**A.**




**B.**




**C.**



**D.**

Figure 2:
**(A)** Canvas relating the existence of the famous Ebers papyrus.

**(B)** Ancient fossil evidence for *Papaver somniferum* in the Swiss Foreland and surrounding areas (Merlin 2003).

**(C)** A Minoan goddess, her hair adorned with poppy-capsules.

**(D)** Apothecary vessel for storage of opium as a pharmaceutical, Germany, 18th or 19th century.

wars in 1839 and 1858, opium use in China continued to increase and in 1905 almost 25 % of the male population was counted as regular drug users.

In 1805, the German pharmacist Friedrich W. Sertürner isolated and described the principal active alkaloid in opium. He named it morphine after Morpheus, the Greek god of dreams. Other active ingredients were isolated from opium in the following years; noscapine (1817), codeine (1832 by Pierre Jean Robiquet), thebaine (1833) and papaverine (1848). During the 19$^{th}$ century, the emergence of medical material like the hypodermic syringe as well as a refine production of morphine allowed a larger clinical use of this alleviating pain drug. However, because of a stronger efficiency than opium, morphine appeared to induce even more addiction problems. Therefore, hundreds of morphine analogues, including heroin which was synthesized in 1874 by Charles Robert Alder Wright, were produced with the intention of developing drugs with analgesic properties without the addictive side effects (Figure 2). Unfortunately, these new compounds failed to be as efficient as morphine to relieve pain and were even more prone to induce addiction.

An increasing interest of chemists and physicians to develop drugs that would be analgesic without being addictive drove the scientific community to better understand the action mechanism of these drugs. The endogenous opioid system was discovered in the 1970's, simultaneously by three groups, and with radiolabeled opiate binding experiments (Pert and Snyder 1973; Simon, Hiller et al. 1973; Terenius 1973).

Subsequently to the discovery of stereospecific binding sites for opiates, the endogenous opioid system was further explored by pharmacological characterization of these receptors. In 1976, a study defined three different opioid receptors (Martin, Eades et al. 1976) based on the ability to reverse their activation with high doses of the opioid antagonist, naloxone. These receptors were named depending on their preferred ligand: mu (μ), kappa (κ) and sigma (σ) for their preferential binding of morphine, ketocyclazocine and SKF 10,047, respectively. However, a few years later a study showed that the sigma receptor was able to bind phencyclidine in a non reversal way and this finding led to the exclusion of this receptor from the opioid receptor family (Vincent and Engelke 1979). At the same time, another group identified a new opioid receptor in the mouse vas deferens which was consequently named delta (δ) (Lord, Waterfield et al. 1977).

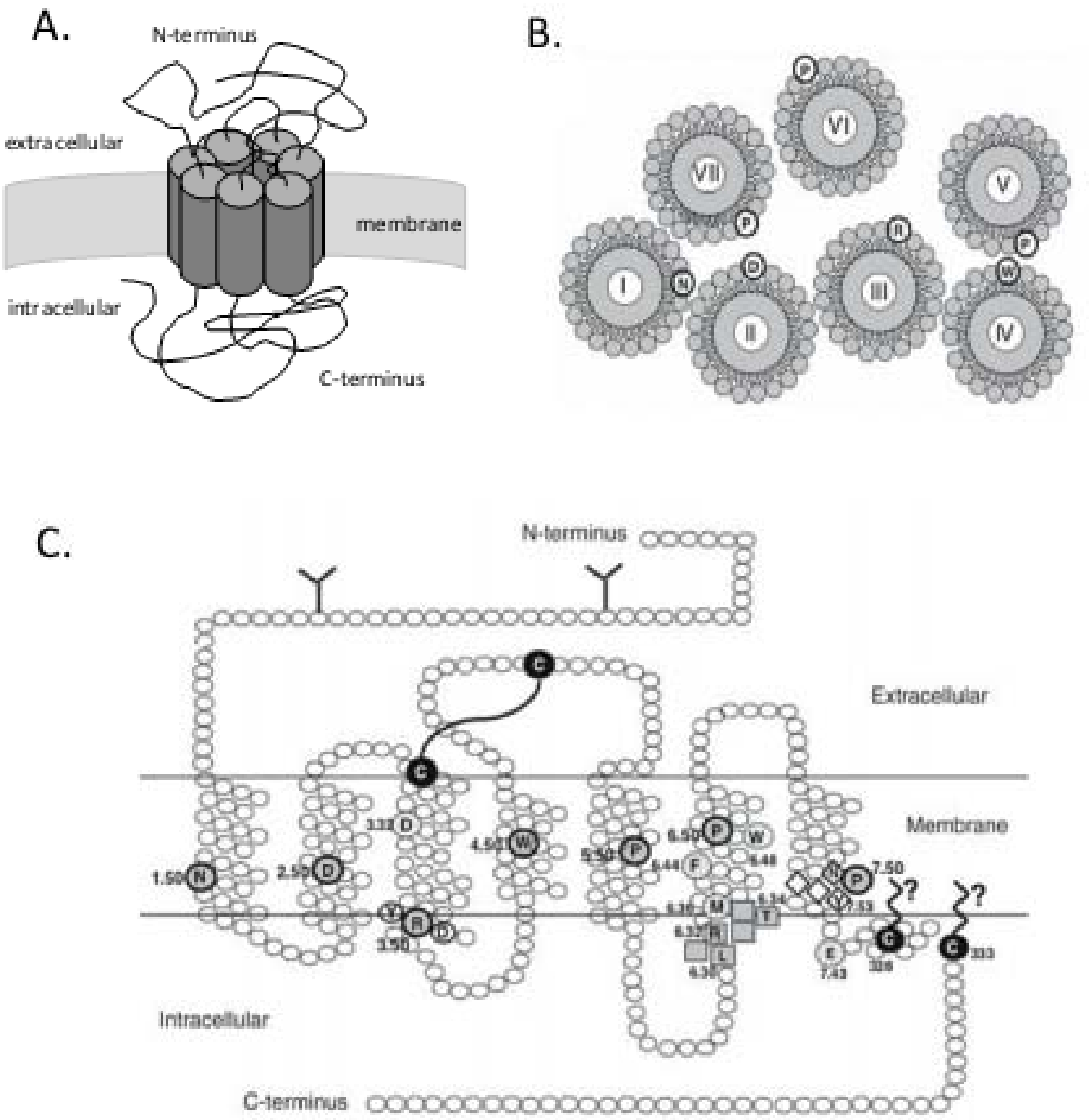



Figure 3: Opioid receptors structure
**(A)** Schematic view in 3D of opioid receptor conformation

**(B)** Seven transmembrane domains organizations. Amino acids (aa) indicated by circles are the conserved aa in class A GPCR receptors.

**(C)** Organization of the delta opioid receptor (Massotte and Kieffer, 2005). Circled aa correspond to highly conserved residues. N-glycosylation in N-terminus are depicted (Y). The disulfide bridge between cysteine of the helix III and the extracellular loop 2 is represented.

At the beginning of the 1980's, specific synthetic agonists for the three opioid receptors were produced (DAMGO for mu opioid receptor (Handa, Land et al. 1981), DPDPE for DOR (Mosberg, Hurst et al. 1983) and U-50488 (Vonvoigtlander, Lahti et al. 1983) for the kappa opioid receptor. Rapidly, these specific agonists permitted to define the pharmacological properties of each opioid receptor as well as their distribution through the nervous system.

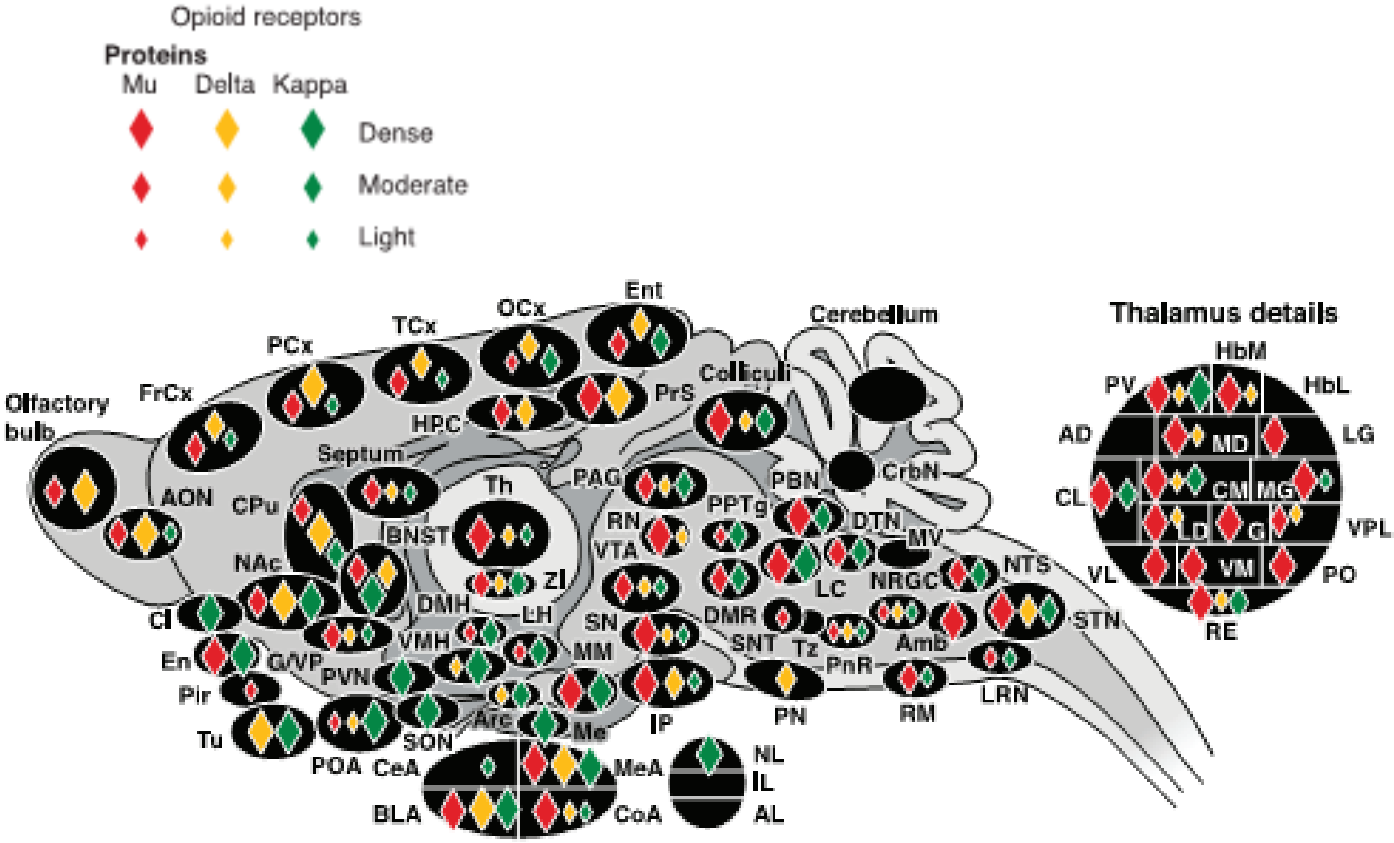
In the early 1990's, two groups simultaneously reported the first expression cloning of an opioid receptor, the mouse delta opioid receptor (Evans, Keith et al. 1992; Kieffer, Befort et al. 1992). The human and rodent cDNA of mu and kappa opioid receptors were cloned based on their homology with the delta opioid receptor (for a review see (Kieffer 1995; Kieffer and Evans 2009)). In 1994, a fourth opioid receptor was cloned by sequence homology, the nociceptin/ORFQ (or ORL-1) receptor (Bunzow, Saez et al. 1994).

## B. The opioid receptors

### *1. Biosynthesis*

The genes coding for the opioid receptors are *Oprm1*, *Oprd1* and *Oprk1*, respectively for mu, delta and kappa receptors (MOR, DOR and KOR in The International Union of Basic and Clinical Pharmacology IUPHAR nomenclature). They are located on separate chromosomes (*Oprm1* on chromosomes 10 in mouse and 6 in human, *Oprd1* on chromosomes 4 in mouse and 1 in human, *Oprk1* on chromosomes 1 in mouse and 8 in human) but exhibit a similar genomic organization. The genes coding for delta and kappa opioid receptors are composed of three exons (Befort, Mattei et al. 1994; Yasuda, Espinosa et al. 1994; Simonin, Gaveriaux-Ruff et al. 1995), while *Oprm1* contains four exons (Wang, Johnson et al. 1994).

The cloning of genes coding for opioid receptors allowed determining their protein sequences. Mu, delta and Kappa receptors are composed of 398, 372 and 380 amino acids (aa), respectively. They belong to the superfamily of the G protein




Abbreviations

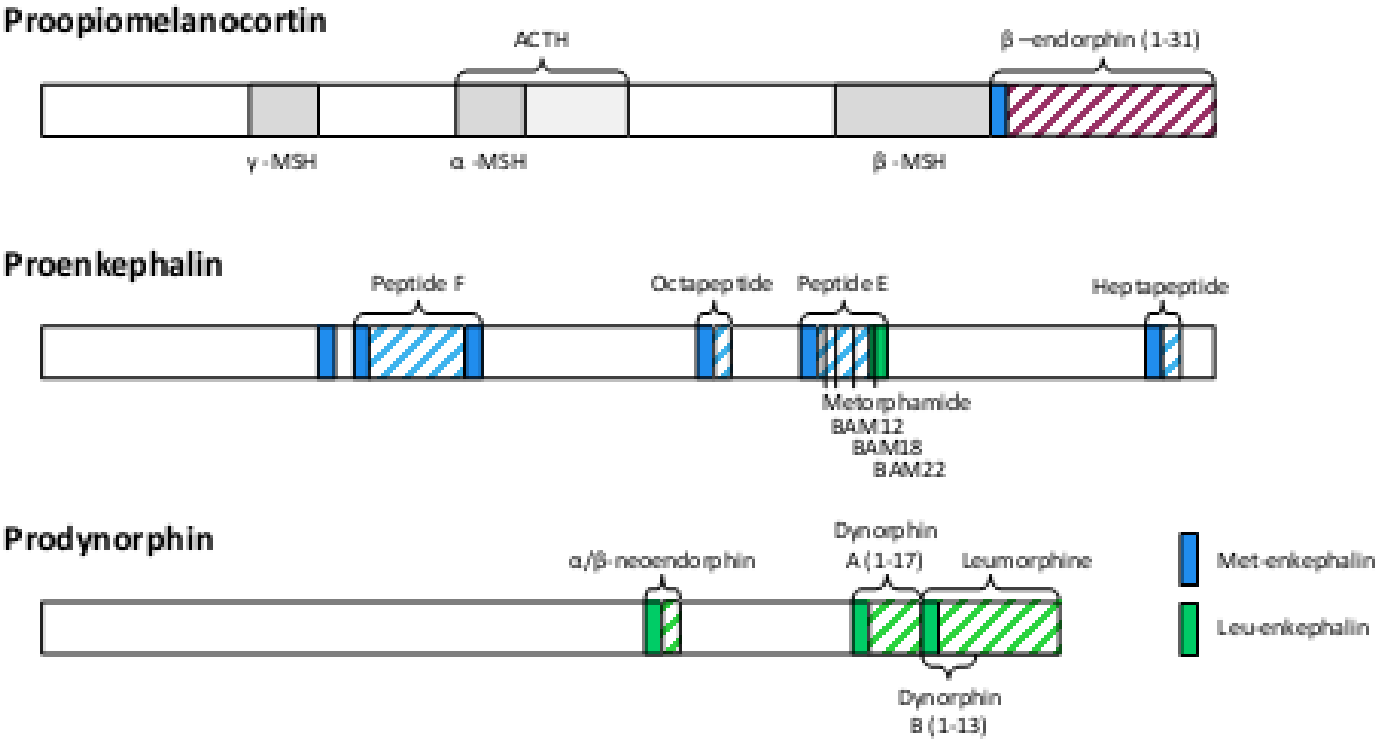
Amb, nucleus ambiguus; AD, anterodorsal thalamus; AL, anterior lobe, pituitary; AON, anterior olfactory nucleus; Arc, arcuate nucleus, hypothalamus; BLA, basolateral nucleus, amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus, amygdala; Cl, claustrum; CL, centrolateral thalamus; CM, centromedial thalamus; CoA, cortical nucleus, amygdala; CPu, caudate putamen; CrbN, cerebellar nuclei; DMH, dorsomedial hypothalamus; DMR, dorsal and medial raphe ; DTN, dorsal tegmental nucleus; En, endopiriform cortex; Ent, entorhinal cortex; FrCx, frontal cortex; G, nucleus gelatinosus, thalamus; G/VP, globus pallidus/ventral pallidum; HbL, lateral habenula; HbM, medial habenula; HPC, hippocampus; IL, intermediate lobe, pituitary; IP, interpeduncular nucleus; LC, locus coeruleus; LD, laterodorsal thalamus; LG, lateral geniculate, thalamus; LH, lateral hypothalamus; LRN, lateral reticular nucleus; MD, mediodorsal thalamus; Me, median eminence; MEA, median nucleus, amygdala; MG, medial geniculate; MM, medial mammillary nucleus; MV, medial vestibular nucleus; NAc, nucleus accumbens; NL, neuronal lobe, pituitary; NRGC, nucleus reticularis gigantocellularis; NTS, nucleus tractus solitarius; OCx, occipital cortex; PAG, periaqueductal gray; PCx, parietal cortex; Pir, piriform cortex; PN, pontine nucleus; PnR, pontine reticular; PO, posterior thalamus; POA, preoptic area; PPTg, pedunculopontine nucleus; PrS, presubiculum; PV, paraventricular thalamus; PVN, paraventricular hypothalamus; RE, reuniens thalamus; RN, red nucleus; RM, raphe magnus; SON, supraoptic nucleus; SN, substancia nigra; SNT, sensory trigeminal nucleus; STN, spinal trigeminal nucleus; TCx, temporal cortex; Th, thalamus; Tu, olfactory tubercle; Tz, trapezoid nucleus; VL, ventrolateral thalamus; VM, ventromedial thalamus; VMH, ventromedial hypothalamus; VPL, ventroposterolateral thalamus; VTA, ventral tegmental area; ZI, zona incerta

Figure 4:
Neuroanatomical distribution of opioid receptor on a sagittal section of rodent brain (Le Merrer et al., 2009).

coupled receptor (GPCR) and were classified within the class A GPCR family because of their sequence homology with rhodopsin (Figure 3) (Fredriksson, Lagerström et al. 2003). As a member of the GPCRs, opioid receptors are characterized by seven transmembrane domains (TM) connected by three intracellular and three extracellular loops, an N-terminal extremity that specifically interacts with ligands and a C-terminal extremity linked to the downstream intracellular effectors such as the G proteins (Surratt and Adams 2005). The transmembrane domains are composed of seven α-helices and present a highly homologous sequence across the three different receptors (73 to 76 %). They participate to ligand binding as well as receptor signaling (Befort, Tabbara et al. 1996). The extra- and intracellular loops also share highly homologous sequences (86 to 100 %) while the N-terminal and C-terminal diverge in the three opioid receptors (N-terminal: 9 to 10 % of identity; C-terminal: 14 to 20 % of identity). The extracellular loops and N-terminal extremity are responsible for selective ligand binding (Gether 2000). The C-terminal extremity contributes to the receptor stability and the intracellular signaling. Moreover, this extremity contains sites for post-translational modifications that modulate the receptor activity as well as the G protein coupling (Decaillot, Befort et al. 2003). Recently, the structure of opioid receptors has been determined at high-resolution by X-Ray crystallography (Granier, Manglik et al. 2012; Manglik, Kruse et al. 2012; Wu, Wacker et al. 2012) and represent an important discovery for better understanding receptor-ligand interactions. In the future, it will also help for the bioinformatic modeling of receptor interactions.

### *2. Neuroanatomical distribution*

The opioid receptors are broadly expressed in the central nervous system (Figure 4). They are also expressed in the peripheral nervous system as well as in other organs and systems (Townsend, Portoghese et al. 2004; Schramm and Honda 2010). For instance, the expression in immune cells has been reported (Gaveriaux, Peluso et al. 1995). Here, we will focus on opioid receptors location within the brain. The anatomical distribution of opioid receptors was determined by in situ hybridization and autoradiographic binding experiments, which allowed for a precise characterization of each mRNA (Mansour, Fox et al. 1994; Kitchen, Slowe et al.




| | |
|---|---|
| β-endorphin (1-31) | **Tyr-Gly-Gly-Phe**-Met-Thr-Ser-Glu-Lys-Ser-Gln-Thr-Pro-Leu-Val-Thr-Leu-Phe-Lys-Asn-Ala-Ile-Ile-Lys-Asn-Ala-Tyr-Lys-Lys-Gly-Glu |
| Met-enkephalin | **Tyr-Gly-Gly-Phe**-Met |
| Leu-enkephalin | **Tyr-Gly-Gly-Phe**-Leu |
| Octapeptide | **Tyr-Gly-Gly-Phe**-Met-Arg-Gly-Leu |
| Heptapeptide | **Tyr-Gly-Gly-Phe**-Met-Arg-Phe |
| Peptide F | **Tyr-Gly-Gly-Phe**-Met-Lys-Lys-Met-Asp-Glu-Leu-Tyr-Pro-Leu-Glu-Val-Glu-Glu-Glu-Ala-Asn-Gly-Gly-Glu-Val-Leu-Gly-Lys-Arg-**Tyr-Gly-Gly-Phe**-Met |
| Peptide E | **Tyr-Gly-Gly-Phe**-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu-Trp-Trp-Met-Asp-Tyr-Gln-Lys-Arg-**Tyr-Gly-Gly-Phe**-Leu |
| Metorphamide | **Tyr-Gly-Gly-Phe**-Met-Arg-Arg-Val-NH2 |
| α-neoendorphin | **Tyr-Gly-Gly-Phe**-Leu-Arg-Lys-Tyr-Pro-Lys |
| β-neoendorphin | **Tyr-Gly-Gly-Phe**-Leu-Arg-Lys-Tyr-Pro |
| Dynorphin A (1-17) | **Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln |
| Dynorphin A (1-8) | **Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Ile |
| Leumorphine | **Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr-Arg-Ser-Gln-Glu-Asp-Pro-Asn-Ala-Tyr-Tyr-Glu-Glu-Leu-Phe-Asp-Val |
| Dynorphin B (1-13) | **Tyr-Gly-Gly-Phe**-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr |

Figure 5:
Endogenous peptides (from Faget L.). Schematic representation of genes coding for endogenous opioid precursor peptides. Pomc, Penk and Pdyn genes are composed of 267, 267 and 254 aa respectively. In the table is represented the sequence of the main endogenous peptides with the common “opioid motif” bolded.

1997; Slowe, Simonin et al. 1999; Goody, Oakley et al. 2002). While the endogenous peptides distribution has been assessed by immunohistochemistry assays, the commercial antibodies used to determine the opioid receptors distribution showed a low specificity. Therefore, this questioned the accuracy of reported receptor distributions. More recently, knockin mutant mice expressing the DORs in fusion with the green fluorescent protein (GFP) were developed (Scherrer, Tryoen-Toth et al. 2006). The emergence of such genetically engineered mice will allow mapping the in vivo location of opioid receptors.

The opioid receptors are mostly rexpressed in the cortex, limbic system and brain stem (Le Merrer, Becker et al. 2009). The mu opioid receptor is abundantly expressed in the thalamus (Th), habenula (Hb), substantia nigra (SN), striatum, ventral tegmental area (VTA) and the nucleus of the solitary tract (NTS). The kappa opioid receptor is enriched in the basal anterior forebrain, olfactory tubercule (Tu), striatum, preoptic area (POA), hypothalamus (Hyp) and pituitary gland. The DOR is highly expressed in the olfactory bulb (OB), striatum (CPu and NAc), the globus pallidus, the nucleus of the diagonal band of Broca, septal nuclei, the hippocampus and in subregions of the amygdala (BLA, CoA and MeA). DOR is also the most represented opioid receptor in the olfactory tract and in cortical regions (including the PFC) in particular in the insular cortex (InsCx).

## C. The endogenous opioid peptides

### *1. Biosynthesis*

The opioid system is composed of three families of endogenous peptides: the enkephalins, endorphins and dynorphins (Figure 5). In 1975, Hughes and colleagues identified two molecules from brain extracts that displayed similar action as morphine and was reversed by naloxone (Hughes, Smith et al. 1975). Following this study, about 30 endogenous opioid peptides were discovered. They are composed of 5 to 31 amino acids (aa) and share a common N-terminal sequence Tyr-Gly-Gly-Phe, so called the “opioid motif” (Akil, Owens et al. 1998). The endogenous peptides are synthesized from three precursors: the preproenkephalin (PENK),

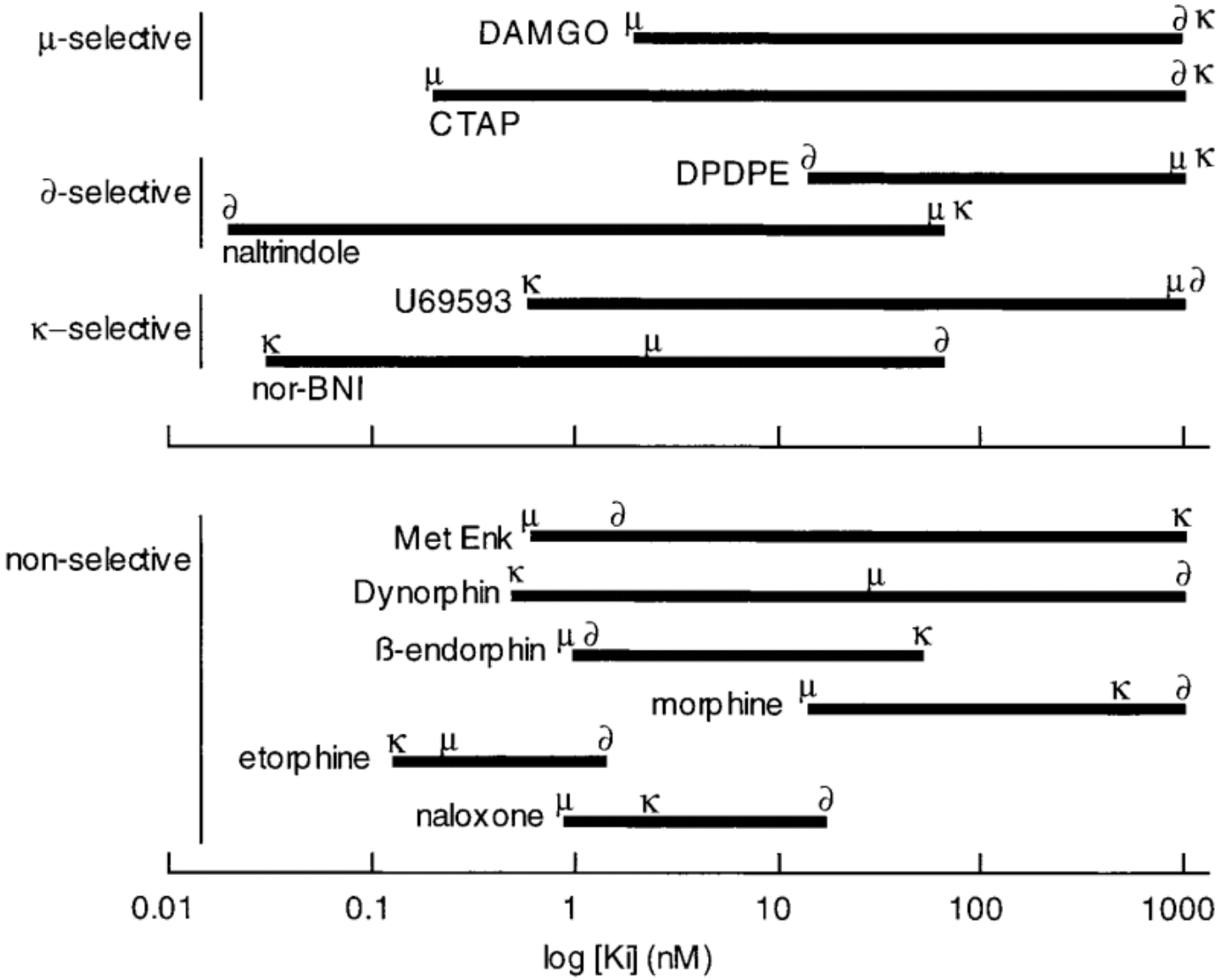


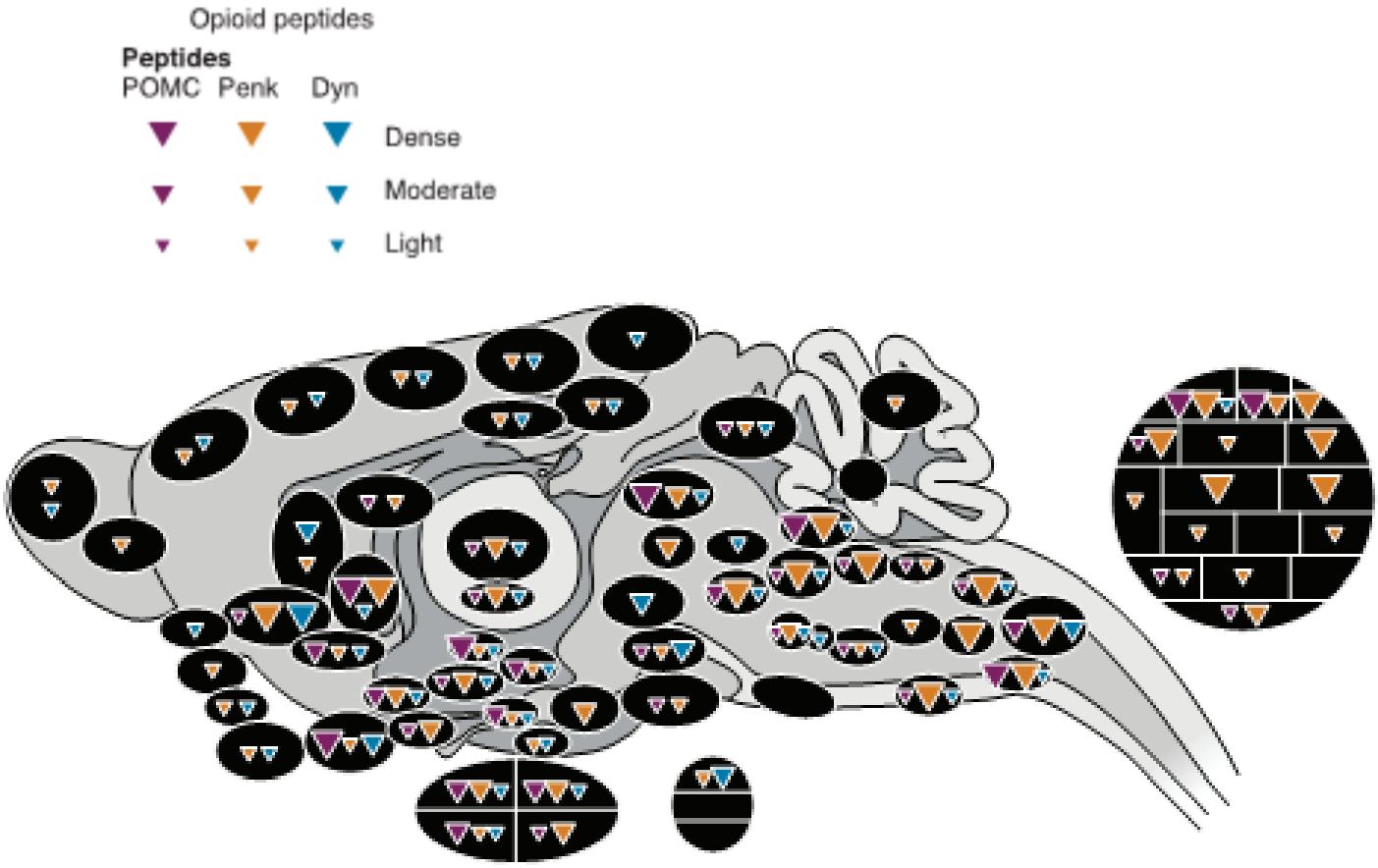

Figure 6:
Selectivity windows of some opioid agonists and antagonists (William et al., 2001). At the top are represented compounds highly selective for each opioid receptor. At the bottom are represented endogenous peptides and other commonly used opioids.

preproopiomelanocortin (POMC) and preprodynorphine (PDYN). The genes encoding these precursors were cloned before the cloning of the genes coding for opioid receptors, in the early 1980's (Nakanishi, Inoue et al. 1979; Comb, Seeburg et al. 1982; Kakidani, Furutani et al. 1982).

The cleavage by endopeptidases of these endogenous peptide precursors occurs during the post-translational maturation, in a tissue dependent manner, and leads to the production of about 30 functional peptides (Fricker and Devi 1993; Akil, Owens et al. 1998). The *Penk* gene codes for a 267 aa polypeptide precursor containing four copies of met-enkephalin, one copy of leu-enkephalin and other enkephalins (Rossier 1993). The *Pomc* gene codes for a 267 aa polypeptide precursor which after proteolytic cleavage provides one copy of β-endorphin, one copy of adrenocorticotropin (ACTH) and several other peptides activating the melanocytes (Young, Bronstein et al. 1993). The *Pdyn* gene encodes a 245 aa polypeptide precursor cleaved in leu-enkephalin, dynorphin A and dynorphin B (Day, trujillo et al. 1993). The enkephalins, β-endorphin and dynorphin are the main endogenous active peptides and each is able to bind the three different opioid receptors (Figure 6). However, enkephalins and β-endorphin present a lower affinity for the kappa opioid receptor (Loh, Tseng et al. 1976) which is more strongly bound by dynorphin (Goldstein, Tachibana et al. 1979). Recently, some studies discovered and characterized a biosynthesis pathway for morphine production in mice suggesting that morphine could be synthesized endogenously (Grobe, Lamshoft et al. 2010; Laux, Muller et al. 2011).

### *2. Neuroanatomical distribution*

The neuroanatomical distribution of *Penk*, *Pomc* and *Pdyn* mRNA has been described using in situ hybridization experiments (for review see (Le Merrer, Becker et al. 2009)). Moreover, distribution of the active peptides has been determined by immunohistochemistry. Opioid peptide immunoreactivity overlaps largely with the localization of opioid receptors (Figure 7). PENK is largely distributed and the most abundant opioid precursor. It is strongly detected in the striatum and globus pallidus where it overlaps with DOR. PDYN is located in most brain regions with the highest




Abbreviations

Amb, nucleus ambiguus; AD, anterodorsal thalamus; AL, anterior lobe, pituitary; AON, anterior olfactory nucleus; Arc, arcuate nucleus, hypothalamus; BLA, basolateral nucleus, amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus, amygdala; Cl, claustrum; CL, centrolateral thalamus; CM, centromedial thalamus; CoA, cortical nucleus, amygdala; CPu, caudate putamen; CrbN, cerebellar nuclei; DMH, dorsomedial hypothalamus; DMR, dorsal and medial raphe ; DTN, dorsal tegmental nucleus; En, endopiriform cortex; Ent, entorhinal cortex; FrCx, frontal cortex; G, nucleus gelatinosus, thalamus; G/VP, globus pallidus/ventral pallidum; HbL, lateral habenula; HbM, medial habenula; HPC, hippocampus; IL, intermediate lobe, pituitary; IP, interpeduncular nucleus; LC, locus coeruleus; LD, laterodorsal thalamus; LG, lateral geniculate, thalamus; LH, lateral hypothalamus; LRN, lateral reticular nucleus; MD, mediodorsal thalamus; Me, median eminence; MEA, median nucleus, amygdala; MG, medial geniculate; MM, medial mammillary nucleus; MV, medial vestibular nucleus; NAc, nucleus accumbens; NL, neuronal lobe, pituitary; NRGC, nucleus reticularis gigantocellularis; NTS, nucleus tractus solitarius; OCx, occipital cortex; PAG, periaqueductal gray; PCx, parietal cortex; Pir, piriform cortex; PN, pontine nucleus; PnR, pontine reticular; PO, posterior thalamus; POA, preoptic area; PPTg, pedunculopontine nucleus; PrS, presubiculum; PV, paraventricular thalamus; PVN, paraventricular hypothalamus; RE, reuniens thalamus; RN, red nucleus; RM, raphe magnus; SON, supraoptic nucleus; SN, substancia nigra; SNT, sensory trigeminal nucleus; STN, spinal trigeminal nucleus; TCx, temporal cortex; Th, thalamus; Tu, olfactory tubercle; Tz, trapezoid nucleus; VL, ventrolateral thalamus; VM, ventromedial thalamus; VMH, ventromedial hypothalamus; VPL, ventroposterolateral thalamus; VTA, ventral tegmental area; ZI, zona incerta

Figure 7:
Neuroanatomical distribution of endogenous opioid peptides on a sagittal section of rodent brain (Adapted from, Le Merrer et al., 2009).

concentration in the nucleus accumbens. POMC distribution is more restricted and absent from cortical structures except for the amygdala. *Penk* and *Pdyn* expressing cell bodies show an extensive distribution in the whole brain, while *Pomc* expressing cell bodies are limited to three regions of the brain; the arcuate nucleus of the hypothalamus (Arc), nucleus of the solitary tract (NTS) and in the pituitary gland. Overall, despite discrepancies in some regions, the anatomical distribution of opioid peptides and receptors is in agreement with the notion that enkephalins and endorphins preferentially bind to delta and mu receptors and that dynorphins preferentially activate kappa receptors.

## D. Physiological roles of the endogenous opioid system

The endogenous opioid system is involved in many different physiological functions. The different functions of the opioid system were explored by using pharmacological approaches and genetically engineered mutant mouse lines. The receptors expressed in peripheral nervous system and other organs are described to be involved in the regulation of autonomic vegetative constants like the cardiovascular responses (Saraiva, Oliveira et al. 2004), regulation of the body temperature (Rawls, Hewson et al. 2005), gastro-intestinal transit (Mehendale and Yuan 2006) as well as hepatic and renal functions (Atici, Cinel et al. 2005).

The three opioid receptors are described as major actors of regulation pain perception, or nociception (Gaveriaux-Ruff 2013). Indeed, three mouse lines genetically deleted for either MOR, DOR or KOR showed modifications of pain perception suggesting a tonic inhibition of pain responses. However, they regulate different aspects of nociception. MORs are involved in acute mechanical and chemical pain (Martin, Matifas et al. 2003; Zollner and Stein 2007). KORs mainly contribute to the regulation of visceral pain (Simonin, Valverde et al. 1998; Chavkin 2011). The DORs participate essentially to the management of chronic pain (Gaveriaux-Ruff and Kieffer 2011). Moreover, it has been recently suggested that DORs specifically expressed on $Na_v$ 1.8-positive nociceptive neurons in the dorsal root ganglia tonically inhibit mechanical hypersensitivity in inflammatory and neuropathic pain (Gaveriaux-Ruff, Nozaki et al. 2011).

The opioid system plays a critical role in the control of behavioral responses stimulated by natural rewards and drugs of abuse (Bodnar 2004; Smith and Berridge 2005). The rewarding properties of addictive drugs are mainly dependent on the mu opioid receptor (Matthes, Maldonado et al. 1996; Ghozland, Matthes et al. 2002; Le Merrer, Becker et al. 2009). KOR is involved in the negative emotional state experienced during withdrawal periods (Shippenberg, Zapata et al. 2007; Gillett, Harshberger et al. 2013). The role of DOR in drug addiction remains poorly studied despite a potential role in drug craving and relapse. However, the contribution of DORs in several processes such as anxiety-related behaviors (Filliol, Ghozland et al. 2000), drug-context association (Le Merrer, Faget et al. 2012) or motor impulsivity (Olmstead, Ouagazzal et al. 2009; Befort, Mahoney et al. 2011) may indirectly participate to the regulation of addictive responses.

Most studies on the role of the opioid system have focused on the regulation of pain and addictive responses mainly because of the reported effects of opiates reported since the latest ages. Nevertheless, the opioid system is also implicated in mood and well-being (Filliol, Ghozland et al. 2000; Chu Sin Chung and Kieffer 2013), learning and memory (Robles, Vivas-Mejia et al. 2003; Holahan, Nichol et al. 2008; Rodefer and Nguyen 2008), motor control (Le Merrer, Rezai et al. 2013).

## E. Opioid receptor / ligand interaction and intracellular signaling

Agonists interact with the receptor at the level of the binding pocket composed of the extracelluar loops and the N-terminal extremity. Granier and colleagues recently proposed that the upper binding pocket that diverges among receptor subtypes is responsible for the ligand selectivity, whereas the lower portion of the binding pocket is well-conserved in both sequence and structure (Granier, Manglik et al. 2012; Manglik, Kruse et al. 2012). Moreover, mutagenesis studies suggested that all ligands were not binding the delta receptor at the same site (Befort, Zilliox et al. 1999; Decaillot, Befort et al. 2003). Then, ligand binding to GPCRs induces conformational changes of the TM domains (Visiers, Ballesteros et al. 2002). These conformational changes ultimately lead to the uncoupling of the G proteins from the

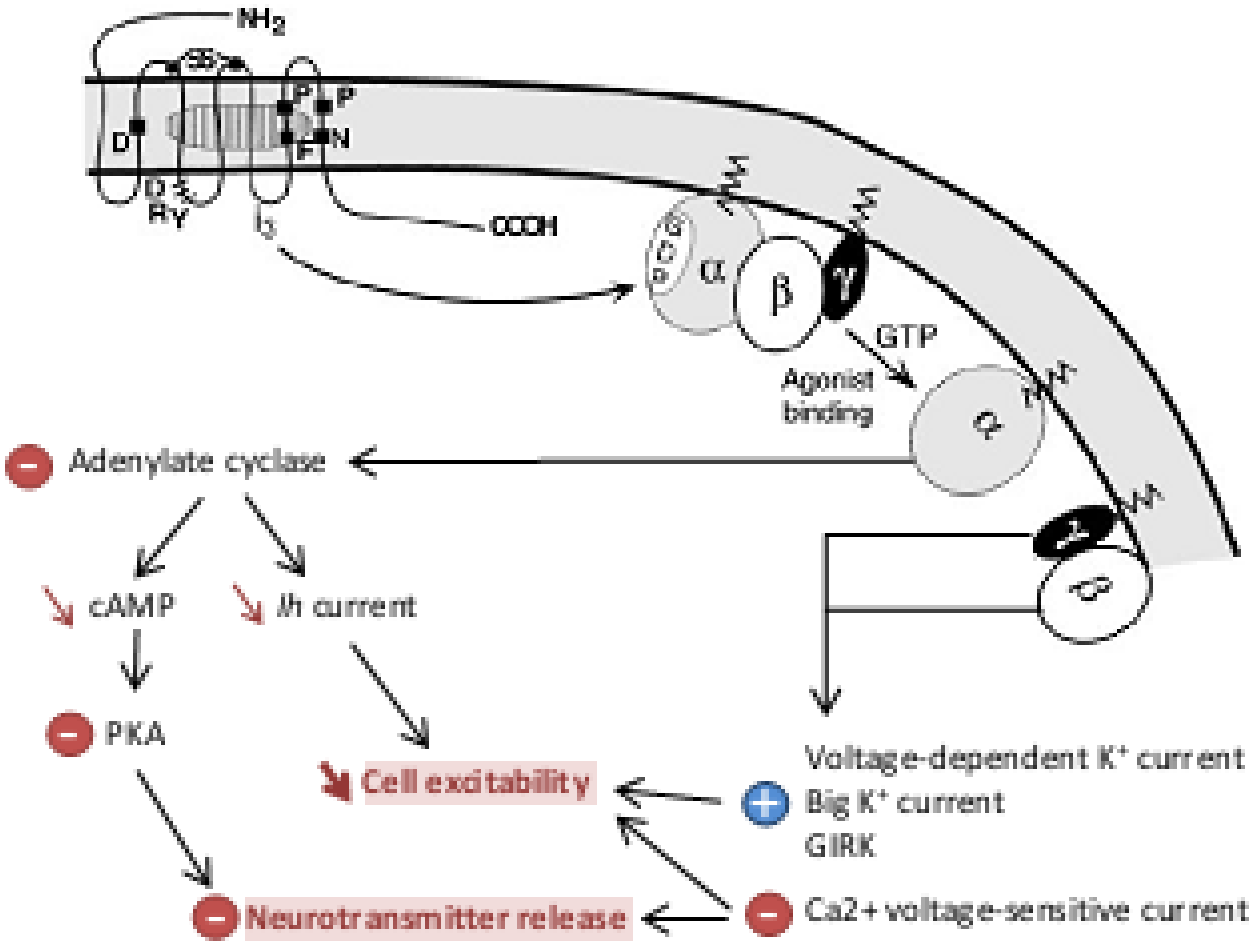


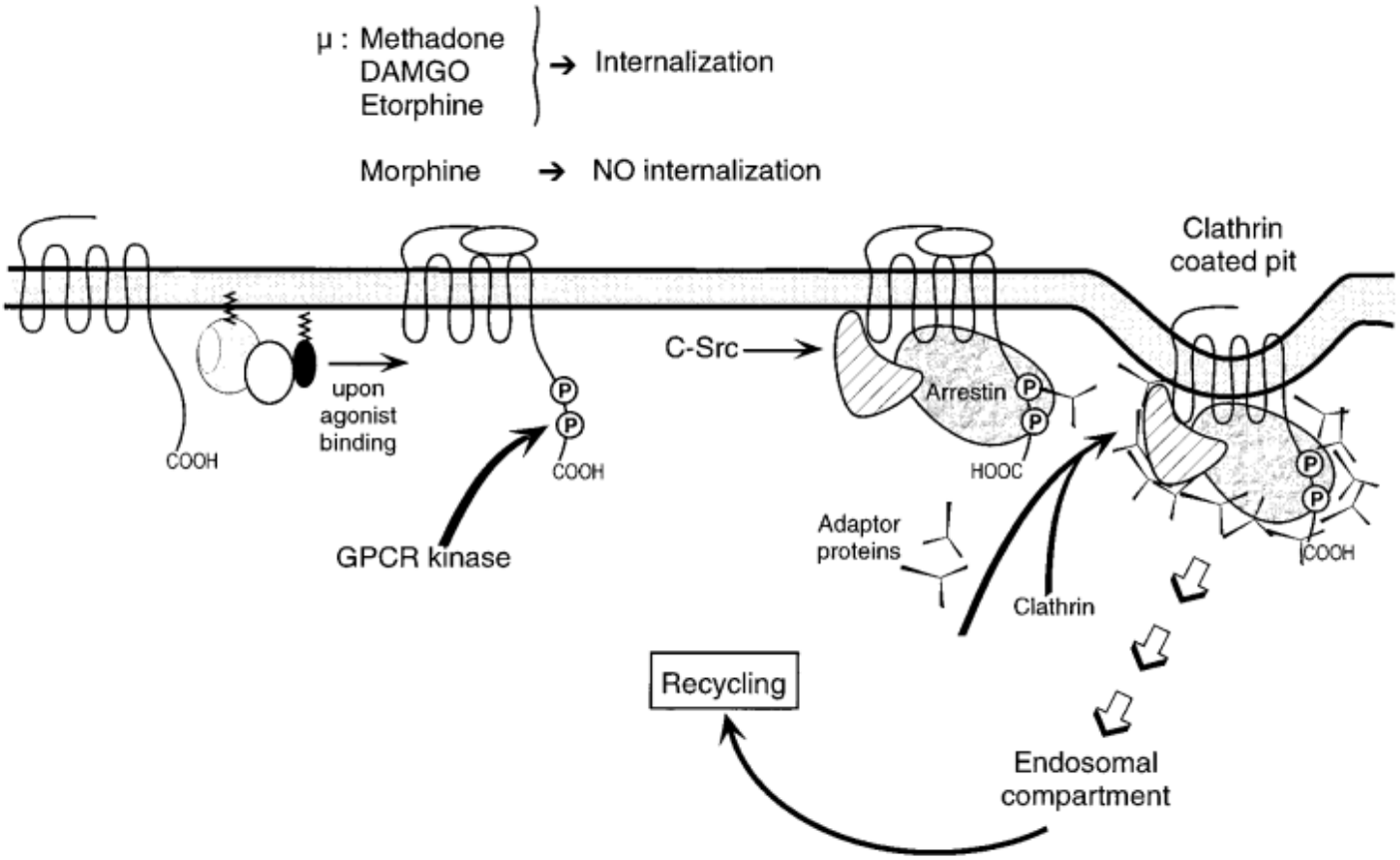

Figure 8:
Intracellular signaling of opioid receptors (from Faget L., adapted from Williams et al., 2001). Opioid receptor activation by ligand binding induces activation of protein $G_{i/o}$ α and β/γ subunits. Then, these protein G subunits modulate numerous channel activity ($K^+$, $Ca^{2+}$ and *Ih* currents) and inhibite the adenylate cyclase activity which in turn lead to a decreased cell excitability.

C-terminal of the receptor and exposure to other effectors proteins (Waldhoer, Bartlett et al. 2004).

Opioid receptors are GPCRs coupled to $G_{i/o}$ proteins and their activation leads to a global decrease of the cell excitability and neurotransmitter release (Figure 8). $G_{i/o}$ proteins are composed of three subunits: α, β and γ. Subsequently to the ligand binding, GTP is hydrolyzed leading to G proteins subunits $\alpha_{i/o}$ and β/γ uncoupling (Oldham and Hamm 2008).

The activation of $\alpha_{i/o}$ subunit inhibits the adenylate cyclase (AC) activity responsible for cAMP production. Decreased cAMP concentration induces a decreased activity of the protein kinase A (PKA) and consequently of many others downstream signaling pathways. The decreased AC activity also modulates the activity of a voltage-dependent current *Ih* (Ingram and Williams 1994; Svoboda and Lupica 1998). This current is normally responsible for the repolarization of the membrane potential after a strong hyperpolarization and then allows future activation of the cell. Opioid receptors activation leads to a diminished amplitude of this potassium inward current (also called pacemaker current). In addition, the AC inhibition may induce a decrease of neurotransmitter release via a PKA-dependent mechanism (Chieng and Williams 1998; Ingram, Vaughan et al. 1998).

The β/γ subunits following opioid receptor activation enhance three potassium conductance: a G-protein inwardly rectifying conductance (GIRK) (Sodickson and Bean 1998), a voltage-dependent potassium current (Madamba, Schweitzer et al. 1999) and a calcium-sensitive potassium conductance (Twitchell and Rane 1993). In parallel, these subunits inhibit voltage-sensitive Ca2+ channels (N, P/Q and T types) (Wilding, Womack et al. 1995). Opioid receptor activation also leads to long term modifications such as changes in gene expression (Bilecki, Wawrzczak-Bargiela et al. 2004). Indeed, β/γ subunits are able to activate the mitogen-activated protein kinases (MAPK) pathway, mainly the Extracellular signal Regulated Kinase 1 and 2 (ERK 1/2), via the Ras-GRF membrane protein, the phosphatidylinositol 3-kinase (Pi-3 kinase) and the phospholipase C (Williams, Christie et al. 2001). Consequently, MAPK phosphorylate several transcription factors such as the CREB (CA2+/cAMP responsive element binding protein), Elk-1 (Ets LiKe gene 1), estrogen receptor, c-jun, c-fos, or AP-1 (activator protein 1 - heterodimeric protein composed of c-fos and c-jun) depending on the opioid receptor subtype. In conclusion, opioid receptor




Internalization see thèse xavier

Figure 9:
Receptor internalization process (Williams et al., 2001). Activated opioid receptor can be phosphorylated at the C-terminal. The phosphorylated sites are bind by arrestin proteins which then recruit c-Src adaptor proteins. Then, the proteic complex is recognized by clathrin to promote endocytosis leading to receptor recycling or degradation.

activation induces some short term effects such as a reduction of the cell excitability as well as a decrease of neurotransmitter release; but also triggers long term modifications of gene expression.

## F. Desensitization and receptor trafficking

### *1. Desensitization mechanisms*

Receptor desensitization is a cellular mechanism that regulates the activity of the GPCRs and likely plays a critical role in some physiological functions (Bohn, Gainetdinov et al. 2004). Following receptor activation by a selective agonist, the desensitization process begins with the phosphorylation of this receptor at the C-terminal extremity. Hence, the phosphorylated receptors are no more able to bind G proteins and enter the internalizing process to be either recycled at the membrane surface or degraded in lysozyme vesicles (Figure 9). This mechanism disrupts the GPCR signal transduction.

The phosphorylation process is mediated by G protein-coupled receptor kinases (GRKs) (Law, Kouhen et al. 2000) or by protein kinase A or C (PKA or PKC) (Xiang, Yu et al. 2001). These two effectors can be recruited differentially depending on agonists and may target separate pathways leading to different physiological responses. Recent studies suggested that the same GPCR activated by different agonists could provide diverse cellular responses (Kenakin 2011; Reiter, Ahn et al. 2012). This concept, called biased agonism, will be reviewed later in the introduction for the delta opioid receptor (see section B.1.2).

The stimulation of delta opioid receptor by an agonist induces the phosphorylation of the Serine and Threonine residues, $Ser^{344}$ and $Ser^{363}$ (Guo, Wu et al. 2000). Moreover, it has been shown that substitution of the Serine and Threonine residues by Alanine prevents the internalization of DORs (Whistler, Tsao et al. 2001). Cytoplasmic proteins called arrestins specifically recognize phosphorylated residues. In vitro binding of arrestins to MORs and DORs has been correlated to desensitization (Kovoor, Nappey et al. 1997). This arrestins-dependent desensitization has been described in vivo for MORs. Indeed, the desensitized

response after morphine injection was not detected in β-arrestin knockout mice (Bohn, Gainetdinov et al. 2000). Binding of arrestins on phosphorylated receptors therefore prevents further coupling to G proteins. This desensitization mechanism has been described as playing an important role in physiological responses such as opioid tolerance (Koch and Hollt 2008).

#### *2. Receptor trafficking*

After agonist stimulation, opioid receptors are subsequently internalized in intracellular vesicles. This internalization phenomenon requires previous binding of arrestins on the activated receptors. MORs and DORs internalize via a clathrin-coated pits (Trapaidze, Keith et al. 1996; Zhang, Xiong et al. 2009). Clathrins are responsible for the stabilization and endocytosis of vesicles containing receptors.
Once GPCRs are internalized in vesicles, they can be either recycled at the cell surface or degraded by fusion with lysosomes vesicles (von Zastrow 2003). Recycling of GPCRs can be a fast or slow process depending on the receptors as well as the activating ligand. MOR has been described as a fast-recycling receptor under peptidic activation (Koch, Widera et al. 2005). In contrast, DOR post-endocytic fate has been shown as slow-recycling process (Pradhan, Becker et al. 2009). Some studies showed that it is recycled after non-peptidic activation (Lecoq, Marie et al. 2004; Marie, Aguila et al. 2006). Therefore, an activated receptor will follow different trafficking pathways depending on the stimulating agonist. This argument in favor of a biased agonism suggests that different post-endocytic fates may induce diverse physiological responses.

# II. The Delta Opioid Receptor

## A. Ligands

#### *1. Biosynthesis and affinity*

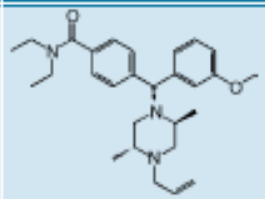
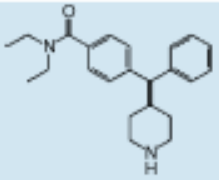
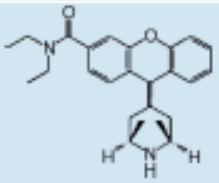
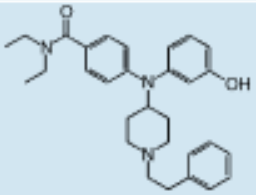
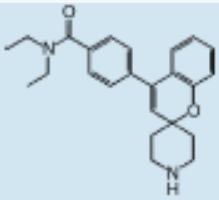
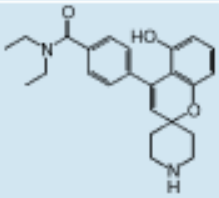
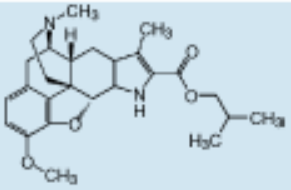
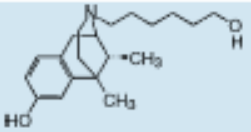
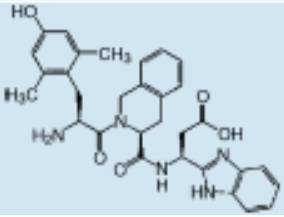
| Small-molecule agonists[a] | Structure | Summary |
|---|---|---|
| SNC80 |  | Classic non-peptide delta opioid receptor agonist<br>Analgesic, antidepressant and anxiolytic effects<br>Induces receptor internalization *in vivo* |
| AR-M100390 |  | Analgesic effect<br>No receptor internalization *in vivo* |
| JNJ-20788560 |  | Analgesic effect |
| Compound 8e |  | Analgesic effect |
| ADL5747 |  | Analgesic effect<br>Phase II clinical trial ongoing |
| ADL5859 |  | Analgesic and antidepressant effects<br>Phase II clinical trial completed |
| SB-235863 |  | Analgesic effect |
| NIH 11082 |  | Analgesic and antidepressant effects |
| UFP-512 |  | Antidepressant and anxiolytic effects |

Figure 10:
DOR agonists chemical structure and main physiological effect (Pradhan et al., 2011).

As previously mentioned, the opioid receptors can be activated by the three endogenous peptides enkephalins, β-endorphins and dynorphins despite differential selectivity (Williams, Christie et al. 2001). It is well accepted that enkephalins present the highest affinity for the DOR (≈ 2 nM) (Figure 10).

Additionally, several exogenous agonists have been described to activate the DORs (Pradhan, Befort et al. 2011). DOR ligand can be divided in two main classes: the peptidic and the alkaloids molecules.

The peptidic ligands correspond to cyclic analogs of the enkephalin and exhibit a very high affinity for the receptor compared to other opioid receptors. One of the most classically used exogenous ligand is the [D-Cys2, L-Pen5]-and [D-Cys2, D-Pen5]-enkephalin (DPDPE) and presents a 100 fold higher affinity for delta than mu opioid receptor (Mosberg, Hurst et al. 1983). The deltorphin I and II are exogenous ligand extracted from frog skin (*Phyllomedusa bicolor*) and show high affinity and specificity for the DOR (Kreil, Barra et al. 1989). Moreover, specific peptidic antagonists were synthetized such as the TIPP-Ψ (Schiller, Weltrowska et al. 1993).

The alkaloid ligands are also exogenous molecules exhibiting a significant affinity and selectivity towards DOR activation. For instance, SNC80 offers affinity Kd values for 1.73, 882 and 442 nM for delta, mu and kappa binding respectively (Bilsky, Calderon et al. 1995). BW373U86 is another delta opioid agonist obtained from the degradation of the SNC80 that exhibits similar affinity and specificity (Chang, Rigdon et al. 1993). Several novel delta opioid agonists have been developed recently (Pradhan, Befort et al. 2011). Regarding the alkaloid antagonists, naltrindole has been described as the compound with the highest affinity for the DORs compared to the more global opioid anatagonists such as the naloxone (Portoghese, Sultana et al. 1988). Later on, more selective DOR antagonists were developed (Bryant, Salvadori et al. 1998).

### *2. Biased agonism*

The biased agonism corresponds to the fact that two different agonists activating the same receptor may produce several different cellular and/or behavioral responses (Kenakin 2007; Pradhan, Walwyn et al. 2010).

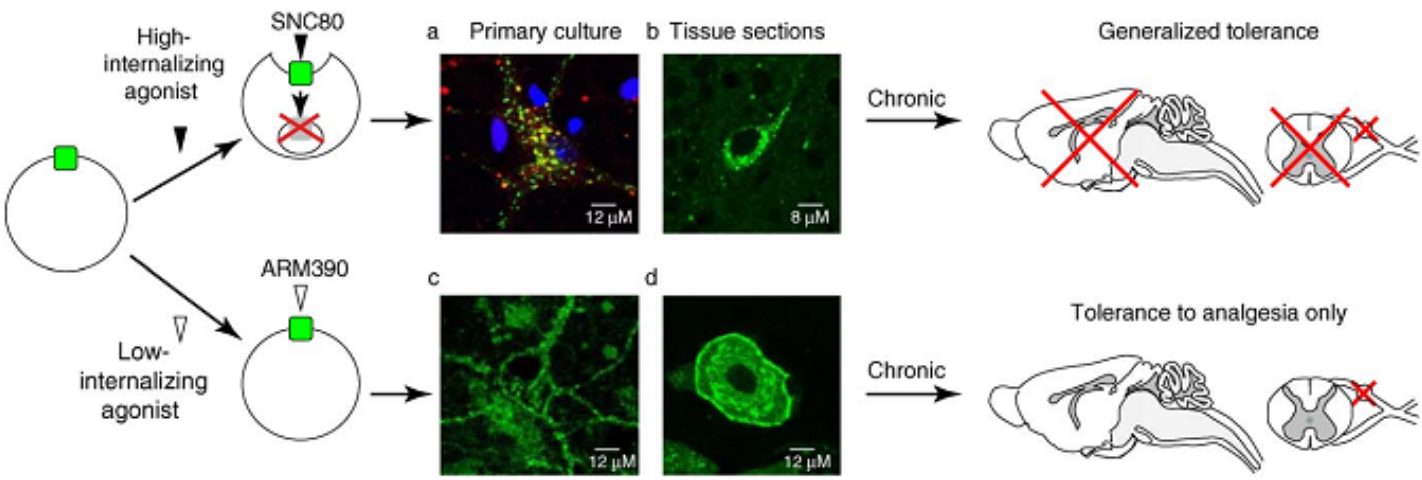



Figure 11:
In vivo example of DOR biased agonism (Pradhan et al., 2011). At the top, SNC80 triggers DOR-eGFP internalization in primary culture and tissue sections. Chronic administration of SNC80 result in a generalized tolerance. At the bottom, ARM-390 does not affect DOR-eGFP internalization in primary culture and tissue sections. Chronic administration of ARM-390 induces only tolerance to analgesia.

SNC80 has been reported to induce DOR internalization. Conversely, the agonist ARM-390, ADL5747 and ADL5859 selectively activate DOR without stimulating receptor internalization (Marie, Lecoq et al. 2003; Pradhan, Becker et al. 2009; Nozaki, Le Bourdonnec et al. 2012). Moreover, the dissociation between high- and low- internalizing agonists has been shown to underlie some physiological processes such as the development of analgesic tolerance (Pradhan, Walwyn et al. 2010). This study provided a significant illustration of biased agonism in vivo (Figure 11). Additionally, ligand-biased agonism at DOR may occur after the internalization process, to favor either receptor recycling or degradation (Audet, Charfi et al. 2012).
New DOR agonists were developed in order to avoid the deleterious consequences of DOR activation by agonists like SNC80. Indeed, while SNC80 induced epileptic seizures and anxiolytic effects, KNT-127 has been shown to produce on anxiolytic effects without provoking any convulsions (Saitoh, Sugiyama et al. 2011). This *in vivo* illustration of biased agonism provides innovative strategies to develop new drugs for the treatment of several pathologies. Interestingly, the Adolor5859 (ADL5859) (Le Bourdonnec, Windh et al. 2008) and Adolor 5747 (ADL5747) (Le Bourdonnec, Windh et al. 2009) are also two DOR agonists currently in phase 2 clinical trials to treat patient suffering from mood disorders.

## B. Genetically engineered mutant mouse lines

The physiological role of DOR has been first assessed following the development of selective ligands such as DPDPE and naltrindole. Nevertheless, the development of genetic models targeting DOR (Filliol, Ghozland et al. 2000; Scherrer, Tryoen-Toth et al. 2006) or PENK (Konig, Zimmer et al. 1996) genes brought new tools to explore the physiological role of DORs. In the future, the construction of refined genetically engineered mouse lines will help to evaluate the participation of DOR in some subtle phenotypes. Additionally, new reporter mouse lines may allow determining more precisely the localization of the receptor in neuronal circuits.

### *1. Null mutant mice line: DOR constitutive KO mice*

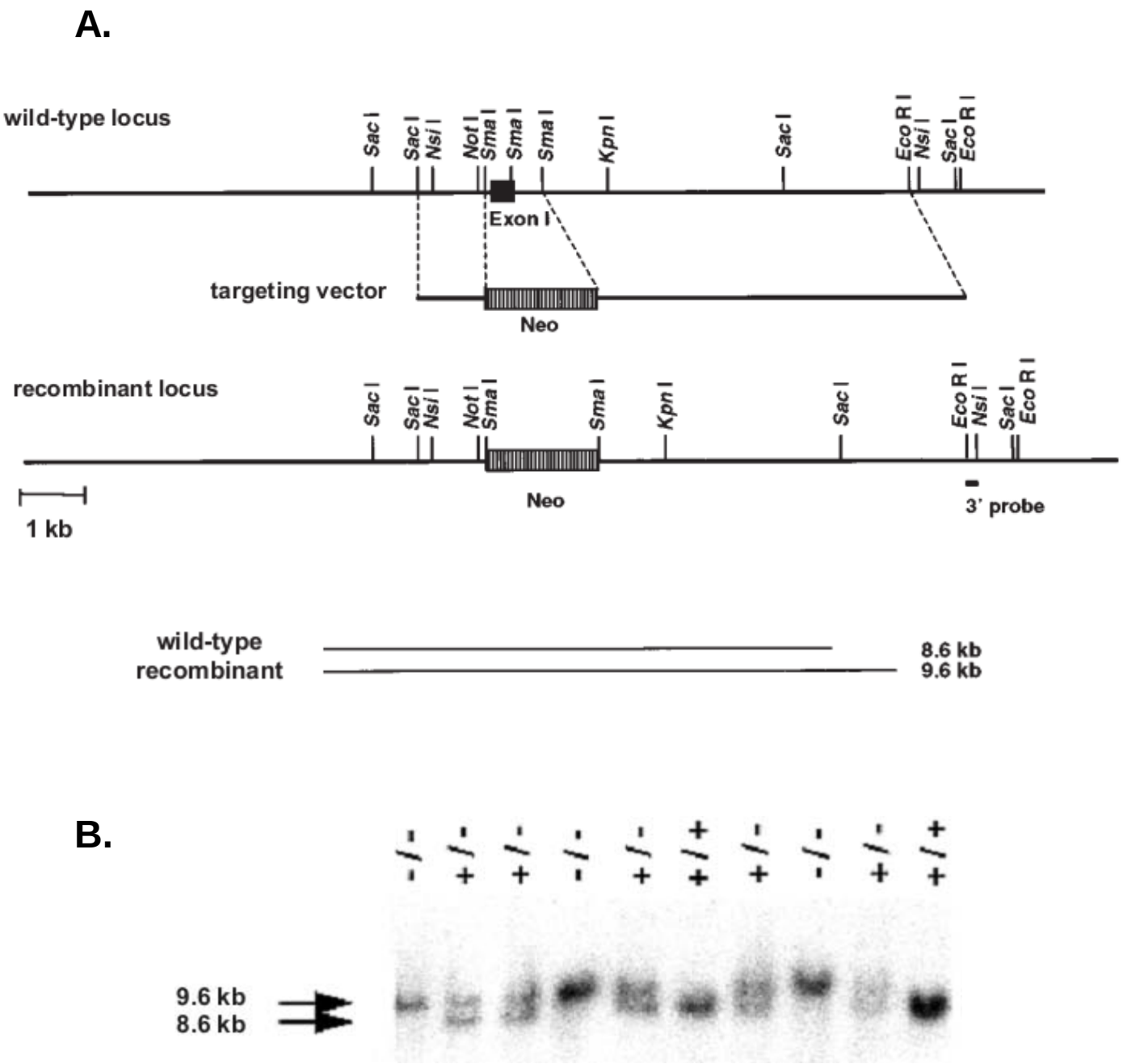



Figure 12:

**(A)** Genetic construction of constitutive DOR knockout mice (Filliol et al., 2000). Exon 1 coding for *Oprd1* gene is replaced by Neomycine cassette. The recombinant allele obtained is a 9.6 kb fragment (wild-type fragment of 8.6 kb).

**(B)** Southern-blot analysis of mouse tail DNA sample from Oprd1$^{-/-}$ offsprings mice.

The DOR constitutive KO mice were generated in our laboratory by using a homologous recombination strategy (Figure 12) (Filliol, Ghozland et al. 2000). In the targeting vector, the first coding exon, encoding the extracellular N-terminal and the first TM, as well as the translation-initiation codon of the *Oprd1* gene were replaced by a Neomycine cassette. Then, the sequence was integrated into embryonic stem cells. The selected embryonic cells were implanted into C57BL/6 blastocysts. Finally, homozygous mutant mice were obtained under a hybrid 129 SvPas/C57BL/6J (50%/50%) genetic background.

Knockout animals have provided crucial informations in the identification of proteins functions involved in variety of pathologies. However, constitutive knockout present several limits.

This technology does not allow having a temporal control over the inactivation of the gene of interest. It might be relevant to assess the contribution of a particular molecule at a precise moment, such as for instance during adolescence. Furthermore, some compensatory mechanisms could take place and then hamper the identification of the protein of interest function. Lastly, the nervous system is highly complex and composed of a variety of neuronal populations and circuits. A given protein could be expressed in many different areas and networks playing different roles, even opposite, depending on its localization. Consequently, the total deletion of a specific protein does not enable to evaluate its contribution in a specific circuit, brain region or neuronal population. Additionally, subtle phenotype may be hard to detect in this fully excised models.

In order to overcome these issues and have a significant spatial and/or temporal control of the gene inactivation, the conditional knockout approach appears as the next relevant strategy. The use of recombinant virus, such as adeno-associated virus, is also an innovative technology to specifically target regions or neuronal populations.

### *2. Conditional knockout mice lines: the DOR floxed and DOR $Na_{v\,1.8}$ mice*

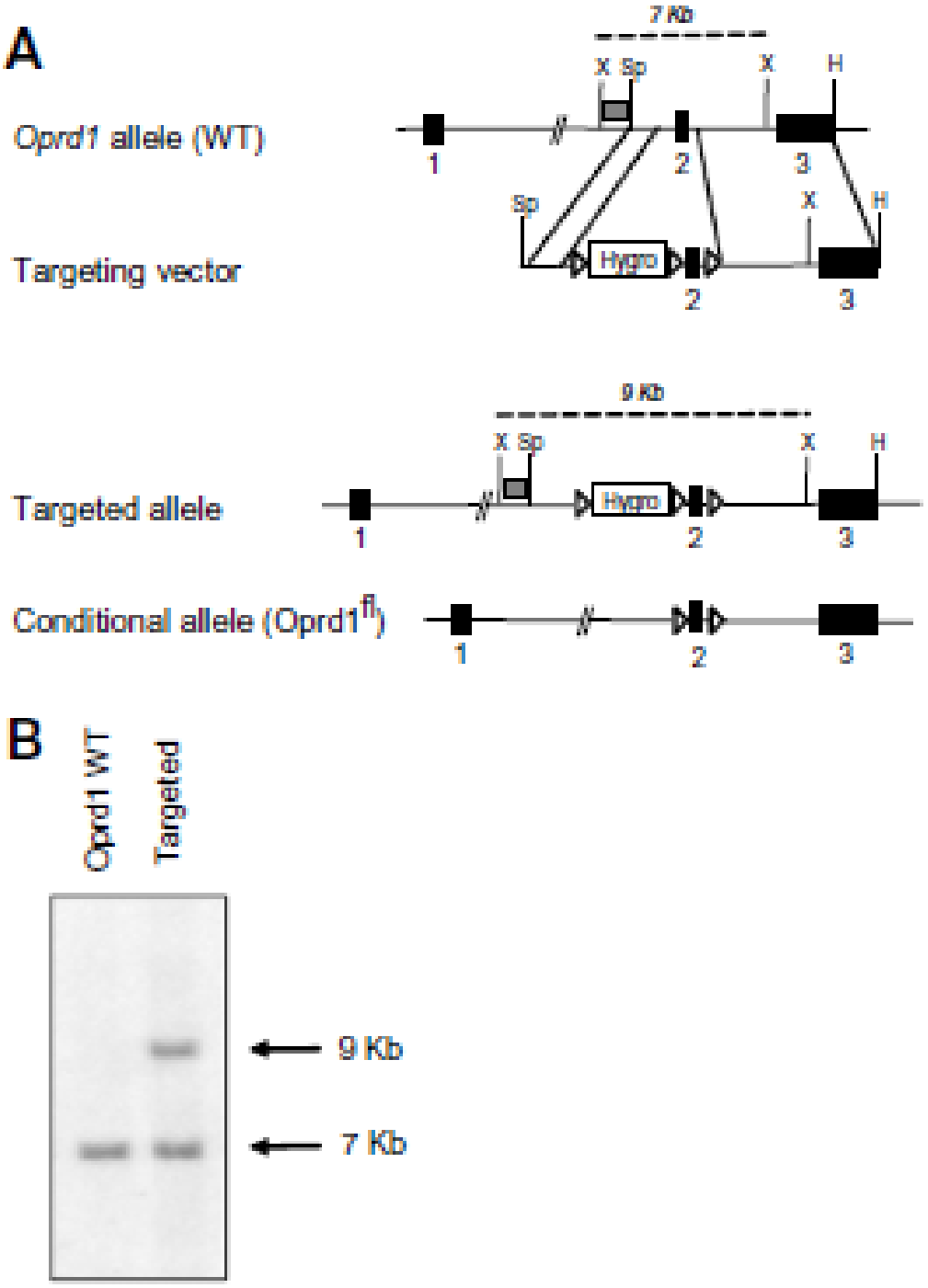



Figure 13:
**(A)** Genetic construction of homozygous floxed mice(Gavériaux-Ruff et al., 2011). Exon 2 of *Oprd1* gene is replaced by homologous recombination with floxed allele containing Hygromycine cassette as well as the exon 2 surounded by loxP sites.

**(B)** Southern blot analysis of embryonic stem cells from wild-type or recombinant cells.

The conditional knockout gene approach has been mainly developed based on the Cre/loxP system. This technology is classically used in neurogenetics. It was developed in order to inactivate gene in precise regions or cell populations (Gaveriaux-Ruff and Kieffer 2007). This tool gives the opportunity to target a gene of interest with a very high spatial control and has been also used to rescue the expression of a gene in specific regions. The Cre recombinase is a tyrosine recombinase enzyme obtained from the bacteriophage P1. The enzyme mediates the specific recombination between two loxP sites. The loxP site corresponds to a 34 base pair sequence composed of two 13 base pair palindromic sequences that flank an 8 base pair spacer region. Depending on the two loxP sites orientation, the surrounded gene can be excised (same loxP sites orientation) or inverted (opposite loxP sites orientation). Additionally, Cre recombinase can also induce translocation between two DNA fragments that both comprise one *lox*P site. The conditional knockout mouse lines are obtained by crossing two different mouse lines. The first mutant mouse line present loxP sites surrounding a part of the gene of interest. The second transgenic mouse line expresses the Cre recombinase in a tissue or cell population specific manner. Currently, more than 500 different transgenic mouse lines expressing the Cre recombinase under the control of a specific promoter have been developed (Nagy, Mar et al. 2009) and among them, about 70 provide a specific targeting of neurons (Gaveriaux-Ruff and Kieffer 2007). A database with all available information about the properties of these Cre transgenic lines has been created (the ‘‘CreXmice’’ database; http://www.mshri.on.ca/nagy/).

Full knockout could also be obtained by taking advantage of the Cre/loxP system. Indeed, the excision of the flanked sequence of the gene can be achieved by breeding the floxed mice with a mutant mouse line expressing the Cre recombinase under the control of a ubiquitously active promoter. For this purpose, the cytomegalovirus (CMV) is classically used (Feil, Brocard et al. 1996).
The *Oprd1* floxed mice were generated in our laboratory (Gaveriaux-Ruff, Nozaki et al. 2011). In this mouse line, the exon 2 of the *Oprd1* gene is flanked by two loxP sites (also called floxed) (Figure 13). The homozygous *Oprd1* floxed mouse line was obtained on a 50% C57BL/6J–50% 129SvPas genetic background. Furthermore,

DOR activation has been checked by [35S]-GTPγS binding experiment and showed a functional DOR.

The $Na_{v\ 1.8}$ conditional knockout mice for DOR represent the first and currently the only reported conditional approaches of DOR (Gaveriaux-Ruff, Nozaki et al. 2011). They were obtained by crossing the *Oprd1* floxed mice described above with a transgenic mouse line expressing the Cre recombinase under the control of $Na_{v\ 1.8}$ promoter. The $Na_{v\ 1.8}$–Cre mutant line specifically expresses the enzyme in peripheral nociceptive neurons, unmyelinated C and thinly myelinated AΔ fibers, and has been previously successfully used (Abrahamsen, Zhao et al. 2008).

Recently, new technologies used for the study of in vivo gene functions emerges such as the zinc-finger nucleases (ZFNs) or Transcription Activator-Like Effector (TALE) Nucleases (TALENs) and will likely provide alternatives to the Cre/lox system in the future (Sung, Baek et al. 2012)

## C. Physiological functions

As previously mentioned, the opioid system is involved in many physiological processes in particular pain control, hedonic homeostasis (maintenance of the rewarding/aversive balance processes in a physiological range), mood and well-being. Studies on the DOR revealed its role in emotional control (Filliol, Ghozland et al. 2000), in processes that may modulate drug addiction (Roberts, Gold et al. 2001; Le Merrer, Plaza-Zabala et al. 2011; Faget, Erbs et al. 2012), in the development of seizures (Broom, Nitsche et al. 2002; Jutkiewicz, Baladi et al. 2006), in the regulation of locomotor activity (Filliol, Ghozland et al. 2000; Le Merrer, Rezai et al. 2013) and in neuroprotective processes (Gao, Niu et al. 2012; He, Sandhu et al. 2013). The contribution of DOR in these different functions will be discussed later in this manuscript.

Moreover, DOR appeared to be involved in the modulation of immune function (Weber, Gomez-Flores et al. 2004), in cardioprotection process (Maslov, Lishmanov et al. 2009; Shen, Ben et al. 2012) and in gastro-intestinal function (Bueno and Fioramonti 1988; Townsend, Portoghese et al. 2004). These regulatory roles will not be discussed here.

In addition, DOR is a major player in pain perception (Gaveriaux-Ruff and Kieffer 2011) and in memory processes (Robles, Vivas-Mejia et al. 2003; Le Merrer, Faget et al. 2012) which will be discussed in the following parts.

### *1. Pain*

Pain is defined by the International Association for the Study Pain's as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Bonica 1979). Acute pain is characterized by different modalities such as thermal, mechanical or chemical pain and can be distinguished from chronic pain such as inflammatory or neuropathic pain.

The constitutive DOR knockout mice showed no differences in acute thermal, mechanical or chemical pain perceptions (Kieffer and Gaveriaux-Ruff 2002), whereas MORs are implicated in the regulation of these responses (Martin, Matifas et al. 2003). However, evidence supports the contribution of DOR in chronic pain. Indeed, it has been shown that pharmacological activation of DOR by SNC80 was able to reduce inflammatory pain perception (Gaveriaux-Ruff, Karchewski et al. 2008). In addition, the DOR knockout mice displayed reduced pain thresholds in a classical inflammatory model using Freund adjuvant injections to induce inflammatory conditions (Gaveriaux-Ruff, Karchewski et al. 2008) and also in a classical neuropathic model using the sciatic nerve injury surgery (Nadal, Banos et al. 2006; Benbouzid, Gaveriaux-Ruff et al. 2008).

Altogether, these studies support a role of DOR in decreasing chronic pain perception.

### *2. Learning and memory*

The DORs are expressed in regions involved in learning and memory such as the hippocampus (Erbs, Faget et al. 2012). The pyramidal cells of hippocampus are regulated by GABAergic interneurons which express DOR, suggesting that DOR participate to the modulation of hippocampal outputs.

Robles and colleagues showed that animals performing successfully in a spatial discrimination paradigm, the holeboard task, present increased DOR mRNA expression (Robles, Vivas-Mejia et al. 2003). This study emphasizes the potential contribution of DOR in spatial memory skills. A recent study in our laboratory showed that pharmacological inactivation or genetic deletion of DOR in mice altered performances in the spatial object recognition task (Le Merrer, Rezai et al. 2013). Moreover, some results obtained in our laboratory indicate that DOR knockout mice displayed decreased context-induced freezing in a fear conditioning task supporting a deficit in fear memory processes (Scherrer et al., in preparation).

In addition, it has been shown that mice deficient for DOR also present a deficit in a drug-context association paradigm. Indeed, they exhibit a decrease of morphine conditioned place preference (CPP) and lithium conditioned place aversion (CPA) tests (Le Merrer, Plaza-Zabala et al. 2011), while they self-administered morphine at a similar level compared to WT mice. Interestingly, the morphine CPP was restored in these animals by exposing them to cues predicting morphine (Le Merrer, Faget et al. 2012). Then, DOR appears crucial for the modulation of spatial contextual cue-related responses.

These data emphasize that DOR may facilitate spatial memory processes and play a major role of DOR in drug-context associations likely crucial in the persistence of addictive behaviors.

### *3. Summary of other DOR functions*

The contribution of DOR in the control of emotional processes, in reward and addiction, in the onset of epileptic seizures, in the control of locomotor activity as well as in hypoxic/ischemic conditions is discussed in the following review (Chu Sin Chung and Kieffer 2013).

# III. Neurobiology of Anxiety

## D. Definition

Anxiety is defined as an unpleasant mental state which breaks out in anticipation of potential threat (Gross and Hen 2004), whereas fear arises in anticipation of a real or imminent threat.

The non-pathological anxiety is a physiological process necessary for the survival and the adaptation of an organism to its environment. Anxiety can be decomposed in two classes: the state anxiety corresponding to the acute reactivity towards a potentially threatening situation and the trait anxiety which reflects the natural tendency of an organism to express an increase anxiety response over time (Endler and Kocovski 2001; Kennedy, Schwab et al. 2001).

Pathological anxiety is responsible for the incidence of several diseases. According to the *Diagnostic and Statistical Manual of American Psychiatric Association* (DSM-4$^{th}$ edition TR-2000), anxiety disorders are divided in 7 major classes: generalized anxiety disorder (GAD), social phobia, simple phobia, panic disorder, agoraphobia, post-traumatic stress disorder (PTSD) and obsessive-compulsive disorder (OCD). In the DSM 5$^{th}$ edition, the latter two are removed from the anxiety disorder category and are defined in their own chapters. The diagnostic criteria remain similar to the previous edition, except that patients do not need to declare their fear as irrational or excessive.

Definitions of anxiety disorders:

1) Generalized Anxiety Disorders are the most largely diagnosed anxiety disorder and usually affects young adults. They are characterized by excessive, uncontrollable and often irrational worry.

2 and 3) Social and simple Phobias are defined as an intense and irrational fear (“out of proportion”) toward a precise object or situation that the individual try to avoid, even at the cost of enormous efforts. The specific object or situation is not necessary threatening or noxious for the individual

4) Panic disorders are mainly characterized by the manifestation of a panic attack associated with the fear of another attack.

5) Agoraphobia is similar to the panic disorders and defined as an irrational fear of places or situations in which another attack may occur and the patient may be unable to leave or find someone to help.

5) Post-traumatic stress disorders are considered as a symptomatic response to a previous traumatic experience.

6) Obsessive Compulsive Disorders are characterized by undesirable, insistent and repetitive behaviors. The individual had to perform these behaviors or else will feel an intense anxiety.

## E. Animal models

Anxiety disorders are among the most prevalent psychiatric diseases in Europe and North America. They represent a dramatic health problem for individual as well as a major cost for societies. Therefore, there is an important need for the development of therapies and for a better understanding about genetic and environmental risk factors that trigger these pathologies (Cryan and Sweeney 2011). Numerous animal tests of anxiety have long been validated to assess anxiolytic potential of novel drugs (Pellow, Chopin et al. 1985; File, Lippa et al. 2004).

These models should present a reasonable analogy to the human disorder in manifestation or symptoms (Face Validity) like for instance an excessive avoidance of threatening situation. They must also induce objective, measurable behavioral changes that are due to similar physiological mechanisms as for the human pathology (Construct Validity). Finally, animal model for anxiety should display sensitivity to effective clinical treatments such as diazepam (Predictive Validity).

Anxiety tests can be divided in three categories: exploratory behavior models, acute behavioral stress responses test and conditioned responses (Cryan and Sweeney 2011; Haller and Alicki 2012; Kumar, Bhat et al. 2013).

In the first category, anxiety tests are generally based on approach-avoidance reflected by natural tendency of rodent to avoid potentially dangerous environment such as open and/or lit environment. They present a strong ethological relevance (i. e. open field, light-dark box, elevated plus maze, elevated zero maze, social interaction, T-maze, hole board tests).

The second category regroups conflict-based tests (i. e. Geller-Seifter test, Vogel punished drinking test, defensive marble burying), hyponeophagia paradigm (i. e. novelty suppressed feeding, novelty induced hypophagia) and physiological tests like stress-induced hyperthermia or autonomic telemetry measures.

The last category of anxiety tests was designed to overcome the effect of motor output and animal reactivity toward conditioned stimuli (i. e. active/passive avoidance, fear potentiated startle, pavlovian fear conditioning, conditioned emotional response, conditioned taste aversion).

As emphasized by the large variety of anxiety tests existing and their variety of stressor applied and parameters measured, animal models of anxiety assess several neurobiological processes involved in anxiety. Therefore, it is inappropriate to consider that one model may serve to detect compounds for a disease that is mediated through multiple and diverse mechanisms. Similarly, it is likely relevant to use several tests in order to evaluate neurobiological processes underlying anxiety in a given study (Ramos 2008).

## F. Neurocircuitry of anxiety

Over the past decades, many studies investigated the neuroanatomical substrates underlying anxiety. Neuroimaging approach has been importantly used to identify brain regions contributing to anxiety disorders (Kent and Rauch 2003). Experiments performed on rodent mainly focused on neurocircuits involved in fear responses. Key brain regions identified in these studies include the amygdala, nucleus accumbens, bed nucleus of the stria terminalis, hippocampus, ventromedial hypothalamus, periaqueductal gray, some brainstem nuclei, thalamus, insular cortex and some prefrontal regions (Davis 2006; Shin and Liberzon 2010). In parallel, *in vivo* electrophysiological recording, tracing and lesions approaches allowed to characterize the specific contribution of these areas in basic components of fear circuitry.

Interestingly, some evidence suggest that fear and anxiety networks might be orchestrated by distinct systems. A contribution of the olfactory bulb (Saitoh, Hirose et al. 2006; Saitoh and Yamada 2012), prefrontal cortex (Bechara, Damasio et al. 2000; Davidson 2002), insular cortex (Paulus and Stein 2006; Lamm and Singer 2010), ventral hippocampus (Deacon, Bannerman et al. 2002; Fournier and Duman 2013) and amygdala (Baxter and Murray 2002; Cardinal, Parkinson et al. 2002) has been evidenced in emotional processing circuits.

Since this will be of interest of the third part of this work, we next reviewed evidence about the contribution of amygdala in emotional responses.

## G. The amygdala

In the early 19th century, Burdach is credited to the first description of the amygdala, a brain area close to the human temporal cortex. The amygdala has long been established to be a key structure for the regulation of emotions as well as for the modulation of memory (LeDoux 2000; Ehrlich, Humeau et al. 2009; Roozendaal, McEwen et al. 2009). In addition, an extensive literature studied the contribution of the amygdala in fear conditioning processes (Johansen, Wolff et al. 2012; Pare and Duvarci 2012). The basolateral and lateral nuclei of the amygdala (BLA) are established as the main site for conditioned stimulus (for instance cues or context) and unconditioned stimulus (reward or punishment) associations. On the other hand, the BLA is transmit informations of such associations to the central et centromedial nuclei of the amygdala CeA which in turn may orchestrate adapted autonomic and behavioral responses (Everitt, Cardinal et al. 2003).

The amygdala has been considered as a major limbic area in the neuronal circuits supporting the anxiety-related behaviors. It has been demonstrated that chronic stress enhances the reactivity of projecting neurons of the amygdala by in vivo electrophysiological recordings of pyramidal neurons of the lateral nucleus (Rosenkranz, Venheim et al. 2010). Three models of anxiety (foot shock avoidance, elevated plus maze and puff-induced ultrasonic vocalization test) (Silveira, Sandner et al. 1993; Duncan, Knapp et al. 1996) as well as four anxiogenic drugs (FG-7142, yohimbine, mCPP and caffeine) (Singewald, Salchner et al. 2003) induced an

increase of c-fos immunoreactivity in the amygdala. Affective sensory stimuli are essentially provided to the amygdala from associative or sensory cortical areas and lead to an increase of dopamine release in the BLA (Inglis and Moghaddam 1999). This increase of dopamine is reversed by the classical anxiolytic drug diazepam (Coco, Kuhn et al. 1992). Moreover, the crosstalk between the amygdala and the PFC has been demonstrated as critical for the modulation of sensory informations, through dopaminergic projections, coming from the temporal cortex (Rosenkranz and Grace 2001; Rosenkranz and Grace 2002). The optogenetic activation of glutamatergic projections from the BLA into the CeA produced a reversible anxiolytic effect measured in the elevated plus-maze and the open-field tests in mice, while the opposite effect has been observed by inhibition of the same connections (Tye, Prakash et al. 2011).

The classical fear conditioning paradigm increases c-fos immunoreactivity in the cingulate cortex and amygdala (Huang, Shyu et al. 2013). Moreover, the same study showed that fear conditioning extinction, known as an active process of learning, is related to the amygdala as well. It is well-accepted that the amygdala is a critical brain structure for the acquisition, storage and retrieval of fear memory. The lesion of the lateral nucleus of the amygdala in rats disrupted the freezing-induced by an auditory conditioned stimulus in the classical fear conditioning paradigm (LeDoux, Cicchetti et al. 1990).

Although the amygdala has been essentially studied in the context of aversive conditioning, evidence also support a major role in appetitive conditioning (Everitt, Cardinal et al. 2003). The lesion of the BLA altered the approach to a conditioned stimulus that predicts the apparition of sucrose reinforcement (Burns, Everitt et al. 1999). Interestingly, the BLA is required for the firing of dopamine neurons in the NAc in response to cue-evoked reward (Ambroggi, Ishikawa et al. 2008). Recently, specific optogenetic activation of the glutamatergic projections from the BLA to the NAc reinforced the self-stimulation of light to reactivate the same pathway, suggesting a role in reward-seeking behaviors. Conversely, the inhibition of the same pathway decreased the cue-evoked intake of sucrose and thus confirmed that this connection is critical for the cue-reward association (Stuber, Sparta et al. 2011). Moreover, a local microinjection of the opioid antagonist, naloxone methiodid, into the

BLA abolished context-induced reinstatement for alcohol seeking (Marinelli, Funk et al. 2010).

Altogether these studies suggest that the amygdala is required to attribute an affective value to aversive or appetitive stimuli as well as associated cues (global internal and external environment). The amygdala may thus be involved in the conditioned motivational processes, especially through its connection with the reward circuit.

The different functions of the amygdala emphasize the large contribution of this brain structure to many physiological processes and suggest that the amygdala may be central to several pathologies. Amygdala-mediated emotional control and learning points to a neural substrate where neuroadaptations may occur during the development of substance use disorders. Indeed, the enhanced reactivity of the brain stress systems plays an important role in addiction, especially during the withdrawal stage, and studies suggested that some modifications arise at the level of the amygdala (Bruchas, Land et al. 2009; Smith, Schindler et al. 2012).

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# Delta opioid receptors in brain function and diseases

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ABSTRACT

Evidence that the delta opioid receptor (DOR) is an attractive target for the treatment of brain disorders has strengthened in recent years. This receptor is broadly expressed in the brain, binds endogenous opioid peptides, and shows as functional profile highly distinct from those of mu and kappa opioid receptors. Our knowledge of DOR function has enormously progressed from in vivo studies using pharmacological tools and genetic approaches. The important role of this receptor in reducing chronic pain has been extensively overviewed; therefore this review focuses on facets of delta receptor activity relevant to psychiatric and other neurological disorders. Beneficial effects of DOR agonists are now well established in the context of emotional responses and mood disorders. DOR activation also regulates drug reward, inhibitory controls and learning processes, but whether delta compounds may represent useful drugs in the treatment of drug abuse remains open. Epileptogenic and locomotor-stimulating effects of delta agonists appear drug-dependent, and the possibility of biased agonism at DOR for these effects is worthwhile further investigations to increase benefit/risk ratio of delta therapies. Neuroprotective effects of DOR activity represent a forthcoming research area. Future developments in DOR research will benefit from in-depth investigations of DOR function at cellular and circuit levels.



**Contents**



## 1. Introduction

Mu, delta and kappa opioid receptors are G protein coupled receptors, which play a central role in pain control, and are key players in hedonic homeostasis, mood and well-being. The three receptors and their endogenous opioid peptides also regulate responses to stress, and a number of peripheral physiological functions including respiratory, gastrointestinal, endocrine and immune processes. Opioid receptors are highly homologous in sequence, and their crystal structure has been recently elucidated at high-resolution by X-Ray crystallography (Granier et al., 2012; Manglik et al., 2012; Wu et al., 2012). All three receptors

*Abbreviations:* Amy, amygdala; CA, continuous access; CeA, central nucleus of the amygdala; Cg, cingulate cortex; CPA, conditioned place aversion; CPP, conditioned place preference; CPu, caudate putamen nucleus; Cx, cortex; DOR, delta opioid receptor; EEG, electroencephalography; Enk, enkephalin; FCx, frontal cortex; GPCR, G protein coupled receptor; Hipp, hippocampus; Hyp, hypothalamus; i.c.v., intracerebroventricular; i.p., intraperitoneal; i.v., intravenous; IA, intermittent access; KO, knockout; Nacc, nucleus accumbens; NTI, naltrindole; OB, olfactory bulb; p.o., pers os; PR, progressive ratio; PVN, paraventricular nucleus; RS, retrosplenial cortex; s.c., subcutaneous; SA, self-administration; SC, spinal cord; Th, thalamus; VTA, ventral tegmental area.

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inhibit neuronal activity, via reduced neuronal firing or lower transmitter release, and a main goal in opioid research is the identification of receptor-mediated signaling pathways that operate in vivo, to regulate physiology and behavior (Pradhan et al., 2012).

In the past two decades, refinement of pharmacological tools and availability of genetic approaches have clarified the specific role of each opioid receptor in many aspects of opioid-related responses (Shippenberg et al., 2008; Gianoulakis, 2009; Sauriyal et al., 2011; Lutz & Kieffer, in press; Gaveriaux-Ruff, in press). Mu opioid receptors mediate both analgesic and addictive properties of clinically useful and abused opiates. Mu opioid receptor activation strongly inhibits severe pain, and is a major target for post-operative and cancer pain management (Zollner & Stein, 2007). Mu receptors are also central for reward processing (Le Merrer et al., 2009), representing a main factor in the initiation of addictive behaviors. Kappa opioid receptors also release pain (Chavkin, 2011) but oppose mu receptors in the regulation of hedonic homeostasis. The notion that kappa receptor blockade alleviates stress responses and depressive states is raising increasing interest (Shippenberg, 2009; Knoll & Carlezon, 2010).

Delta opioid receptors (also known as δ receptors, DORs or DOP receptors in the IUPHAR nomenclature) have emerged as an attractive target in many respects. In accordance with the rodent mRNA distribution, DOR in the human central nervous system is expressed in cortical regions and limbic structures such as hippocampus and amygdala, as well as basal ganglia and hypothalamus (Simonin et al., 1994; Peckys & Landwehrmeyer, 1999; Smith et al., 1999; Peng et al., 2012).

The development of highly selective delta opioid agonists and rapid progress in mouse mutagenesis approaches targeting the *Oprd1* gene (Filliol et al., 2000; Scherrer et al., 2006, 2009; Gaveriaux-Ruff et al., 2011) have set delta receptors as a model system for the analysis of G protein coupled receptor (GPCR) trafficking and biased signaling in vivo, and established this receptor as a promising target to treat chronic pain and mood disorders (Pradhan et al., 2011). The stimulation of delta opioid receptors strongly reduces pain, specifically under situations of persistent pain, and mechanisms of delta agonist analgesia have been extensively overviewed recently (Gaveriaux-Ruff & Kieffer, 2011). Here we will focus on non-nociceptive facets of delta receptor function, and summarize accumulating preclinical data supporting the key role of delta receptors in emotional processes (Tables 1 and 2), drug reward and addiction (Table 3), and other aspects of potential therapeutic relevance (Table 4). Both genetic approaches and behavioral pharmacology concur to support an implication of delta receptors in psychiatric and neurological disorders, and delta agonists have entered clinical trials (Table 5).

## 2. Delta opioid receptor and the control of emotional processes

Genetic studies have revealed a prominent role for DORs in emotional processing more than a decade ago. Knockout of the *Oprd1* gene, encoding DOR, led to higher anxiety-related responses and depressive-like behaviors (Filliol et al., 2000). This activity was clearly DOR-selective, since neither mu receptor knockout mice nor kappa receptor knockout mice showed a similar phenotype (Filliol et al., 2000). Mice deficient for *Penk* gene, encoding the pre-proenkephalin precursor, also showed increased levels of anxiety using a large number of experimental testing conditions (Konig et al., 1996; Ragnauth et al., 2001), suggesting that DOR/enkephalinergic systems exert control over anxiety-related behaviors. This was later supported by experiments performed in wild-type and mu receptor mutant mice, which both showed similar decreased levels of anxiety upon systemic administration of RB101, an enkephalinase inhibitor (Mas Nieto et al., 2005). Interestingly, over-expression of enkephalin by a virus approach in the amygdala potentiates the anxiolytic effect of benzodiazepines and this effect is abolished by systemic naltrindole (NTI)

**Table 1**
Delta opioid receptor function in anxiety-related behavior control.

| Approach | Model/compound | Test | Delta compound administration (route/dose) | Anxiety level (vs control) | References |
|---|---|---|---|---|---|
| Genetic | | | | | |
| | DOR KO mice | Elevated plus maze | | ↑ | Filliol et al., 2000 |
| | | Light-dark box | | ↑ | Filliol et al., 2000 |
| | | Open field | | ↔ | Filliol et al., 2000 |
| | Enk KO mice | Open field | | ↑ | Konig et al., 1996; Ragnauth et al., 2001 |
| | | Elevated O-maze | | ↑ | Konig et al., 1996 |
| | | Resident-intruder test | | ↑ | Konig et al., 1996 |
| | | Light-dark box | | ↑ | Ragnauth et al., 2001 |
| | | Fear conditioning | | ↑ | Ragnauth et al., 2001 |
| DOR antagonist | Rats/NTI | Elevated plus maze | s.c. (1, 3 or 5 mg/kg) | ↑ | Saitoh et al., 2004; Saitoh et al., 2005; Perrine et al., 2006 |
| | | Elevated plus maze | Local into Hipp (0.5, 1 or 2 μg/rat) | ↑ | Solati et al., 2010 |
| | | Light-dark box | Local into BLA (10 pmol/rat) | ↑ | Narita et al., 2006a |
| | Mice/NTI | Light-dark box | i.c.v. (1 nmol/mouse) | ↑ | Narita et al., 2006a |
| | | Light-dark box | s.c. (1 mg/kg) | ↑ | Narita et al., 2006b |
| | | Light-dark box | Local into cingulate Cx (1 pmol/mouse) | ↑ | Narita et al., 2006b |
| | | Elevated plus maze | s.c. (1 mg/kg) | ↑ | Narita et al., 2006b |
| | | Elevated plus maze | Local into cingulate Cx (1 pmol/mouse) | ↑ | Narita et al., 2006b |
| DOR agonist | Rats/SNC80 | Fear conditioning | s.c. (1 or 3 mg/kg) | ↓ | Saitoh et al., 2004 |
| | | Elevated plus maze | s.c. (1-20 mg/kg) | ↓ | Saitoh et al., 2004; Perrine et al., 2006 |
| | | Open field | s.c. (1 or 3 mg/kg) | ↔ | Saitoh et al., 2004 |
| | | Defensive burying paradigm | s.c. (5 mg/kg) | ↓ | Perrine et al., 2006 |
| | | Elevated O-maze | s.c. (5 mg/kg) | ↓ | Ambrose-Lanci et al., 2008 |
| | Rats/DPDPE | Elevated plus maze | Local into CeA (0.5 or 1.5 μg/μl; 1 μl/CeA) | ↓ | Randall-Thompson et al., 2010 |
| | Mice/UFP-512 | Light-dark box | i.p. (1 mg/kg) | ↓ | Vergura et al., 2008 |
| | | Elevated plus maze | i.p. (0.1 or 1 mg/kg) | ↓ | Vergura et al., 2008 |
| | | Open Field | i.p. (0.1 or 1 mg/kg) | ↔ | Vergura et al., 2008 |
| | Rat/enkephalin | Elevated plus maze | Local into Hipp (1, 2 or 5 μg/rat) | ↓ | Solati et al., 2010 |
| | Mice/RB101 | Elevated O-maze | i.p. (80 mg/kg) | ↓ | Mas Nieto et al., 2005 |
| | Rats/opiorphin | Defensive burying paradigm | i.v. (1 mg/kg) | ↔ | Javelot et al., 2010 |
| | Rats/AZD2327 | Modified Geller-Seifter conflict test | p.o. (0.5, 1 or 5 mg/kg) | ↓ | Hudzik et al., 2011 |

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**Table 2**
Delta opioid receptor function in depressive-like behavior control.

| Approach | Model/compound | Test | Delta compound administration (dose/route) | Despair level (vs control) | References |
|---|---|---|---|---|---|
| Genetic | | | | | |
| | DOR KO mice | Forced swim test | | ↑ | Filliol et al., 2000 |
| | | Motility conditioned suppression test | | ↔ | Filliol et al., 2000 |
| | Enk KO mice | Forced swim test | | ↔ | Bilkei-Gorzo et al., 2007 |
| | | Tail suspension test | | ↔ | Bilkei-Gorzo et al., 2007 |
| DOR antagonist | Mice/NTI | Forced swim test | s.c. (1 or 3 mg/kg) | ↔ | Saitoh et al., 2004 |
| DOR agonist | Rats/SNC80 | Forced swim test | s.c. (3.2, 10 or 32 mg/kg) | ↓ | Jutkiewicz et al., 2005a; Jutkiewicz et al., 2005b |
| | Mice/SNC80 | Forced swim test | s.c. (1 or 3 mg/kg) | ↓ | Saitoh et al., 2004 |
| | Rats/DPDPE | Forced swim test | i.c.v. (155 nmol/rat) | ↓ | Torregrossa et al., 2006 |
| | Rats/Deltorphin II | Forced swim test | i.c.v. (0.03 or 0.1 nmol/rat) | ↓ | Torregrossa et al., 2006 |
| | Rats/JOM-13 | Forced swim test | i.v. (32 mg/kg) | ↓ | Torregrossa et al., 2006 |
| | Mice/NIH 11082 | Tail suspension test | i.p. (16 or 32 mg/kg) | ↓ | Naidu et al., 2007 |
| | Mice/UFP-512 | Forced swim test | i.p. (0.1 or 0.3 mg/kg) | ↓ | Vergura et al., 2008 |
| | Rats/RB101 | Forced swim test | i.v. (32 mg/kg) | ↓ | Jutkiewicz et al., 2006b |
| | Mice/RB101 | Forced swim test | i.p. (80 mg/kg) | ↓ | Mas Nieto et al., 2005 |
| | Rats/Opiorphin | Forced swim test | i.v. (1 mg/kg) | ↓ | Javelot et al., 2010 |
| | Rats/AZD2327 | Learned helplessness | p.o. (1 or 10 mg/kg) | ↓ | Hudzik et al., 2011 |
| | Mice/KNT-127 | Forced swim test | s.c. (0.1, 0.3 or 1 mg/kg) | ↓ | Saitoh et al., 2011 |

administration (Primeaux et al., 2006). Altogether therefore, genetic approaches have opened the way to explore DOR function in the areas of anxiety (Table 1) and depression (Table 2).

Pharmacological studies using both delta agonists and antagonists in rodents confirmed anxiolytic activity of the opioid tone mediated by DOR. As observed for knockout mice, receptor blockade by NTI administration, a selective DOR antagonist, increased anxiety-related behaviors in mice (Narita et al., 2006b) and rats (Saitoh et al., 2004, 2005; Perrine et al., 2006). DOR activation by selective agonists such as SNC80 (Saitoh et al., 2004; Perrine et al., 2006; Ambrose-Lanci et al.,

**Table 3**
Delta opioid receptor function in reward and addiction.

| Drug of abuse/approach | Model/compound | Test | Delta compound administration (dose/route) | Behavioral level (vs control) | References |
|---|---|---|---|---|---|
| ***Morphine*** | | | | | |
| Genetic | DOR KO mice | CPP | | ↓ | Chefer and Shippenberg, 2009; Le Merrer et al., 2011 |
| | | CPA (lithium) | | ↓ | Le Merrer et al., 2011 |
| | | SA | | ↔ | Le Merrer et al., 2011 |
| | | SA | | ↔ | David et al., 2008 |
| DOR antagonist | Mice/NTI | CPP | s.c. (0.3 mg/kg) | ↓ | Chefer and Shippenberg, 2009 |
| | Rats/naltriben | CPP | i.p. (1 mg/kg) | ↓ | Billa et al., 2010 |
| DOR agonist | Mice/TAN-67 | CPP | s.c. (10 or 20 mg/kg) | ↑ | Suzuki et al., 1996 |
| ***Ethanol*** | | | | | |
| Genetic | DOR KO mice | SA (two bottle choice CA) | | ↑ | Roberts et al., 2001 |
| | | Operant SA | | ↑ | Roberts et al., 2001 |
| | Enk KO mice | SA (two bottle choice CA) | | ↔ | Racz et al., 2008 |
| DOR antagonist | Rats/NTI | Cue or context induced drug-seeking | i.p. (1, 5, 7.5 or 15 mg/kg) | ↓ | Marinelli et al., 2009 |
| | | SA (two bottle choice CA) | i.p. (5 or 10 mg/kg) | ↓ | Nielsen et al., 2008 |
| | | CPP | Intra-CeA (2 nM) | ↓ | Bie et al., 2009 |
| | | SA (two bottle choice IA) | Intra-striatal (1 or 2 μg) | ↓ | Nielsen et al., 2012 |
| | Mice/naltriben | SA (two bottle choice IA) | s.c. (6 or 10 mg/kg) | ↓ | van Rijn and Whistler, 2009 |
| | Rats/TIPPΨ | SA (two bottle choice CA) | Intra-VTA (5 μM) | ↓ | Margolis et al., 2008 |
| | Rats/SORI-9409 | SA (two bottle choice CA and IA) | i.p. (5, 15 or 30 mg/kg) | ↓ | Nielsen et al., 2008 |
| DOR agonist | Rats/SNC80 | SA (two bottle choice IA) | i.p. (20 mg/kg) | ↑ | van Rijn et al., 2010a |
| | | | Intra-striatal (5 ng) | ↑ | Nielsen et al., 2012 |
| | Rats/DPDPE | SA (two bottle choice CA) | Intra-VTA (10 mM) | ↓ | Margolis et al., 2008 |
| | Rats/DALA | SA (two bottle choice IA) | Intra-PVN (7.1 or 14.2 nM) | ↑ | Barson et al., 2010 |
| ***Cannabinoids*** | | | | | |
| Genetic | DOR KO mice | CPP | | ↔ | Ghozland et al., 2002 |
| ***Nicotine*** | | | | | |
| Genetic | DOR KO mice | Nicotine CPP | | ↓ | Berrendero et al., 2012 |
| | | Nicotine SA | | ↓ | Berrendero et al., 2012 |
| DOR antagonist | Rats/NTI | Nicotine SA (0.03 mg/kg/infusion) | s.c. (0.3, 1 or 3 mg/kg) | Trend ↓ | Ismayilova and Shoaib, 2010 |
| | Mice/NTI | Nicotine SA (30 μg/kg/infusion) | i.p. (5 mg/kg) | ↓ | Berrendero et al., 2012 |
| ***Psychostimulant*** | | | | | |
| DOR antagonist | Mice/NTI | Amphetamine-induced CPP | s.c. (5 mg/kg) | ↓ | Belkai et al., 2009 |
| | Rats/NTI | Cocaine SA (PR) (1.5 mg/kg/infusion) | Intra-NAc (5 nM/side) | ↓ | Ward and Roberts, 2007 |
| | | | Intra-VTA (5 nM/side) | ↑ | Ward and Roberts, 2007 |
| | | | Intra-amygdala (5 nM/side) | ↔ | Ward and Roberts, 2007 |
| | Rats/NTI | Cocaine reinstatement | Intra-NAc (300, 1000 or 3000 ng/side) | ↔ | Simmons and Self, 2009 |

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**Table 4**
Delta opioid receptor role in epileptic seizures, hypoxia/ischemia and Parkinson disease.

| Condition/ pathology | Model/compound | Test/measures | Delta compound administration (dose/route) | Results | References |
|---|---|---|---|---|---|
| Epileptic seizures | DOR KO mice / | Ethological observations | s.c. (10–100 mg/kg) | DOR agonist-mediated seizures abolished | Broom et al., 2002 |
| | Mice/SNC80 | Ethological observations | s.c. (10–100 mg/kg) | Seizures↑ | Broom et al., 2002 |
| | Mice/BW373U86 | Ethological observations | s.c. (1–32 mg/kg) | Seizures↑ | Broom et al., 2002 |
| | Rats/SNC80 | Ethological observations/EEG recording | s.c. or i.v. (1–100 mg/kg) | Seizures↑ | Jutkiewicz et al., 2005b, 2006a |
| | Rats/NTI | Ethological observations of SNC80-induced convulsions | s.c. (0.1–10 mg/kg) | Seizures↓ | Jutkiewicz et al., 2005b |
| | Mice/KNT-127 | Ethological observations | s.c. (30 or 100 mg/kg) | No seizures | Saitoh et al., 2011 |
| | Mice/RB101 | Ethological observations/EEG recording | i.v. (32 mg/kg) | No seizures | Jutkiewicz et al., 2006b |
| | Rats/ADL5859 | EEG recording | i.v. (10 or 30 mg/kg) | No seizures | Le Bourdonnec et al., 2008 |
| | Rats/ADL5747 | EEG recording | i.v. (10 or 30 mg/kg) | No seizures | Le Bourdonnec et al., 2009 |
| Motor control | Mice/SNC80 | Spontaneous locomotor activity | s.c. (1, 5 or 10 mg/kg) | ↑ | Nozaki et al., 2012; Saitoh et al., 2011 |
| | Rats/SNC80 | Spontaneous locomotor activity | s.c. (3.2, 10 or 32 mg/kg) | ↑ | Jutkiewicz et al., 2005a |
| | Rats/RB101 | Spontaneous locomotor activity | i.v. (32 mg/kg) | ↑ | Jutkiewicz et al., 2006b |
| | Rats/$DV^{2}_{L}DA^{5}_{L}$LanEnk | Ethological observations | i.t. (0.1–30 μg) i.p. (0.1, 1 or 3 mg/kg) | ↔ | Svensson et al., 2003 |
| | Mice/KNT-127 | Spontaneous locomotor activity | s.c. (1 or 10 mg/kg) | ↔ | Saitoh et al., 2011 |
| | Mice/ADL5747 and ADL 5859 | Spontaneous locomotor activity | p.o. (10–300 mg/kg) | ↔ | Nozaki et al., 2012 |
| | Rats/ADL5859 | Spontaneous locomotor activity | p.o. (up to 1000 mg/kg) | ↔ | Le Bourdonnec et al., 2008 |
| | Rats/ADL5747 | Spontaneous locomotor activity | p.o. (30, 100 or 300 mg/kg) | ↔ | Le Bourdonnec et al., 2009 |
| Parkinson's disease | Rats/UFP-512 | Hemiparkinsonian 6-OHDA-induced unilateral lesions/drag test-rotarod | i.p. (0.1–1000 μg/kg) | Low dose UFP-512 Motor coordination ↑ High dose UFP-512 Motor coordination ↓ | Mabrouk et al., 2009 |
| | Rats/DPDPE | Hemiparkinsonian 6-OHDA-induced unilateral lesions/ethological observation | i.c.v. (10 μg/5 μl/rat) | Abnormal movements ↑ | Billet et al., 2012 |
| | Rats/NTI | Hemiparkinsonian 6-OHDA-induced unilateral lesions/ethological observation | i.c.v. (10 μg/5 μl/rat) | Abnormal movements ↓ | Billet et al., 2012 |

2008), UFP-512 (Vergura et al., 2008) and ARM390 (Pradhan et al., 2010) decreased anxiety-related behaviors in most classical experimental paradigms (Table 1).

Regarding depressive states, and as predicted from knockout mice data, most currently existing DOR agonists (Pradhan et al., 2011) consistently decreased despair-like behaviors in a large number of tests (summarized in Table 2) in both mice (Saitoh et al., 2004; Naidu et al., 2007; Vergura et al., 2008) and rats (Jutkiewicz et al., 2005a, 2005b; Torregrossa et al., 2006; Le Bourdonnec et al., 2008). Although no depression-related phenotype could be detected in animals lacking preproenkephalin (Bilkei-Gorzo et al., 2007), systemic administration of enkephalinase inhibitors had an antidepressant effect (Jutkiewicz et al., 2006b; Javelot et al., 2010). These studies suggest that the DOR/enkephalinergic system plays an important role in the control of depressive-like behaviors.

The circuitry of emotional processing has been extensively studied (LeDoux, 2000; Price & Drevets, 2012). Sensory information reaches cortical regions mostly through the thalamus and is integrated in limbic structures such as prefrontal cortex, hippocampus and amygdala. These brain areas, which attribute emotional value to internal and external stimuli show high DOR densities (Fig. 1). Stereotaxic microinjection of several DOR agonists in the hippocampus (Solati et al., 2010), amygdala (Narita et al., 2006a; Randall-Thompson et al., 2010) and cingulate cortex (Narita et al., 2006b) reduced anxiety, and conversely, NTI administration at these brain sites increased levels of anxiety (Table 1). These data together suggest that DOR acting at the level of amygdala–cortico-hippocampal circuitry regulates emotional responses. Gene conditional approaches may be instrumental in the future to elucidate neural processes underlying DOR-controlled emotional responses at the cellular level.

## 3. Delta opioid receptor, reward and addiction

Drugs of abuse activate brain reward systems, and initially produce pleasurable effects. Repeated drug exposure may lead to loss of control over drug intake, and drug dependence. A well-accepted view describes drug abuse as a three-stage vicious circle involving intoxication/withdrawal/craving episodes (Koob & Volkow, 2010). Animal studies have demonstrated the development of altered reward processes and enhanced stress responses (Koob & Le Moal, 2008), the

**Table 5**
Clinical trials targeting the delta opioid receptor.

| Sponsor | Drug | Condition | Clinical phase | References (ID) |
|---|---|---|---|---|
| AstraZeneca | AZD2327 | Anxious major depressive disorder | 2 | NCT00759395 |
| Cubist Pharmaceuticals | ADL5859 | Acute pain | 2 | NCT00993863 |
| Cubist Pharmaceuticals; Pfizer | ADL5859 | Osteoarthritis of the knee | 2 | NCT00979953 |
| | ADL5747 | Osteoarthritis of the knee | 2 | NCT00979953 |
| Cubist Pharmaceuticals; Pfizer | ADL5747 | Postherpetic neuralgia | 2 | NCT01058642 |
| Cubist Pharmaceuticals | ADL5945 | Opioid-induced constipation | 2 | NCT01207427 |
| Diamyd Inc. | NP2 | Intractable pain | 2 | NCT01291901 |
| Penn State University | NP2 | Hepatocellular cancer | 1 | NCT00706576 |
| | | Head and neck squamous cell carcinoma | 2 | NCT00905099 |

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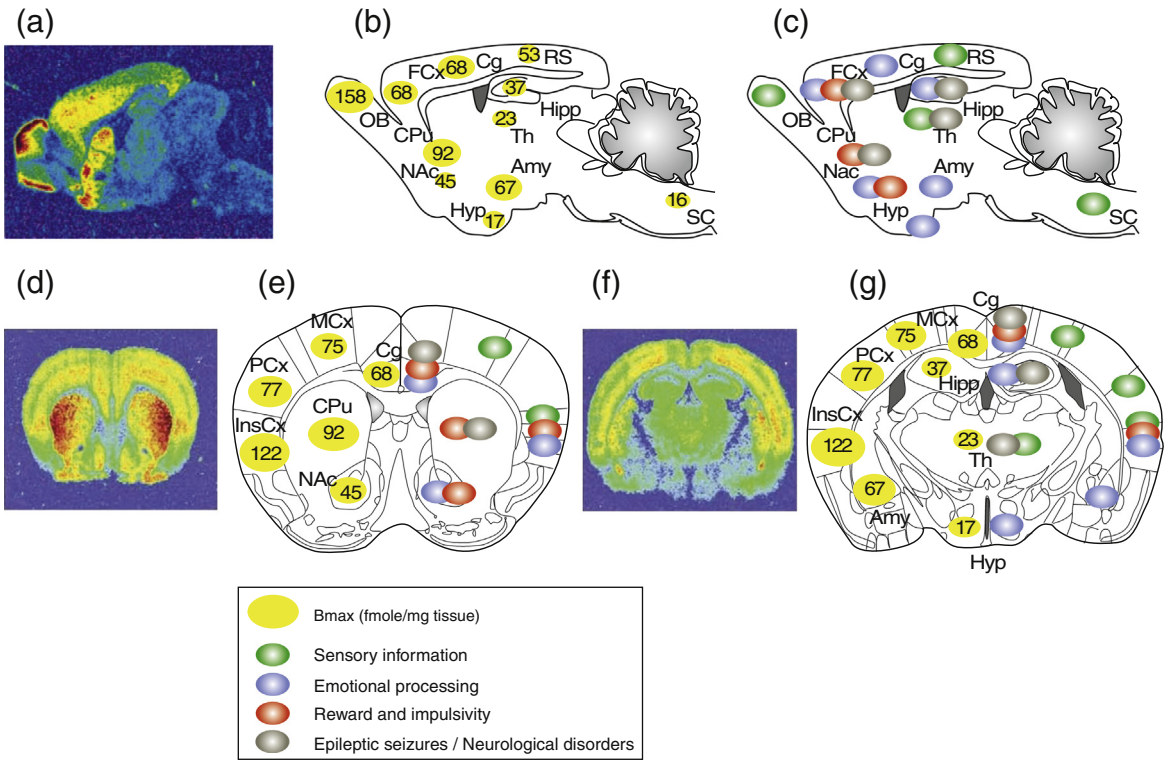


**Fig. 1.** Anatomical distribution of delta opioid receptors and relevant brain functions. Top panels, sagittal sections; bottom panels, coronal sections at 2 different anterio-posterior positions ((**e**) bregma 0.98 mm; (**g**) bregma −1.46 mm). (**a**, **d** and **f**) ([$^3$H]deltorphin ligand autoradiography reveals delta opioid receptor binding sites (courtesy of Ian Kitchen)). (**b**, **e** left part and **g** left part) Quantification of DOR expression levels in fmole/mg of tissue (means from Kitchen et al., 1997; Simonin et al., 1998; Slowe et al., 1999; Goody et al., 2002). DORs are particularly abundant in the OB, cortical regions (FCx, Cg, MCx, PCx and InsCx), amygdala and striatum (CPu and NAc). DORs are also expressed at moderate levels in the Hipp, RS, and at much lower levels in Hyp, Th and SC. (**c**, **e** right part and **g** right part). Schematic representation of potential neural sites for DOR function. DORs are expressed in sensory regions (green circles), brain areas important for the regulation of anxiety and depression (blue circles adapted from File et al., 2000; LeDoux, 2000; Cardinal et al., 2002; Everitt et al., 2003; Paulus & Stein, 2006; Rodrigues et al., 2009; Etkin et al., 2011; Gross & Canteras, 2012; Steenland et al., 2012), brain sites for reward processing and inhibitory controls (red circles adapted from Robbins & Everitt, 1996; Balleine & Dickinson, 1998; Kesner & Gilbert, 2007; Paton & Louie, 2012; Richard et al., in press) and areas relevant to epileptic seizures (gray circles adapted from Andre et al., 1998; Brevard et al., 2006). Abbreviations: Amy, amygdala; Cg, cingulate cortex; CPu, caudate putamen; FCx, frontal cortex; Hipp, hippocampus; Hyp, hypothalamus; InsCx, insular cortex; MCx, motor cortex; NAc, nucleus accumbens; OB, olfactory bulb; PCx, parietal cortex; RS, retrosplenial; SC, spinal cord; Th, thalamus.

setting of aberrant learning mechanisms (Belin et al., 2009) and habitual behaviors (Everitt et al., 2008), the disruption of self-control (Baler & Volkow, 2006) and the engagement of cue-induced relapse mechanisms (Pickens et al., 2011), which all contribute to maintaining drug use. All three opioid receptors are largely expressed in reward and associated neural circuits (Le Merrer et al., 2009; Koob & Volkow, 2010), which adapt to chronic drug exposure, and are involved in both recreational drug use (reward) and the many aspects of addictive behaviors.

Animal and human studies have clearly established that mu opioid receptors are essential to mediate rewarding properties of both natural stimuli and drugs of abuse, and that kappa receptors mediate dysphoria, particularly under stressful conditions (Lutz & Kieffer, in press). The implication of DOR in drug reward is more complex and differs across drugs of abuse. Data from conditioned place preference (CPP) and self-administration (SA) experiments for four distinct classes of drugs of abuse are compiled in Table 3. Beyond drug reward, delta receptors also contribute to the development of adaptations upon chronic drug exposure, mainly examined for morphine.

### 3.1. Morphine

DOR knockout mice showed decreased morphine-induced CPP in two studies (Chefer & Shippenberg, 2009; Le Merrer et al., 2011). However this effect was independent from rewarding properties of the drug, since mutant mice also exhibited decreased conditioned place aversion to lithium, as well as normal motivation to obtain morphine in a SA paradigm (David et al., 2008; Le Merrer et al., 2011). The association of stimuli that predict morphine administration was able to restore full expression of morphine CPP in these KO animals (Le Merrer et al., 2012). This set of experiments strongly suggests that DOR does not mediate morphine reward per se, but rather modulates learning processes in a place conditioning setting. Pharmacological studies using CPP experiments in rodents also support a role for DOR involvement in place conditioning paradigms (Suzuki et al., 1996; Shippenberg et al., 2009; Billa et al., 2010). A potential implication from all these data is that DOR may facilitate opiate-context association, which may be critical clinically in situations of context-induced relapse. A recent study, combining gene knockout and pharmacology, suggests that DOR is required to assign hedonic value to a reward-associated stimulus, a process that might influence motivation to get a reward (Laurent et al., 2012). The latter study, involving sucrose reward provides another indication for DOR-mediated associative processes.

Regarding chronic morphine effects, DOR knockout mice showed enhanced sensitization to locomotor effects of morphine (Chefer & Shippenberg, 2009), and pharmacological blockade of DOR by NTI (Chefer & Shippenberg, 2009) or naltriben (Billa et al., 2010) increased morphine-induced locomotor sensitization. Notably, morphine acts at mu opioid receptors in vivo (Contet et al., 2004) and does not directly activate DORs, as suggested by intact morphine analgesia (Zhu et al., 1999; Scherrer et al., 2009) and reward (Table 3) in DOR knockout mice. Therefore the exact nature of delta-mu opioid receptor

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interactions in vivo and mechanisms underlying DOR-regulated chronic morphine effects remain to be clarified.

### 3.2. Ethanol

Pharmacological blockade of DOR systemically by NTI, naltriben or SORI-9409 decreased voluntary ethanol consumption (Nielsen et al., 2008; van Rijn & Whistler, 2009) and also cue-mediated drug seeking (Marinelli et al., 2009). Those studies suggested that DOR is likely involved in both rewarding properties of alcohol and learning processes responsible for the context-drug consumption association. Local administration of DOR antagonists into the ventral tegmental area (VTA) (Margolis et al., 2008), the dorsal striatum (Nielsen et al., 2012) or the central nucleus of the amygdala (Bie et al., 2009) also disrupted ethanol self-administration or ethanol-induced CPP. In accordance, systemic or local administration (dorsal striatum and paraventricular nucleus of the hypothalamus) of DOR agonists stimulated ethanol SA (Barson et al., 2010; van Rijn et al., 2010a; Nielsen et al., 2012). Therefore, pharmacology approaches concur to indicate that DOR activation at several brain sites, and overall, facilitates ethanol drinking in rodents.

Paradoxically, DOR knockout mice showed increased ethanol consumption in a two bottle choice test (SA paradigm) (Roberts et al., 2001). Because these mutant mice exhibit high levels of anxiety (Filliol et al., 2000), and ethanol SA reduced their innate high anxiety levels (Roberts et al., 2001), high voluntary ethanol intake in mutant mice may reflect a self-medication approach. No alcohol phenotype could be detected in animals lacking the *Penk* gene in two-bottle-choice and ethanol-induced conditioned place preference paradigms (Racz et al., 2008).

### 3.3. Psychostimulants

DOR knockout mice showed decreased nicotine-induced CPP and SA (Berrendero et al., 2012). Systemic DOR blockade by NTI produced a similar effect in rats and mice (Ismayilova & Shoaib, 2010; Berrendero et al., 2012), and also abolished amphetamine-induced CPP (Belkai et al., 2009). Endogenous DOR activity therefore seems to contribute to reinforcing properties of these two drugs, as for alcohol. NTI infused locally in the nucleus accumbens, VTA and amygdala had contrasting effects on cocaine SA (Ward & Roberts, 2007; Simmons & Self, 2009), suggesting differing roles of DORs at distinct brain sites of reward processing (Fig. 1). Finally, a recent SNP study showed association between an *Oprd1* variant and cocaine addiction in the African American population (Crist et al., 2013), providing support for a role of DOR in psychostimulant dependence in humans.

In sum, both genetic and pharmacologic approaches suggest a regulatory role for DOR in drug intake, seeking and dependence, which vary depending on the drug and testing paradigm. DOR activity seems to facilitate alcohol and psychostimulant reward, but does not contribute to rewarding properties of morphine. Examination of reinforcing effects of cannabinoids showed no difference between DOR knockout and their control mice (Ghozland et al., 2002), and a contribution of DOR to cannabinoid reward has not been established. DORs are also involved in other aspects contributing to the development of drug abuse, including context learning and the development of tolerance (morphine), or the regulation of emotional responses (alcohol). The latter aspects may be critical in the development of therapeutic strategies. Indeed, targeting aspects of DOR function other than reward, which contribute to maintaining drug dependence, to the negative mood of protracted abstinence or to context-induced relapse, might be of particular interest. Finally, DOR was shown to regulate inhibitory controls in mice (Olmstead et al., 2009) and rats (Befort et al., 2011), revealing yet another facet of DOR function in cognitive processes with potential implication in substance abuse disorders.

## 4. Delta opioid receptor and epileptic seizures

Early studies showed that the first developed non-peptidic DOR agonists, BW373U86 and SNC80 exhibit convulsive properties (Broom et al., 2002; Jutkiewicz et al., 2005b) and data are overviewed in Table 4. Convulsions induced by the agonists SNC80 are abolished both in DOR knockout mice and after pharmacological blockade of DOR with NTI (Jutkiewicz et al., 2005b). Notably, electroencephalographic and behavioral changes elicited by acute SNC80 administration remain brief and non-lethal as compared to those obtained with the reference seizurogenic GABA antagonist pentylenetetrazole (Jutkiewicz et al., 2006a). Mechanisms underlying DOR-mediated convulsions remain poorly understood, but likely relate to the neural circuitry involved in absence epilepsy (Jutkiewicz et al., 2006a).

SNC80-induced convulsions, but not anti-depressant effects, were greatly diminished when slowing the rate of administration (Jutkiewicz et al., 2005b), indicating a possible dissociation between proconvulsant and antidepressant activities of SNC80. Importantly also, recently developed delta agonists showed no detectable convulsing effects. ADL5859 in both rats and mice at doses up to 1000 mg/kg (p. o.) induced no seizures and no EEG disturbances (Le Bourdonnec et al., 2008), and a similar result was found for ADL5747 (Le Bourdonnec et al., 2009). Therefore the pro-epileptic activity of DOR seems agonist-dependent and opens the way to developing therapeutic compounds with a better benefit/risk profile. Whether this is a pharmacokinetics issue or another indication of biased-agonism at DOR in vivo (Pradhan et al., 2011) remains to be determined.

## 5. Delta opioid receptor and motor control

The DOR receptor is strongly expressed in the striatum (Fig. 1) and the agonist SNC80 shows locomotor-stimulating properties (Fraser et al., 2000; Jutkiewicz et al., 2005a; Saitoh et al., 2011; Nozaki et al., 2012). On the other hand, DOR knockout mice showed hyperactivity in actimetry boxes (Filliol et al., 2000), and deficient striatal-dependent responses in a cross-maze assessing the hippocampal/striatal balance (Le Merrer et al., in press). These data suggest a significant but complex implication of DOR in the regulation of motor activity and this facet of DOR function is of potential interest in diseases involving impaired motor control such as Parkinson 's disease (PD). Indeed, DOR activation by the agonist UFP-512 at low dose increased locomotor coordination in a hemiparkinsonian rat model (Mabrouk et al., 2009), and had opposing effects at a high dose (Mabrouk et al., 2009). The antagonist NTI diminished abnormal movements classically described in the 6-OHDA model (Billet et al., 2012). More studies are necessary to understand DOR-mediated mechanisms regulating direct and indirect striatal output pathways.

Notably, recently developed DOR agonists do not show locomotor-activating properties (Svensson et al., 2003; Le Bourdonnec et al., 2008, 2009; Saitoh et al., 2011; Nozaki et al., 2012). Therefore, as for epileptic seizures, DOR-mediated locomotor effects appear agonist-dependent. Further investigations are required to define whether DOR agonist-mediated epileptic seizures and locomotor activity may share common neural circuitry and signaling pathway mechanisms.

## 6. Delta opioid receptor in hypoxia/ischemia

Hypoxic/ischemic conditions are characterized by reduced oxygen availability and trigger broad physiological alterations leading to cell death. The neuroprotective function of DOR activation has emerged recently, and offers interesting clinical perspectives for hypoxic/ischemic stress (Chao & Xia, 2010; Johnson & Turner, 2010).

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Beneficial effects of DOR activity deduced from in vivo models of hypoxia and ischemia are summarized in Table 4. Pharmacological studies showed that DOR activation by DADLE, a specific agonist, significantly increased neuronal survival in a model of asphyxia cardiac arrest, and that NTI opposed neuroprotective effects of hypoxic preconditioning in this model (Gao et al., 2010, 2012). DADLE also showed significant protective effects on astrocyte death in the hippocampus in another model of global ischemia (Duan et al., 2011). Studies in cell cultures suggested a critical role in ionic homeostasis in DOR-mediated neuroprotection (Chao et al., 2008, 2009). In a mitochondrial respiratory chain injury model, DOR activation protected neurons by decreasing pro-apoptotic factor expression levels like cytochrome c and caspase-3 (Zhu et al., 2009, 2011). Altogether, these data strongly support a role for DOR to maintain cellular metabolic homeostasis and counteract detrimental effects of hypoxic/ischemic injury.

DOR may also minimize consequences of hypoxia on autonomic neural responses. In models of panic attack, CO2 exposure produces acute dyspnea. This response is alleviated by diazepam in wild-type but not DOR knockout mice, suggesting a role for DOR in diazepam-regulated respiratory responses (Borkowski et al., 2011). Also, low oxygen-evoked decrease in body temperature returned to normal levels more slowly upon DOR blockade by NTI (Scarpellini Cda et al., 2009). Altogether these data indicate that DOR agonists may be beneficial under ischemic conditions via multiple, direct and indirect, mechanisms.

## 7. Clinical perspectives

The pain-reducing (Gaveriaux-Ruff & Kieffer, 2011) and mood-enhancing (Tables 1 and 2) properties of delta opioid agonists in animal models have attracted lots of interest, and efforts are being developed to bring delta drugs to the clinic (Table 5). Several agonists are being tested for pain, including a number of indications in chronic pain patients. The AstraZeneca compound ADZ2327 went successfully through Phase II trials in patients with anxiety-associated major depressive disorder (NCT00759395) (Hudzik et al., 2011). Clinical trials with delta agonists are only at their beginning. Potential convulsant effects need to be carefully controlled, and whether delta agonists could be useful for neuroprotection or to treat Parkinson's disease will require additional validation from animal research.

With regard to drug design, the notion that DOR may heterodimerize with MOR, KOR, or another GPCR in vivo has fostered the development of dimer-specific drugs endowed with pharmacological properties distinct from agonists acting at DOR homomers (Panetta & Greenwood, 2008; van Rijn et al., 2010b; Costantino et al., 2012; Kleczkowska et al., in press). Also, the recent demonstration of biased agonism at DOR in vivo may have clinical implications. The “biased agonism” concept (Galandrin et al., 2007; Kenakin, 2011), also referred to as functional selectivity, stems from the observation that distinct agonists acting at the same GPCR can engage different active receptor conformations and/or complexes with other GPCRs or intracellular effectors, leading to agonist-specific signaling responses. Opioid receptors were among the first GPCRs for which agonist-biased responses in vivo were demonstrated (Pradhan et al., 2012). The observation that delta opioid receptor agonists causing high (SNC80) or low (ARM00390) receptor internalization lead to distinct forms of tolerance (Pradhan et al., 2010) opens novel avenues towards drug design for therapeutic effects with limited side effects.

## 8. Concluding remarks

Delta opioid receptors and opioid peptides are broadly expressed across the brain. Our understanding of DOR function has tremendously progressed from in vivo studies using pharmacological tools and genetic approaches. Beneficial effects of DOR agonists are of a particular interest in the case of emotional responses and mood disorders. DOR regulates drug reward, and also plays a significant role in inhibitory controls and learning processes whose dysfunction contributes to the development of addiction. Whether delta compounds will represent useful drugs in addiction treatment remains open. DOR control over epileptic seizure mechanisms deserves further studies to enable the development of delta drugs with limited side effects. The neuroprotective role of DOR represents an emerging research field, with potential new opportunities for delta opioid drugs in the clinic. In the future, the development of improved delta drugs will also benefit from a better understanding of DOR function at distinct brain sites within neural circuits of emotion and cognition.

## Conflict of interest

The authors declare that there are no conflicts of interest.

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## References

Ambrose-Lanci, L. M., Sterling, R. C., & Van Bockstaele, E. J. (2008). Cocaine withdrawal-induced anxiety in females: impact of circulating estrogen and potential use of delta-opioid receptor agonists for treatment. *J Neurosci Res 88*, 816–824.

Andre, V., Pineau, N., Motte, J. E., Marescaux, C., & Nehlig, A. (1998). Mapping of neuronal networks underlying generalized seizures induced by increasing doses of pentylenetetrazol in the immature and adult rat: a c-Fos immunohistochemical study. *Eur J Neurosci 10*, 2094–2106.

Baler, R. D., & Volkow, N. D. (2006). Drug addiction: the neurobiology of disrupted self-control. *Trends Mol Med 12*, 559–566.

Balleine, B. W., & Dickinson, A. (1998). Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. *Neuropharmacology 37*, 407–419.

Barson, J. R., Carr, A. J., Soun, J. E., Sobhani, N. C., Rada, P., Leibowitz, S. F., et al. (2010). Opioids in the hypothalamic paraventricular nucleus stimulate ethanol intake. *Alcohol Clin Exp Res 34*, 214–222.

Befort, K., Mahoney, M. K., Chow, C., Hayton, S. J., Kieffer, B. L., & Olmstead, M. C. (2011). Effects of delta opioid receptors activation on a response inhibition task in rats. *Psychopharmacology (Berl) 214*, 967–976.

Belin, D., Jonkman, S., Dickinson, A., Robbins, T. W., & Everitt, B. J. (2009). Parallel and interactive learning processes within the basal ganglia: relevance for the understanding of addiction. *Behav Brain Res 199*, 89–102.

Belkai, E., Scherrmann, J. M., Noble, F., & Marie-Claire, C. (2009). Modulation of MDMA-induced behavioral and transcriptional effects by the delta opioid antagonist naltrindole in mice. *Addict Biol 14*, 245–252.

Berrendero, F., Plaza-Zabala, A., Galeote, L., Flores, A., Bura, S. A., Kieffer, B. L., et al. (2012). Influence of delta-opioid receptors in the behavioral effects of nicotine. *Neuropsychopharmacology 37*, 2332–2344.

Bie, B., Zhu, W., & Pan, Z. Z. (2009). Ethanol-induced delta-opioid receptor modulation of glutamate synaptic transmission and conditioned place preference in central amygdala. *Neuroscience 160*, 348–358.

Bilkei-Gorzo, A., Michel, K., Noble, F., Roques, B. P., & Zimmer, A. (2007). Preproenkephalin knockout mice show no depression-related phenotype. *Neuropsychopharmacology 32*, 2330–2337.

Billa, S. K., Xia, Y., & Moron, J. A. (2010). Disruption of morphine-conditioned place preference by a delta2-opioid receptor antagonist: study of mu-opioid and delta-opioid receptor expression at the synapse. *Eur J Neurosci 32*, 625–631.

Billet, F., Costentin, J., & Dourmap, N. (2012). Influence of corticostriatal delta-opioid receptors on abnormal involuntary movements induced by L-DOPA in hemiparkinsonian rats. *Exp Neurol 236*, 339–350.

Borkowski, A. H., Barnes, D. C., Blanchette, D. R., Castellanos, F. X., Klein, D. F., & Wilson, D. A. (2011). Interaction between delta opioid receptors and benzodiazepines in CO(2)-induced respiratory responses in mice. *Brain Res 1396*, 54–59.

Brevard, M. E., Kulkarni, P., King, J. A., & Ferris, C. F. (2006). Imaging the neural substrates involved in the genesis of pentylenetetrazol-induced seizures. *Epilepsia 47*, 745–754.

Broom, D. C., Nitsche, J. F., Pintar, J. E., Rice, K. C., Woods, J. H., & Traynor, J. R. (2002). Comparison of receptor mechanisms and efficacy requirements for delta-agonist-induced convulsive activity and antinociception in mice. *J Pharmacol Exp Ther 303*, 723–729.

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Cardinal, R. N., Parkinson, J. A., Hall, J., & Everitt, B. J. (2002). Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neurosci Biobehav Rev 26*, 321–352.

Chao, D., & Xia, Y. (2010). Ionic storm in hypoxic/ischemic stress: can opioid receptors subside it? *Prog Neurobiol 90*, 439–470.

Chao, D., Bazzy-Asaad, A., Balboni, G., Salvadori, S., & Xia, Y. (2008). Activation of DOR attenuates anoxic K+ derangement via inhibition of Na+ entry in mouse cortex. *Cereb Cortex 18*, 2217–2227.

Chao, D., Balboni, G., Lazarus, L. H., Salvadori, S., & Xia, Y. (2009). Na+ mechanism of delta-opioid receptor induced protection from anoxic K+ leakage in the cortex. *Cell Mol Life Sci 66*, 1105–1115.

Chavkin, C. (2011). The therapeutic potential of kappa-opioids for treatment of pain and addiction. *Neuropsychopharmacology 36*, 369–370.

Chefer, V. I., & Shippenberg, T. S. (2009). Augmentation of morphine-induced sensitization but reduction in morphine tolerance and reward in delta-opioid receptor knockout mice. *Neuropsychopharmacology 34*, 887–898.

Contet, C., Kieffer, B. L., & Befort, K. (2004). Mu opioid receptor: a gateway to drug addiction. *Curr Opin Neurobiol 14*, 370–378.

Costantino, C. M., Gomes, I., Stockton, S. D., Lim, M. P., & Devi, L. A. (2012). Opioid receptor heteromers in analgesia. *Expert Rev Mol Med 14*, e9.

Crist, R. C., Ambrose-Lanci, L. M., Vaswani, M., Clarke, T. K., Zeng, A., Yuan, C., et al. (2013). Case–control association analysis of polymorphisms in the delta-opioid receptor, OPRD1, with cocaine and opioid addicted populations. *Drug Alcohol Depend 127*, 122–128.

David, V., Matifas, A., Gavello-Baudy, S., Decorte, L., Kieffer, B. L., & Cazala, P. (2008). Brain regional Fos expression elicited by the activation of mu- but not delta-opioid receptors of the ventral tegmental area: evidence for an implication of the ventral thalamus in opiate reward. *Neuropsychopharmacology 33*, 1746–1759.

Duan, Y. L., Wang, S. Y., Zeng, Q. W., Su, D. S., Li, W., Wang, X. R., et al. (2011). Astroglial reaction to delta opioid peptide [D-Ala2, D-Leu5] enkephalin confers neuroprotection against global ischemia in the adult rat hippocampus. *Neuroscience 192*, 81–90.

Etkin, A., Egner, T., & Kalisch, R. (2011). Emotional processing in anterior cingulate and medial prefrontal cortex. *Trends Cogn Sci 15*, 85–93.

Everitt, B. J., Cardinal, R. N., Parkinson, J. A., & Robbins, T. W. (2003). Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning. *Ann N Y Acad Sci 985*, 233–250.

Everitt, B. J., Belin, D., Economidou, D., Pelloux, Y., Dalley, J. W., & Robbins, T. W. (2008). Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. *Philos Trans R Soc Lond B Biol Sci 363*, 3125–3135.

File, S. E., Kenny, P. J., & Cheeta, S. (2000). The role of the dorsal hippocampal serotonergic and cholinergic systems in the modulation of anxiety. *Pharmacol Biochem Behav 66*, 65–72.

Filliol, D., Ghozland, S., Chluba, J., Martin, M., Matthes, H. W., Simonin, F., et al. (2000). Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses. *Nat Genet 25*, 195–200.

Fraser, G. L., Parenteau, H., Tu, T. M., Ducharme, J., Perkins, M. N., & Clarke, P. B. (2000). The effects of delta agonists on locomotor activity in habituated and non-habituated rats. *Life Sci 67*, 913–922.

Galandrin, S., Oligny-Longpre, G., & Bouvier, M. (2007). The evasive nature of drug efficacy: implications for drug discovery. *Trends Pharmacol Sci 28*, 423–430.

Gao, C. J., Li, J. P., Wang, W., Lu, B. C., Niu, L., Zhu, C., et al. (2010). Effects of intracerebroventricular application of the delta opioid receptor agonist [D-Ala2, D-Leu5] enkephalin on neurological recovery following asphyxial cardiac arrest in rats. *Neuroscience 168*, 531–542.

Gao, C. J., Niu, L., Ren, P. C., Wang, W., Zhu, C., Li, Y. Q., et al. (2012). Hypoxic preconditioning attenuates global cerebral ischemic injury following asphyxial cardiac arrest through regulation of delta opioid receptor system. *Neuroscience 202*, 352–362.

Gaveriaux-Ruff, C. (2013). Opiate-induced analgesia: contributions from mu, delta and kappa opioid receptors mouse mutants. *Curr Pharm Des* (in press).

Gaveriaux-Ruff, C., & Kieffer, B. L. (2011). Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches. *Behav Pharmacol 22*, 405–414.

Gaveriaux-Ruff, C., Nozaki, C., Nadal, X., Hever, X. C., Weibel, R., Matifas, A., et al. (2011). Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia. *Pain 152*, 1238–1248.

Ghozland, S., Matthes, H. W., Simonin, F., Filliol, D., Kieffer, B. L., & Maldonado, R. (2002). Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors. *J Neurosci 22*, 1146–1154.

Gianoulakis, C. (2009). Endogenous opioids and addiction to alcohol and other drugs of abuse. *Curr Top Med Chem 9*, 999–1015.

Goody, R. J., Oakley, S. M., Filliol, D., Kieffer, B. L., & Kitchen, I. (2002). Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice. *Brain Res 945*, 9–19.

Granier, S., Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Weis, W. I., et al. (2012). Structure of the delta-opioid receptor bound to naltrindole. *Nature 485*, 400–404.

Gross, C. T., & Canteras, N. S. (2012). The many paths to fear. *Nat Rev Neurosci 13*, 651–658.

Hudzik, T. J., Maciag, C., Smith, M. A., Caccese, R., Pietras, M. R., Bui, K. H., et al. (2011). Preclinical pharmacology of AZD2327: a highly selective agonist of the delta-opioid receptor. *J Pharmacol Exp Ther 338*, 195–204.

Ismayilova, N., & Shoaib, M. (2010). Alteration of intravenous nicotine self-administration by opioid receptor agonist and antagonists in rats. *Psychopharmacology (Berl) 210*, 211–220.

Javelot, H., Messaoudi, M., Garnier, S., & Rougeot, C. (2010). Human opiorphin is a naturally occurring antidepressant acting selectively on enkephalin-dependent delta-opioid pathways. *J Physiol Pharmacol 61*, 355–362.

Johnson, S. M., & Turner, S. M. (2010). Protecting motor networks during perinatal ischemia: the case for delta-opioid receptors. *Ann N Y Acad Sci 1198*, 260–270.

Jutkiewicz, E. M., Kaminsky, S. T., Rice, K. C., Traynor, J. R., & Woods, J. H. (2005a). Differential behavioral tolerance to the delta-opioid agonist SNC80 ([(+)-4-[(alphaR)-alpha-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl ]-(3-methoxyphenyl)methyl]-N,N-diethylbenzamide) in Sprague–Dawley rats. *J Pharmacol Exp Ther 315*, 414–422.

Jutkiewicz, E. M., Rice, K. C., Traynor, J. R., & Woods, J. H. (2005b). Separation of the convulsions and antidepressant-like effects produced by the delta-opioid agonist SNC80 in rats. *Psychopharmacology (Berl) 182*, 588–596.

Jutkiewicz, E. M., Baladi, M. G., Folk, J. E., Rice, K. C., & Woods, J. H. (2006a). The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with pentylenetetrazol. *J Pharmacol Exp Ther 317*, 1337–1348.

Jutkiewicz, E. M., Torregrossa, M. M., Sobczyk-Kojiro, K., Mosberg, H. I., Folk, J. E., Rice, K. C., et al. (2006b). Behavioral and neurobiological effects of the enkephalinase inhibitor RB101 relative to its antidepressant effects. *Eur J Pharmacol 531*, 151–159.

Kenakin, T. (2011). Functional selectivity and biased receptor signaling. *J Pharmacol Exp Ther 336*, 296–302.

Kesner, R. P., & Gilbert, P. E. (2007). The role of the agranular insular cortex in anticipation of reward contrast. *Neurobiol Learn Mem 88*, 82–86.

Kitchen, I., Slowe, S. J., Matthes, H. W., & Kieffer, B. (1997). Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene. *Brain Res 778*, 73–88.

Kleczkowska, P., Lipkowski, A. W., Tourwe, D., & Ballet, S. (2013). Hybrid opioid/non-opioid ligands in pain research. *Curr Pharm Des* (in press).

Knoll, A. T., & Carlezon, W. A., Jr. (2010). Dynorphin, stress, and depression. *Brain Res 1314*, 56–73.

Konig, M., Zimmer, A. M., Steiner, H., Holmes, P. V., Crawley, J. N., Brownstein, M. J., et al. (1996). Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature 383*, 535–538.

Koob, G. F., & Le Moal, M. (2008). Review. Neurobiological mechanisms for opponent motivational processes in addiction. *Philos Trans R Soc Lond B Biol Sci 363*, 3113–3123.

Koob, G. F., & Volkow, N. D. (2010). Neurocircuitry of addiction. *Neuropsychopharmacology 35*, 217–238.

Laurent, V., Leung, B., Maidment, N., & Balleine, B. W. (2012). Mu- and delta-opioid-related processes in the accumbens core and shell differentially mediate the influence of reward-guided and stimulus-guided decisions on choice. *J Neurosci 32*, 1875–1883.

Le Bourdonnec, B., Windh, R. T., Ajello, C. W., Leister, L. K., Gu, M., Chu, G. H., et al. (2008). Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: discovery of N, N-diethyl-4-(5-hydroxyspiro[chromene-2,4′-piperidine]-4-yl)benzamide (ADL5859). *J Med Chem 51*, 5893–5896.

Le Bourdonnec, B., Windh, R. T., Leister, L. K., Zhou, Q. J., Ajello, C. W., Gu, M., et al. (2009). Spirocyclic delta opioid receptor agonists for the treatment of pain: discovery of N, N-diethyl-3-hydroxy-4-(spiro[chromene-2,4′-piperidine]-4-yl) benzamide (ADL5747). *J Med Chem 52*, 5685–5702.

Le Merrer, J., Becker, J. A., Befort, K., & Kieffer, B. L. (2009). Reward processing by the opioid system in the brain. *Physiol Rev 89*, 1379–1412.

Le Merrer, J., Plaza-Zabala, A., Del Boca, C., Matifas, A., Maldonado, R., & Kieffer, B. L. (2011). Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement. *Biol Psychiatry 69*, 700–703.

Le Merrer, J., Faget, L., Matifas, A., & Kieffer, B. L. (2012). Cues predicting drug or food reward restore morphine-induced place conditioning in mice lacking delta opioid receptors. *Psychopharmacology (Berl) 223*, 99–106.

Le Merrer, J., Rezai, X., Scherrer, G., Becker, J. A., & Kieffer, B. L. (2013). Impaired hippocampus-dependent and facilitated striatum-dependent behaviors in mice lacking the delta opioid receptor. *Neuropsychopharmacology* (in press).

LeDoux, J. E. (2000). Emotion circuits in the brain. *Annu Rev Neurosci 23*, 155–184.

Lutz, P. E., & Kieffer, B. L. (2013). Opioid receptors: distinct roles in mood disorders. *Trends Neurosci* (in press).

Mabrouk, O. S., Marti, M., Salvadori, S., & Morari, M. (2009). The novel delta opioid receptor agonist UFP-512 dually modulates motor activity in hemiparkinsonian rats via control of the nigro-thalamic pathway. *Neuroscience 164*, 360–369.

Manglik, A., Kruse, A. C., Kobilka, T. S., Thian, F. S., Mathiesen, J. M., Sunahara, R. K., et al. (2012). Crystal structure of the micro-opioid receptor bound to a morphinan antagonist. *Nature 485*, 321–326.

Margolis, E. B., Fields, H. L., Hjelmstad, G. O., & Mitchell, J. M. (2008). Delta-opioid receptor expression in the ventral tegmental area protects against elevated alcohol consumption. *J Neurosci 28*, 12672–12681.

Marinelli, P. W., Funk, D., Harding, S., Li, Z., Juzytsch, W., & Le, A. D. (2009). Roles of opioid receptor subtypes in mediating alcohol-seeking induced by discrete cues and context. *Eur J Neurosci 30*, 671–678.

Mas Nieto, M. M., Guen, S. L., Kieffer, B. L., Roques, B. P., & Noble, F. (2005). Physiological control of emotion-related behaviors by endogenous enkephalins involves essentially the delta opioid receptors. *Neuroscience 135*, 305–313.

Naidu, P. S., Lichtman, A. H., Archer, C. C., May, E. L., Harris, L. S., & Aceto, M. D. (2007). NIH 11082 produces anti-depressant-like activity in the mouse tail-suspension test through a delta-opioid receptor mechanism of action. *Eur J Pharmacol 566*, 132–136.

Narita, M., Kaneko, C., Miyoshi, K., Nagumo, Y., Kuzumaki, N., Nakajima, M., et al. (2006). Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala. *Neuropsychopharmacology 31*, 739–750.


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Narita, M., Kuzumaki, N., Kaneko, C., Hareyama, N., Miyatake, M., Shindo, K., et al. (2006). Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction. *J Neurochem 97*, 1369–1378.

Nielsen, C. K., Simms, J. A., Pierson, H. B., Li, R., Saini, S. K., Ananthan, S., et al. (2008). A novel delta opioid receptor antagonist, SoRI-9409, produces a selective and long-lasting decrease in ethanol consumption in heavy-drinking rats. *Biol Psychiatry 64*, 974–981.

Nielsen, C. K., Simms, J. A., Li, R., Mill, D., Yi, H., Feduccia, A. A., et al. (2012). Delta-opioid receptor function in the dorsal striatum plays a role in high levels of ethanol consumption in rats. *J Neurosci 32*, 4540–4552.

Nozaki, C., Le Bourdonnec, B., Reiss, D., Windh, R. T., Little, P. J., Dolle, R. E., et al. (2012). Delta-opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion, and receptor internalization. *J Pharmacol Exp Ther 342*, 799–807.

Olmstead, M. C., Ouagazzal, A. M., & Kieffer, B. L. (2009). Mu and delta opioid receptors oppositely regulate motor impulsivity in the signaled nose poke task. *PLoS One 4*, e4410.

Panetta, R., & Greenwood, M. T. (2008). Physiological relevance of GPCR oligomerization and its impact on drug discovery. *Drug Discov Today 13*, 1059–1066.

Paton, J. J., & Louie, K. (2012). Reward and punishment illuminated. *Nat Neurosci 15*, 807–809.

Paulus, M. P., & Stein, M. B. (2006). An insular view of anxiety. *Biol Psychiatry 60*, 383–387.

Peckys, D., & Landwehrmeyer, G. B. (1999). Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study. *Neuroscience 88*, 1093–1135.

Peng, J., Sarkar, S., & Chang, S. L. (2012). Opioid receptor expression in human brain and peripheral tissues using absolute quantitative real-time RT-PCR. *Drug Alcohol Depend 124*, 223–228.

Perrine, S. A., Hoshaw, B. A., & Unterwald, E. M. (2006). Delta opioid receptor ligands modulate anxiety-like behaviors in the rat. *Br J Pharmacol 147*, 864–872.

Pickens, C. L., Airavaara, M., Theberge, F., Fanous, S., Hope, B. T., & Shaham, Y. (2011). Neurobiology of the incubation of drug craving. *Trends Neurosci 34*, 411–420.

Pradhan, A. A., Walwyn, W., Nozaki, C., Filliol, D., Erbs, E., Matifas, A., et al. (2010). Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance. *J Neurosci 30*, 16459–16468.

Pradhan, A. A., Befort, K., Nozaki, C., Gaveriaux-Ruff, C., & Kieffer, B. L. (2011). The delta opioid receptor: an evolving target for the treatment of brain disorders. *Trends Pharmacol Sci 32*, 581–590.

Pradhan, A. A., Smith, M. L., Kieffer, B. L., & Evans, C. J. (2012). Ligand-directed signalling within the opioid receptor family. *Br J Pharmacol 167*, 960–969.

Price, J. L., & Drevets, W. C. (2012). Neural circuits underlying the pathophysiology of mood disorders. *Trends Cogn Sci 16*, 61–71.

Primeaux, S. D., Wilson, S. P., McDonald, A. J., Mascagni, F., & Wilson, M. A. (2006). The role of delta opioid receptors in the anxiolytic actions of benzodiazepines. *Pharmacol Biochem Behav 85*, 545–554.

Racz, I., Schurmann, B., Karpushova, A., Reuter, M., Cichon, S., Montag, C., et al. (2008). The opioid peptides enkephalin and beta-endorphin in alcohol dependence. *Biol Psychiatry 64*, 989–997.

Ragnauth, A., Schuller, A., Morgan, M., Chan, J., Ogawa, S., Pintar, J., et al. (2001). Female preproenkephalin-knockout mice display altered emotional responses. *Proc Natl Acad Sci U S A 98*, 1958–1963.

Randall-Thompson, J. F., Pescatore, K. A., & Unterwald, E. M. (2010). A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors. *Psychopharmacology (Berl) 212*, 585–595.

Richard, J. M., Castro, D. C., Difeliceantonio, A. G., Robinson, M. J., & Berridge, K. C. (2013). Mapping brain circuits of reward and motivation: in the footsteps of Ann Kelley. *Neurosci Biobehav Rev* (in press).

Robbins, T. W., & Everitt, B. J. (1996). Neurobehavioural mechanisms of reward and motivation. *Curr Opin Neurobiol 6*, 228–236.

Roberts, A. J., Gold, L. H., Polis, I., McDonald, J. S., Filliol, D., Kieffer, B. L., et al. (2001). Increased ethanol self-administration in delta-opioid receptor knockout mice. *Alcohol Clin Exp Res 25*, 1249–1256.

Rodrigues, S. M., LeDoux, J. E., & Sapolsky, R. M. (2009). The influence of stress hormones on fear circuitry. *Annu Rev Neurosci 32*, 289–313.

Saitoh, A., Kimura, Y., Suzuki, T., Kawai, K., Nagase, H., & Kamei, J. (2004). Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents. *J Pharmacol Sci 95*, 374–380.

Saitoh, A., Yoshikawa, Y., Onodera, K., & Kamei, J. (2005). Role of delta-opioid receptor subtypes in anxiety-related behaviors in the elevated plus-maze in rats. *Psychopharmacology (Berl) 182*, 327–334.

Saitoh, A., Sugiyama, A., Nemoto, T., Fujii, H., Wada, K., Oka, J., et al. (2011). The novel delta opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions. *Behav Brain Res 223*, 271–279.

Sauriyal, D. S., Jaggi, A. S., & Singh, N. (2011). Extending pharmacological spectrum of opioids beyond analgesia: multifunctional aspects in different pathophysiological states. *Neuropeptides 45*, 175–188.

Scarpellini Cda, S., Gargaglioni, L. H., Branco, L. G., & Bicego, K. C. (2009). Role of preoptic opioid receptors in the body temperature reduction during hypoxia. *Brain Res 1286*, 66–74.

Scherrer, G., Tryoen-Toth, P., Filliol, D., Matifas, A., Laustriat, D., Cao, Y. Q., et al. (2006). Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo. *Proc Natl Acad Sci U S A 103*, 9691–9696.

Scherrer, G., Imamachi, N., Cao, Y. Q., Contet, C., Mennicken, F., O'Donnell, D., et al. (2009). Dissociation of the opioid receptor mechanisms that control mechanical and heat pain. *Cell 137*, 1148–1159.

Shippenberg, T. S. (2009). The dynorphin/kappa opioid receptor system: a new target for the treatment of addiction and affective disorders? *Neuropsychopharmacology 34*, 247.

Shippenberg, T. S., LeFevour, A., & Chefer, V. I. (2008). Targeting endogenous mu- and delta-opioid receptor systems for the treatment of drug addiction. *CNS Neurol Disord Drug Targets 7*, 442–453.

Shippenberg, T. S., Chefer, V. I., & Thompson, A. C. (2009). Delta-opioid receptor antagonists prevent sensitization to the conditioned rewarding effects of morphine. *Biol Psychiatry 65*, 169–174.

Simmons, D., & Self, D. W. (2009). Role of mu- and delta-opioid receptors in the nucleus accumbens in cocaine-seeking behavior. *Neuropsychopharmacology 34*, 1946–1957.

Simonin, F., Befort, K., Gaveriaux-Ruff, C., Matthes, H., Nappey, V., Lannes, B., et al. (1994). The human delta-opioid receptor: genomic organization, cDNA cloning, functional expression, and distribution in human brain. *Mol Pharmacol 46*, 1015–1021.

Simonin, F., Valverde, O., Smadja, C., Slowe, S., Kitchen, I., Dierich, A., et al. (1998). Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal. *EMBO J 17*, 886–897.

Slowe, S. J., Simonin, F., Kieffer, B., & Kitchen, I. (1999). Quantitative autoradiography of mu-, delta- and kappa1 opioid receptors in kappa-opioid receptor knockout mice. *Brain Res 818*, 335–345.

Smith, J. S., Zubieta, J. K., Price, J. C., Flesher, J. E., Madar, I., Lever, J. R., et al. (1999). Quantification of delta-opioid receptors in human brain with N1′-([11C]methyl) naltrindole and positron emission tomography. *J Cereb Blood Flow Metab 19*, 956–966.

Solati, J., Zarrindast, M. R., & Salari, A. A. (2010). Dorsal hippocampal opioidergic system modulates anxiety-like behaviors in adult male Wistar rats. *Psychiatry Clin Neurosci 64*, 634–641.

Steenland, H. W., Li, X. Y., & Zhuo, M. (2012). Predicting aversive events and terminating fear in the mouse anterior cingulate cortex during trace fear conditioning. *J Neurosci 32*, 1082–1095.

Suzuki, T., Tsuji, M., Mori, T., Misawa, M., Endoh, T., & Nagase, H. (1996). Effect of the highly selective and nonpeptide delta opioid receptor agonist TAN-67 on the morphine-induced place preference in mice. *J Pharmacol Exp Ther 279*, 177–185.

Svensson, C. I., Rew, Y., Malkmus, S., Schiller, P. W., Taulane, J. P., Goodman, M., et al. (2003). Systemic and spinal analgesic activity of a delta-opioid-selective lanthionine enkephalin analog. *J Pharmacol Exp Ther 304*, 827–832.

Torregrossa, M. M., Jutkiewicz, E. M., Mosberg, H. I., Balboni, G., Watson, S. J., & Woods, J. H. (2006). Peptidic delta opioid receptor agonists produce antidepressant-like effects in the forced swim test and regulate BDNF mRNA expression in rats. *Brain Res 1069*, 172–181.

van Rijn, R. M., & Whistler, J. L. (2009). The delta(1) opioid receptor is a heterodimer that opposes the actions of the delta(2) receptor on alcohol intake. *Biol Psychiatry 66*, 777–784.

van Rijn, R. M., Brissett, D. I., & Whistler, J. L. (2010). Dual efficacy of delta opioid receptor selective ligands for ethanol drinking and anxiety. *J Pharmacol Exp Ther 335*, 133–139.

van Rijn, R. M., Whistler, J. L., & Waldhoer, M. (2010). Opioid-receptor-heteromer-specific trafficking and pharmacology. *Curr Opin Pharmacol 10*, 73–79.

Vergura, R., Balboni, G., Spagnolo, B., Gavioli, E., Lambert, D. G., McDonald, J., et al. (2008). Anxiolytic- and antidepressant-like activities of H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512), a novel selective delta opioid receptor agonist. *Peptides 29*, 93–103.

Ward, S. J., & Roberts, D. C. (2007). Microinjection of the delta-opioid receptor selective antagonist naltrindole 5′-isothiocyanate site specifically affects cocaine self-administration in rats responding under a progressive ratio schedule of reinforcement. *Behav Brain Res 182*, 140–144.

Wu, H., Wacker, D., Mileni, M., Katritch, V., Han, G. W., Vardy, E., et al. (2012). Structure of the human kappa-opioid receptor in complex with JDTic. *Nature 485*, 327–332.

Zhu, Y., King, M. A., Schuller, A. G., Nitsche, J. F., Reidl, M., Elde, R. P., et al. (1999). Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. *Neuron 24*, 243–252.

Zhu, M., Li, M. W., Tian, X. S., Ou, X. M., Zhu, C. Q., & Guo, J. C. (2009). Neuroprotective role of delta-opioid receptors against mitochondrial respiratory chain injury. *Brain Res 1252*, 183–191.

Zhu, M., Li, M., Yang, F., Ou, X., Ren, Q., Gao, H., et al. (2011). Mitochondrial ERK plays a key role in delta-opioid receptor neuroprotection against acute mitochondrial dysfunction. *Neurochem Int 59*, 739–748.

Zollner, C., & Stein, C. (2007). Opioids. *Handb Exp Pharmacol*, 31–63.


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# Aims of the thesis

The role of the opioid system and the neuroadaptations following exposure to drugs of abuse represent the main focus of investigation in our laboratory. While MOR is the most studied in the context of addiction, the contribution of DOR remains poorly understood.

DORs are largely expressed in the central nervous system more particularly in the anterior part of the brain also called the forebrain. DORs have been described more strongly expressed in the olfactory bulb, the striatum, cortical areas notably the prefrontal and the insular cortex, limbic regions such as the amygdala and the hippocampus, as well as in the lateral hypothalamus (Mansour, Fox et al. 1995; Le Merrer, Becker et al. 2009). Brain areas with high DOR expression are involved in several neural processes, whose dysfunction may lead to neurological or psychiatric disorders. The expression of DOR especially in the amygdala, striatum and cortical areas, suggests an implication for this receptor respectively in anxiety, depression, addictive and impulse disorders (Pradhan, Befort et al. 2011; Chu Sin Chung and Kieffer 2013). Our team has shown a role for DOR to reduce emotional responses (Filliol, Ghozland et al. 2000) and impulsive behaviors (Olmstead, Ouagazzal et al. 2009; Befort, Mahoney et al. 2011). Interestingly, a link has been suggested between DOR anxiolytic effect and ethanol consumption (Roberts, Gold et al. 2001). Moreover, recent studies suggest that DOR plays a critical role in drug-context associations in a pavlovian place conditioning paradigm (Faget, Erbs et al. 2012; Le Merrer, Faget et al. 2012) as well as in pavlovian instrumental transfer for food reward (Laurent, Leung et al. 2012). While the contribution of the opioid system to the hedonic value of reward has been attributed to the MORs, much evidence indicates that DORs are involved in the emotional and mnemonic processes via which they could participate to the development and maintenance of substance use disorders.

The present thesis work has been initiated in order to gather informations that would provide further understanding of the contribution of DOR in these physiological processes, in particular anxiety-related behaviors and epileptic seizures.

My project aims at the identification of neural areas or circuits underlying DOR functions. To this aim we used refined genetic approaches whereby the DOR gene is inactivated in targeted brain areas or neuronal populations, based on the Cre-Lox system. This can be achieved using either transgenic Cre mouse lines (to target selected neuronal types) or an AAV-Cre viral approach (to target selected brain areas). We have generated a conditional knockout mice line (Dlx-DOR) by breeding a floxed delta receptor gene mice, created in our laboratory (Gaveriaux-Ruff, Nozaki et al. 2011), with a Dlx-5/6-Cre line expressing the Cre recombinase specifically in forebrain GABAergic neurons (Monory, Massa et al. 2006).

The work was divided in three parts: (Aim 1) we studied the role of DOR expessed in GABAergic neurons of the forebrain with a specific focus on their contribution to the regulation of emotional responses; (Aim 2) using the same Dlx-DOR model, we assessed the proconvulsing activity of DORs as well as further physiological processes regulated by DOR activity; (Aim 3) using AAV technology and retrograde tracing experiment, we investigate DOR at the level of the basolateral amygdala.

***Aim 1**: DOR in forebrain GABAergic neurons and the regulation of emotions*. The objective was to better understand the role of DOR expressed on GABAergic neurons in the forebrain. DOR expression in GABA interneurons is long established in rodents, especially in the hippocampus (Svoboda, Adams et al. 1999; Stumm, Zhou et al. 2004; Erbs, Faget et al. 2012; Rezai, Faget et al. 2012). Our hypothesis was that DOR in forebrain GABAergic neurons could be involved in both motivational and emotional regulations. We first determined the pattern of DOR deletion in our Dlx-DOR mouse line at both mRNA and protein levels (in collaboration with Ian Kitchen, University of Surrey, Guildford, UK). We demonstrated that our gene targeting approach was successful and that most receptors are selectively deleted in forebrain areas.

Then, we performed behavioural characterization of the Dlx-DOR line in comparison with control floxed littermates (Ctrl). Animals were tested in several paradigms assessing anxiety (Light/Dark box LD, Elevated Plus-Maze EPM and Open Field OF) and depressive-like behaviours (Forced Swim FST and Tail Suspension test TST). The same mouse lines were assessed in a hyponeophagia

paradigm (the novelty suppressed feeding NSF). Furthermore, we investigated the contribution of DOR expressed in the olfactory bulb by exploring behavioral responses of Dlx-DOR mice toward neutral, appetitive or aversive olfactive stimuli. Finally, to address the neuronal activity underlying the anxiety related-behaviors observed in the NSF test, we measured the expression level of the c-fos early gene immunoreactivity as a maker of neuronal activity.

This work is presented in Part I in a manuscript under submission: "**A new anxiogenic function for the delta opioid receptor expressed in forebrain GABAergic neurons**", Chu Sin Chung P., Keyworth H.L., Befort K., Bailey A., Filliol D., Matifas A., Gaveriaux-Ruff C., Kitchen I. and Kieffer B.L..

***Aim 2****: DOR in forebrain GABAergic neurons and non-emotional functions.* In the second part of my thesis work, we investigated the involvement of DOR expressed by forebrain GABAergic neurons in DOR agonists-stimulated convulsions. The neuroanatomical and neurochemical characterization of DOR involved in this effect has never been precisely defined. The pro-epileptic seizure potential of DOR agonists has been previously assessed in rodents (Comer, Hoenicke et al. 1993; Broom, Nitsche et al. 2002; Jutkiewicz, Rice et al. 2005) and described as brief and mild convulsions which are similar to absence-like seizures (Broom, Nitsche et al. 2002; Jutkiewicz, Baladi et al. 2006). However, only a few studies looked at the electroencephalography (EEG) recordings following DOR agonist administration, whereas the absence-like seizures may be difficult to measure only by ethological observations. Therefore, we assessed the convulsions-induced by different DOR agonists using *in vivo* EEG recordings. This work also allowed us to explore an *in vivo* biased agonism effect following the administration of low- (ARM-100390 and ADL5747) or high- (SNC80) internalizing delta drugs.

This work is presented in Part II in a manuscript under submission: "**Delta opioid receptor on GABAergic neurons of the forebrain are responsible for SNC80-induced seizures**", Chu Sin Chung P., Boehrer A., Stephan A., Tatarau C., Befort K., Matifas A. and Kieffer B.L..

Constitutive DOR knockout mice showed spatial memory deficits and improved motor learning skills (Le Merrer, Rezai et al. 2013). In this part, we will also

present further experiment used to explore the contribution of DOR expressed in forebrain GABAergic neurons on non-emotional processes. We tested Dlx-DOR animals in behavioural tests that mainly recruit cortical, striatal or hippocampal circuits. We chose paradigms known to assess locomotion (actimetry boxes) and memory (novel object recognition NOR). Finally, mutant mice were examined in fear conditioning paradigm.

***Aim*** 3: *DOR in the basolateral nucleus of the amygdala.* In this part, we will present supplementary experiments initiated to identify the DOR population that could be involved in the regulation of anxiety and depressive-like behaviours. We focused on the basolateral amygdala (BLA), a brain region pivotal for the control of fear responses and anxiety-related behaviours (Tye, Prakash et al. 2011). We hypothesized that DORs expressed in the BLA are responsible for the anxiolytic role of DOR. We first initiated AAV-Cre viral stereotaxic injections in the BLA of DOR floxed mice to induce a specific deletion of the receptors expressed locally. Then, knockdown animals were assessed for anxiety and despair-like behaviours.

Neuroanatomical (Mansour, Fox et al. 1995) and imaging data (Scherrer, Tryoen-Toth et al. 2006) show strong DOR expression in the BLA and suggest that DORs are mainly pre-synaptic in the BLA. In order to better determine DOR location in the BLA circuitry, we developed a retrograde tracing experiment. Using the DOR-eGFP mice, we performed stereotaxic injections of the cholera toxin subunit B, a classical retrograde tracer, in the BLA of these knockin mice. We performed a relative quantification of DOR-eGFP positive neurons retrogradely labelled in the BLA-afferent regions to identify presynaptic regions expressing DORs and their contributions to the BLA activity.

# First Part

# Delta opioid receptors expressed on forebrain GABAergic neurons

# New function in emotional regulation

# Introduction

Genetic and pharmacological inactivation of delta opioid receptors previously demonstrated the anxiolytic function of the receptor (Chu Sin Chung and Kieffer 2013). This role sustained many interest for the development of delta drugs in the treatment of mood disorders. Therefore, a further challenge is to elucidate the precise mechanisms as well as brain substrates underlying this function.

Recent evidence demonstrated that delta opioid receptors are expressed on GABAergic neurons in the hippocampus, mostly on parvalbumin-immunopositive cells at the presynaptic level on glutamatergic pyramidal cells (Erbs, Faget et al. 2012; Rezai, Faget et al. 2012). Broad expression in the striatum also suggests that receptors are located on GABAergic medium spiny neurons (Le Merrer, Becker et al. 2009). In addition, pharmacological drugs targeting the GABAergic neurons have been long used to help patient suffering from anxiety disorders (Lydiard 2003). Hence, targeting delta opioid receptors specifically on GABAergic neurons would be of great interest to better evaluate their contribution on emotional responses as well as on other physiological processes (see Part II).

Morphologic and functional maturation of GABAergic neurons takes place in mice between P16 and P21 (Del Rio, Soriano et al. 1992). This process requires several genes activity such as DISC-1, ErbB4, NRG or DLX. Two enhancer elements (I56i and I56ii) were identified in the intergenic region of the Dlx5/Dlx6 genes and are highly conserved between zebrafish, mouse, and human (Zerucha, Stuhmer et al. 2000). Conditional knockout mice with a selective deletion of cannabinoid receptor 1 on GABAergic neurons have been successfully generated by expressing the Cre recombinase under the control of the I56i and I56ii intergenic sequences (Marsicano, Goodenough et al. 2003; Monory, Massa et al. 2006).

Therefore, we decided to use the Dlx5/6-Cre mouse to conditionally target delta opioid receptors in forebrain GABAergic neurons and assess emotional responses in this mutant mouse line.

# Manuscript 1

## Delta opioid receptors expressed in forebrain GABAergic neurons increase anxiety and limit risk-taking behaviour.

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*Abbreviations:* Amy, amygdala; BLA, basolateral nucleus of the amygdala; CMV, cytomegalovirus; CPu, caudate-putamen nucleus; DAR, dopamine receptor; DOR, delta opioid receptor; EPM, elevated plus maze; Hipp, hippocampus; KO, knockout; NAc, nucleus accumbens; NSF, novelty suppressed feeding; OB, olfactory bulb; PFC, prefrontal cortex; SC, spinal cord.

*Keywords*: Delta opioid receptor; Conditional Knockout; Locomotion; Emotion; in vivo

## Abstract

**Background:** Delta opioid receptors (DORs) are broadly expressed in the nervous system. This receptor regulates chronic pain, emotional responses, motivational processes and cognition, and both pain reducing and mood-enhancing effects of DOR agonists are well established. At present however, circuit mechanisms underlying DOR function in the brain have been poorly explored, and were not examined by genetic approaches.
**Methods:** We inactivated the DOR gene in forebrain GABAergic neurons using the Cre-LoxP system. We first characterized DOR distribution in conditional mutant (Dlx-DOR) mice. We then tested olfaction, basal locomotor responses and locomotor activation upon treatment with DOR and D1 agonists. We finally evaluated emotional responses of Dlx-DOR using several paradigms, and examine neural activation after novelty suppressed feeding in areas of high DOR density.
**Results:** Dlx-DOR mice showed complete absence of DOR binding sites in olfactory bulb and striatum, and partial deletion in hippocampus. DORs were otherwise intact in cortex and basolateral amygdala, the latter with highest DOR density. There was no change in olfactory perception and basal activity, but locomotor stimulant effects of SNC80 and SKF81297 were abolished and increased, respectively. Despair-like behaviors were unchanged in both forced swim and tail suspension tests. In contrast, Dlx-DOR mice showed lower levels of anxiety in the elevated plus maze and remarkably low latencies to eat in the novelty suppressed feeding test. Modifications of C-fos staining supported the low anxiety/high risk taking phenotype of Dlx-DOR mice within cognition-emotion circuitry.
**Conclusions:** Our data show that DORs expressed in GABAergic forebrain neurons mediate the well-described locomotor effect of SNC80 and inhibit D1-mediated hyperactivity. Our data also reveal an unanticipated function for this particular DOR subpopulation, which increases levels of anxiety and reduces risk-taking behaviors, and has potential protective effect under threat. DORs therefore exert dual roles in mood control that operate in distinct brain circuits, and this finding has important implications in the area of anxiety disorders.

## Introduction

Mu, delta and kappa opioid receptors are largely distributed throughout the nervous system and play a central role in pain control, hedonic homeostasis and emotions (Sauriyal, Jaggi et al. 2011; Lutz and Kieffer 2012). In the last decade, the delta opioid receptor (DOR) has emerged as an attractive target to reduce chronic pain (Gaveriaux-Ruff and Kieffer 2011; Pradhan, Befort et al. 2011). This receptor is also a key player in several brain processes (Chu Sin Chung and Kieffer 2013), including the regulation of emotional responses (Filliol, Ghozland et al. 2000), impulsivity (Befort, Mahoney et al. 2011) or learning and memory (Le Merrer, Faget et al. 2012), and altogether this opioid receptor has raised interest in areas of both neurologic and psychiatric disorders. Mood control represents a most important aspect of DOR function. Preclinical studies have established a general beneficial role for DOR in reducing levels of anxiety and depressive-like behavior, and delta agonists are in clinical trial for the treatment of mood disorders (Pradhan, Befort et al. 2011; Chu Sin Chung and Kieffer 2013).

DORs are broadly expressed in central and peripheral nervous systems. In the mouse, quantitative autoradiographic binding (Kitchen, Slowe et al. 1997; Slowe, Simonin et al. 1999; Goody, Oakley et al. 2002) shows particularly abundant expression in the olfactory bulb, cortex, striatum (caudate putamen and nucleus accumbens) and amygdala. DORs are also expressed at moderate levels in the interpeduncular and pontine nuclei, hippocampus, spinal cord and dorsal root ganglia, and at a much lower level in hypothalamus, thalamus, mesencephalon and brain stem. In situ hybridization and immunohistochemical studies have confirmed this distribution (reviewed in (Le Merrer, Becker et al. 2009)). Analyses of DOR distribution in the human brain shows expression concordant with rodent studies in cortical regions and limbic structures such as hippocampus and amygdala, as well as basal ganglia and hypothalamus (Simonin, Befort et al. 1994; Peckys and Landwehrmeyer 1999; Smith, Zubieta et al. 1999; Peng, Sarkar et al. 2012). Recently, a newly generated knock-in mouse line expressing functional fluorescent DORs (Scherrer, Tryoen-Toth et al. 2006) has allowed anatomical studies of DOR expression with cellular and subcellular details in dorsal root ganglia (Scherrer, Imamachi et al. 2009), enteric neurons (Scherrer, Imamachi et al. 2009; Poole, Pelayo et al. 2011; Erbs, Faget et al. 2012; Rezai, Faget et al. 2012) and the hippocampus (Erbs, Faget et al. 2012; Rezai, Faget et al. 2012). Therefore, refined mapping of DOR expression is now possible (Erbs et al., www.ics….submitted) and provides a basis for understanding DOR activities in the brain and periphery.

At present, neuron populations and brain circuits where delta opioid receptors operate in the nervous system have been poorly explored. In the context of pain research, local pharmacology at the level of dorsal root ganglias and spinal cord has indicated a role for peripheral DORs in pain control (Gaveriaux-Ruff 2013). Recently, a first conditional genetic approach has demonstrated that DORs expressed in small primary nociceptive neurons are essential to reduce persistent pain and mediate

delta opioid analgesia (Gaveriaux-Ruff, Nozaki et al. 2011). In the brain, local pharmacology has provided evidence for an anxiolytic role of DORs at the level of cingulate cortex (Narita, Kuzumaki et al. 2006), hippocampus (Solati, Zarrindast et al. 2010) and amygdala (Narita, Kaneko et al. 2006; Randall-Thompson, Pescatore et al. 2010). However neural populations engaged in DOR-mediated mood control have not been examined by genetic approaches, and DOR-mediated mechanisms underlying motivational and emotional responses, or learning and memory remain largely unexplored.

In this study we genetically inactivated the DOR gene in forebrain GABAergic neurons. We obtained a conditional knockout mouse line that lacks DORs in two main expression sites for the receptor, i. e. the olfactory bulb (OB) and striatum, including caudate putamen (CPu) and nucleus accumbens (NAc). Interestingly these mice retain full receptor density in the basolateral amygdala (BLA), which represents a third main site with densest DOR expression levels. We then examined these mice in behaviors known to engage these brain structures and may recruit DOR-mediated controls. Our data reveal an unexpected anxiogenic role for DORs expressed in forebrain GABAergic neurons, which potentially limits risk-taking behaviors.

## Methods and Materials

### Animals

The DOR-floxed (Oprd1$^{fl/fl}$ or Ctrl mice) mouse line was described previously (Gaveriaux-Ruff, Nozaki et al. 2011). Mice were crossed with CMV-Cre mice or Dlx5/6-Cre mice to produce constitutive knockout (CMV-CreXOprd1$^{fl/fl}$ or CMV-DOR) and conditional knockout (Dlx5/6- Oprd1$^{fl/fl}$ or Dlx-DOR) mouse lines, see details in Supplementary. For all behavioral experiment, the Dlx-DOR mice are compared to their control littermates Ctrl mice. In addition, the CMV-DOR mice were also tested in the anxiety-related tests (see Supplementary). Experiments were performed on animals aged between 6 and 18 weeks old, housed 2-4 per cage under standard laboratory conditions (12h dark/light cycle light on at 7am). Food and water were available ad libitum. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).

### Quantitative Reverse Transcriptase-PCR

Sampling of brain regions, RNA extraction and quantification were performed according to a previous study (Livak and Schmittgen 2001; Befort, Filliol et al. 2008) and briefly described in Supplementary.

**Autoradiographic Binding Assay**

Sections were cut from Ctrl, Dlx-DOR and CMV-DOR brains (n = 3) for determination of total DOR binding using [$^{3}$H] deltorphin-1 as the radiolabeled ligand. On the day of the experiment, sections were thawed and processed according to established protocols (Kitchen, Leslie et al. 1995; 1997), with minor modifications. Films exposure, development and analyze were performed as previously described by Kitchen et al. (1997). Further details are described in Supplementary.

**Agonist-Stimulated [$^{35}$S]-GTPγS Binding Assays**

Membrane preparations and [$^{35}$S]GTPγS binding assays were performed on brain regions from Ctrl, Dlx-DOR and CMV-DOR mice as described (Pradhan, Becker et al. 2009) (see Supplementary).

**Behavioral Assays**

Locomotion, depressive-like behaviors (forced swim and tail suspension tests), anxiety-related behaviors (light/dark box, elevated plus maze and open field tests) and novelty-suppressed feeding tests were performed as described in Supplementary.

**Drugs**

The non-peptidic DOR agonist SNC80 and the dopamine D1 receptor agonist SKF-81297 were used at doses according to previous studies (Nozaki, Le Bourdonnec et al. 2012; Le Merrer, Rezai et al. 2013). See preparation in Supplementary.

**c-Fos immunoreactivity**

Measures of c-fos protein expression were performed as reported (Le Merrer, Gavello-Baudy et al. 2007). Further details about sections processing are provided in Supplementary.

**Statistical analysis**

Statistical differences were determined by analysis of variance (ANOVA) (StatView 5, SAS Institute Inc., Cary, North Carolina) followed by Bonferroni/Dunn post hoc analysis. The F values and experimental degrees of freedom are included in the Results Section. For experiments with two groups, a Student *t*-test was used. The level of statistical significance was set at $p < 0.05$. For the behavioral tests during which data were obtained on several periods during the same session (locomotor tests, the Open Field test and despair-like behavior paradigms), the analysis of variance repeated measures was used.

## Results

### Dlx-DOR mice show massive DOR deletion in olfactory bulb and striatum

We used a Cre-*LoxP* strategy to inactivate the DOR gene in forebrain areas, and maintain intact receptors in the midbrain and hindbrain. Because DORs are reported to be mainly expressed in GABAergic neurons (Stumm, Zhou et al. 2004; Margolis, Fields et al. 2008; Rezai, Faget et al. 2012), we mated floxed-DOR mice (Gaveriaux-Ruff, Nozaki et al. 2011) with Dlx-Cre5/6 mice that express Cre recombinase in forebrain GABAergic neurons (Monory, Massa et al. 2006). We first analyzed DOR transcripts throughout the nervous system using quantitative reverse transcriptase-PCR analysis (Fig. 1A) of microdissected brain areas from double mutant offspring. The DOR mRNA was undetectable in olfactory bulb (OB) and striatum, including both caudate-putamen (CPu) and nucleus accumbens (NAc) of Dlx-DOR mice. We also observed a partial transcript reduction in the prefrontal cortex (PFC) and amygdala (Amy), with no change in the spinal cord (SC). As expected, CMV-DOR mice (total knockout mice) showed no detectable DOR mRNA in any sample. Thus, consistent with the Dlx-Cre expression pattern the genetic deletion mainly impacts forebrain areas (Monory, Massa et al. 2006).

We next fine-mapped and quantified DOR protein distribution in Dlx-DOR mice, using autoradiographic binding (Fig. 1B-C and Table 1). Two-way ANOVA revealed significant effect of Genotype ($F(1, 444) = 70.97$; $p<0.001$), Region ($F(50, 444) = 10.98$; $p<0.001$) and Genotype x Region interaction ($F(50, 444) = 4.00$; $p<0.001$). There was a remarkably strong reduction of [3H] deltorphin-1 binding in external plexiform and internal granular layers of OB, as well as lateral and medial CPu and olfactory tubercles from Dlx-DOR mice in comparison with Ctrl mice (t-test student; all $p < 0.001$). Significant reduction of [3H] deltorphin-1 binding was also found in the NAc shell ($p < 0.01$), and CA2/3 regions of the ventral hippocampus ($p < 0.05$). In contrast there was no significant modification of DOR binding sites throughout cortical areas and BLA subdivisions, suggesting that partial mRNA deletion observed at these sites by qRT-PCR (Fig 1A) may impact distant rather than local receptors (see discussion). Finally, DOR protein levels were unchanged at the level of SC (Fig. 1C). MCID analysis of CMV-DOR samples confirmed complete DOR deletion in CMV-DOR mice throughout the nervous system (Fig. 1B-C).

To further examine protein function, we measured DOR-mediated G protein activation in selected brain areas showing reduced receptor binding sites. The agonist-induced [$^{35}$S]-GTP$\gamma$S binding assay (Suppl Fig S2 and Table S1) confirmed massive suppression of DOR activity in membrane preparations from OB ($E_{max}$ 253.6 $\pm$ 3.5% in Ctrl; 121.7 $\pm$ 2.8% in Dlx-DOR; 107.8 $\pm$ 2.9% in CMV-DOR mice) and CPu ($E_{max}$ 183 $\pm$ 5.7% in Ctl; 116.5 $\pm$ 3.9% in Dlx-DOR; 107.5 $\pm$ 4.4% in CMV-DOR mice). In addition, decreased [$^{35}$S]-GTP$\gamma$S binding was found in hippocampal membranes

preparation from conditional mutant mice ($E_{max}$ 164.5 ± 7.9% in Ctrl; 144 ± 4.2% in Dlx-DOR; 119.1 ± 6.2% in CMV-DOR mice). SC samples showed similar dose-dependent receptor activation across genotypes ($E_{max}$ 150.6 ± 4.3% in Ctrl; 140.4 ± 6.6% in Dlx-DOR; 116.3 ± 3.9% in CMV-DOR mice). Receptor signaling therefore fully matches receptor binding in mutant mice.

In sum (Fig 1D), Dlx-DOR mice show complete mRNA and protein DOR deletion in primary olfactory regions and the entire striatum, indicating that DORs are mainly expressed in local GABAergic neurons in these brain regions. The receptor protein is otherwise intact in the cortex and partially decreased in hippocampus. Low or no receptor deletion in these forebrain regions could be due to either partial Cre-mediated excision or main DOR expression in non-GABAergic neurons at these sites, or could indicate that receptors are transported from distant mid/hindbrain sites to these brain areas. Finally DOR expression is fully preserved in the BLA, a main site for the control of emotional responses.

**Dlx-DOR mice show altered locomotor responses to DOR and D1/D3 DAR agonists**

We first examined whether DOR loss in caudate putamen and nucleus accumbens leads to changes in spontaneous locomotor activity and feeding behavior (Table 2). Dlx-DOR mice and their wildtype littermates (Ctrl) were thus submitted to circadian locomotor activity test (Table 2). Dlx-DOR mice displayed normal locomotor habituation to the novel environment and a normal pattern of circadian activity (data not shown). Analysis of total locomotor activity levels during light and dark phases revealed no significant difference between genotypes ($p>0.05$, Student's *t*-test, Table 2). Similarly, no difference in food consumption was detected between Dlx-DOR and Ctrl mice ($p>0.05$, Student's *t*-test, Table 2).

We then examined locomotor stimulant effects of the prototypal DOR agonist, SNC80 (Jutkiewicz, Kaminsky et al. 2005) in Dlx-DOR mice (Fig 2A) in actimetry cages. No difference in basal locomotor activity (habituation) was detected between genotypes (data not shown), thus confirming previous findings. SNC80 treatment (10 mg/kg) induced the expected locomotor stimulation in Ctrl mice. By contrast, this agonist was inefficient in Dlx-DOR mice. Two-way ANOVA performed on total locomotor activity scores revealed a significant effect of treatment ($F_{(1, 42)} = 14.58$; $p<0.001$) and genotype ($F_{(2, 42)} = 10.39$; $p<0.001$), and a significant treatment x genotype interaction ($F_{(2, 42)} = 4.31$; $p<0.05$). *Post hoc* analysis confirmed that SNC80 treatment significantly enhanced locomotor activity in Ctrl ($p<0.001$, Bonferronni/Dunn test) but not in Dlx-DOR mice ($p>0.05$, Bonferronni/Dunn test). These results show that DORs in forebrain GABAergic neurons are necessary for the expression of locomotor stimulant effect of SNC80, most likely at the level of striatum.

To further explore integrity of the basal ganglia locomotor circuitry, we examined locomotor stimulant effects of a D1 dopamine receptor agonist (Fig 2B). We challenged Dlx-DOR mice and their control littermates with SKF-81297 (1 and 2.5 mg/kg, Fig 2B). At the low dose (1 mg/kg), SKF-81297 induced a slight locomotor

stimulation in both Ctrl and Dlx-DOR mice, and no significant difference was detected between genotypes (P>0.05, Two-way ANOVA). At the high dose (2.5 mg/kg), SKF-81297 induced a significant locomotor hyperactivity in Ctrl mice and this stimulant effect was potentiated in Dlx-DOR mice. Two-way ANOVA performed on total locomotor activity scores showed a significant effect of treatment ($F_{(1, 37)} = 22.23$; p<.0001) and a significant genotype x treatment interaction ($F_{(1, 37)} = 5.54$; p<0.05). *Post hoc* analysis confirmed that Dlx-DOR mice treated with the 2.5 mg/kg dose showed significantly higher locomotor activity compared to the control group (p<0.001, Bonferronni/Dunn test). These results indicate that DORs, which we have deleted in conditional mutant mice, normally inhibit striatal D1/D3 DAR function.

**Dlx-DOR mice display normal olfaction and despair behaviors**

We previously showed that constitutive DOR KO mice display a depressive-like phenotype revealing a key role for DORs in despair behaviors and mood control (Filliol, Ghozland et al. 2000). Alteration of nucleus accumbens function has long been associated with mood disorders and deep brain stimulation of this structure is currently investigated for patients suffering from treatment-resistant depression. Dysfunction of olfactory function is also associated with major depression and olfactory bulbectomy is a widely used strategy to induce depressive-like symptoms in rodents. We therefore examined whether the major DOR loss in olfactory bulb and nucleus accumbens in Dlx-DOR mice would lead to a phenotype similar to constitutive KO mice.

Because lack of DORs in olfactory bulb may perturb basal olfactory perception, we first tested olfaction in Dlx-DOR mice. Mutant animals and their wildtype mice were submitted to an olfactory test that involves discrimination between neutral (water) and attractive odors (social or lemon odor). Figure 3A shows the behavioral response of mice following repeated presentation of social odor. During the first exposure, Dlx-DOR and wildtype mice showed a high preference for social compared to neutral odor (P<0.001, vs habituation and chance level). When animal were reexposed to the same odor, both genotypes showed a clear decline in preference, reflecting the habituation phenomenon. No difference was detected between genotypes during the first or second exposure to social odor (P>0.05, Two-way ANOVA). A similar pattern was obtained following repeated animal exposure to non-social odor (lemon odor, data not shown). These data indicate that Dlx-DOR mice show no alteration in olfactory skills that may confound behavioral testing for emotional responses.

Dlx-DOR and control littermates were then submitted to forced swim and tail suspension tests (Figures 3B), two paradigms classically used to assess despair behaviors in rodents (Porsolt, Anton et al. 1978; Steru, Chermat et al. 1985). Both tests involve exposure of animals to inescapable aversive situations and the immobility observed during testing is considered to reflect a despair state, assuming that animals have given up hope to escape. Dlx-DOR displayed a normal behavioral response (time of floating) in the forced swim test, illustrated by lack of difference

between genotypes (p>0.05, one-way ANOVA and Student *t*-test). Dlx-DOR mutants also showed normal behavior (time of immobility) in the tail suspension test (p>0.05, one-way ANOVA and Student t-test). Mice lacking DORs in forebrain GABAergic neurons, therefore, display normal emotional response under classical despair conditions.

**Dlx-DOR mice show reduced anxiety and higher risk-taking behaviors**

We previously showed that constitutive DOR knockout mice manifest enhanced anxiety-like behavior (Filliol, Ghozland et al. 2000). To determine whether DORs in forebrain GABAergic neurons contributes to this phenotype, animals were submitted to a test battery assessing risk taking and anxiety-like behaviors. In the open field (Figure 4A), Dlx-DOR mice showed similar level of general activity as Ctrl mice (p>0.05, Student *t*-test), and time spent in the arena center, used as an index of anxiety state, was also comparable between genotypes (p>0.05, Student *t*-test).

In contrast, a behavioral phenotype was clearly detectable in the elevated plus-maze test Figure 4B). Dlx-DOR mice displayed lower fear/anxiety-related behavior compared to controls, manifested by increased time spent in open arms, the most aversive part of the maze (p<0.05, Student *t*-test). Mutant mice also made more entries into open arms, although this effect did not reach statistical significance (p>0.05, Student *t*-test). The number of entries in closed arms, used as an index of locomotor activity in the maze, was otherwise unchanged (Ctrl: 11.75 ± 0.72 and Dlx-DOR: 11.19 ±0.79; p>0.05, Student's *t*-test). These data indicate that mice lacking DORs in forebrain GABAergic neurons display low levels of anxiety, a phenotype that opposes the classically described increased anxiety-like behaviors in constitutive DOR knockout mice.

To further examine this unexpected phenotype, we tested Dlx-DOR mice in the novelty suppressed feeding task (Figure 4C). In this paradigm, latency to start eating in a novel environment reflects reduced fear behavior and enhanced risk taking. Remarkably, Dlx-DOR mice showed a shorter latency to feed compared to control littermates (p<0.01, Student *t*-test). Consequently to this decrease time to feed, mutant mice also made fewer approaches compared to control mice (p<0.001, Student *t*-test). Both parameters, therefore, indicate strong behavioral modifications in mutant mice. Together with increased anxiety in the elevated plus maze, our data demonstrate that selective deletion of DORs in forebrain GABAergic neurons produce an emotional phenotype characterized by reduced fear/anxiety-related and enhanced risk-taking behaviors.

**Dlx-DOR mice show abnormal neuronal activity in cortex, amygdala and nucleus accumbens following NSF**

To gain insight into circuit mechanisms underlying the emotional phenotype of Dlx-DOR mice, we assessed Fos protein expression following animal exposure to the novelty suppressed feeding task (Figure 5 and Table 3). The c-fos protein expression is used routinely as a marker of neuronal activity (Dragunow and Faull 1989).

As compared to Ctrl animals, Dlx-DOR mice showed a significant decrease of c-fos protein expression in a set of brain regions involved in central integration of emotional components of fear/aversive stimuli, including the insular cortex ($p<0.05$, Student *t*-test), basolateral amygdala ($p<0.01$, Student *t*-test) and central amygdala ($p<0.001$, Student *t*-test, Figure 5). On the other hand, a significant increase of c-fos protein expression was found in the nucleus accumbens shell and core ($p<0.05$, Student *t*-test), a brain region interfacing emotion, motivation and action. C-Fos expression was otherwise unchanged in all subregions of the caudate putamen. Similarly, no difference between Dlx-DOR and Ctrl mice was detected in cingulate cortex, basomedial nucleus of the amygdala and ventral tegmental area of ($p>0.05$, Student *t*-test, Table 3).

Together these data reveal altered neuronal activity in specific cortico-limbic circuits of Dlx-DOR mice. Thus mutant mice show decreased activation of insular cortex and amygdala together with increased activation of the nucleus accumbens, associated to their low anxiety/high risk-taking phenotype behavior after novelty suppressed feeding.

## Discussion

In the present study, we targeted the DOR gene in forebrain GABAergic neurons and obtained conditional knockout mice with a complete deletion of DORs in olfactory bulb, caudate-putamen and nucleus accumbens. The receptor was otherwise partially deleted in hippocampus, and preserved in the cortex, basolateral amygdala, as well as more rostral brain areas and spinal cord. Behavioral analysis of mutant mice provided first genetic evidence that DORs expressed in striatal GABAergic neurons inhibit D1R-mediated locomotor activity, and uncovered a novel role for DOR in the regulation of fear/anxiety-related behaviors.

The driver Dlx5/6-Cre mouse line was used previously to delete CB1 receptors from GABAergic neurons of the forebrain (Monory, Massa et al. 2006). Based on the notion that opioid receptors are mostly expressed in GABAergic neurons (Erbs, Faget et al. 2012; Rezai, Faget et al. 2012), we anticipated strong decrease of DOR expression throughout the forebrain. Almost complete deletion was indeed observed in the olfactory bulb and entire striatum. Partial reduction of DOR mRNA and protein in hippocampus and preserved protein levels in cortical areas and amygdala were somewhat surprising. In these brain areas, remaining receptor expression could be explained by partial Cre-mediated excision, although crossing Dlx5/6-Cre mice with ROSA26 reporter mice showed strong Cre activity at these sites (data not shown). Another possibility is that DOR are expressed partially or predominantly in non-GABAergic neurons of hippocampus, cortex and amygdala. Similarly, remaining DOR protein in caudate putamen and nucleus accumbens may arise from DOR expression in striatal cholinergic interneurons, at least partially. A third likely possibility is that DOR proteins detected in these brain regions are synthesized and transported from

more posterior brain structures. In support of this, amygdala showed decreased DOR mRNA, indicating local Cre-mediated DOR gene excision. However, DOR protein levels were maintained, suggesting that the majority of amygdalar receptors are localized presynaptically on afferent terminals. Within this line, part of residual protein binding in CPu and NAc may reflect presynaptic receptors on glutamatergic neurons that massively project from cortex and amygdala to the striatum (Christie, Summers et al. 1987; Stuber, Sparta et al. 2011; Buot and Yelnik 2012).

DORs were fully removed from the olfactory bulb in Dlx-DOR mice. Our assessment of odor discrimination, however, shows no main alteration in basic olfactory perception. DORs in the olfactory bulb, therefore, are not necessary to the detection of olfactory stimuli. Olfactory bulbectomy is a classical model of despair-like behavior (Kelly, Wrynn et al. 1997), and we tested whether Dlx-DOR mice would show any despair-like phenotype. Under our standard experimental conditions, mutant mice showed no sign of despair behavior, suggesting that DORs at this site do not tonically regulate emotional circuits associated to olfaction. Despair-like behavior in constitutive DOR KO mice (Filliol, Ghozland et al. 2000) therefore results from lack of receptor activity elsewhere in the brain. In the future, it will be interesting to assess Dlx-DOR mice reactivity to stressful odors, i. e. facing an aversive odor such as a predator odor, in order to determine whether DOR plays any role in olfactory circuitry where the receptor is most densely expressed.

Constitutive DOR KO show enhanced spontaneous locomotor activity, suggesting a tonic inhibitory role of DOR on mouse basal activity (Filliol, Ghozland et al. 2000). In this study, we showed that selective DOR deletion in GABAergic forebrain neurons does not alter this behavior, suggesting that this DOR activity is not mediated by forebrain GABAergic neurons, or alternatively could not be detected under our experimental conditions. We also evaluated the effect of SNC80, a DOR agonist, on locomotor activity (Saitoh, Sugiyama et al. 2011; Nozaki, Le Bourdonnec et al. 2012) and observed that SNC80-induced hyperlocomotion effect was abolished in Dlx-DOR mice, demonstrating that DORs expressed in forebrain GABAergic neurons mediate stimulating effects of the agonist. It is likely that this DOR activity operates at the level of the striatum, known to control locomotor activation (Durieux, Schiffmann et al. 2012), and where DOR genetic inactivation was most effective. Finally, we also show that DOR excision in forebrain GABAergic neurons facilitates locomotor stimulant effects of the dopamine D1 receptor agonist SKF-81297. We have previously reported that constitutive deletion of the DOR gene, and DOR blockage by systemic DOR antagonist treatment, both produce a similar higher sensitivity to SKF-stimulating effects (Le Merrer, Rezai et al. 2013). Together with the present study, therefore, data strongly suggest that DORs expressed in striatal GABAergic neurons exert a tonic suppressive effect on the striatonigral D1 pathway and associated locomotor response. Whether DOR/D1R interactions occur directly at

the level of D1R-expressing medium spiny neurons or via intrastriatal microcircuitry remains to be determined.

Dlx-DOR mice show an intriguing low anxiety/fear phenotype. Although no modification of anxiety levels was detected in the open field, mutant mice spent significantly more time in open arms of the elevated plus maze and showed strongly reduced latency to reach the food in the novelty suppressed feeding test. In the two latter paradigms, the Dlx-DOR mouse phenotype reflects reduced anxiety-related behavior, together with a enhanced risk-taking component. The absence of detectable phenotype in the open field may relate to distinct stress levels applied in the different paradigms (e.g. novelty, brightness, openness, privation, elevation) (File, Lippa et al. 2004; Ramos 2008), and it is likely that hypoanxiety and risk-taking in mutant mice are detectable only under specific stress conditions. The open field test is performed under mild light intensity, which triggers lower stress compared to conditions of both elevated plus maze and novelty suppressed feeding tests. Modification of mutant mice behavior was most obvious in the latter test, which involves an additional food deprivation stress. Our comparison of neural activation in Dlx-DOR mice and their controls, immediately after the novelty suppressed feeding test, further supports the reduced anxiety/high risk taking phenotype of mutant mice. C-fos immunoreactivity indeed was reduced in amygdala and insular cortex, and increased in the nucleus accumbens, perfectly correlating with behavioral modifications. Altogether therefore, the data suggest that DOR activity may exert an adaptive protective role under threatening situations, which develops upon increasing stress and contributes to limit at-risk behaviors. It would be worthy testing mutant mice under even more stressful conditions, to determine whether this phenotype persists or even increases.

The remarkable low anxiety/high risk phenotype of Dlx-DOR mice seems discordant with the well-established high anxiety-related behavior reported for constitutive DOR KO animals (Filliol, Ghozland et al. 2000; Roberts, Gold et al. 2001). Indeed, several studies have consistently shown a general anxiolytic role for DORs. Both genetic deletion and systemic pharmacologic blockade of DOR increase levels of anxiety (Filliol, Ghozland et al. 2000; Saitoh, Kimura et al. 2004; Saitoh, Yoshikawa et al. 2005; Narita, Kaneko et al. 2006; Perrine, Hoshaw et al. 2006) and treatment with DOR agonists causes a reduction of anxiety-related behaviors (Saitoh, Kimura et al. 2004; Perrine, Hoshaw et al. 2006; Vergura, Balboni et al. 2008). A probable explanation for this apparent discrepancy is that full DOR KO mice lack a particular receptor population, whose anxiolytic activity prevails in the brain under classical experimental conditions (open field). These receptors likely operate at the level of basolateral amygdala, where the receptors are most heavily expressed, a hypothesis supported by local pharmacology (Randall-Thompson, Pescatore et al. 2010). This amygdalar DOR receptor population, however, has remained intact in conditional Dlx-DOR mice, which show anxiety-related behavior at control levels

under standard anxiety testing (no phenotype in the open field). Another distinct and anxiogenic-like activity, normally masked by the general strong anxiolytic DOR activity, is now detectable in these mutant mice, particularly under conflicting situations (novelty suppressed feeding). Our study demonstrates that this anxiogenic/fear-inducing DOR activity operates in forebrain GABAergic circuits and is associated with a risk-taking suppressive component, possibly at the level of inhibitory controls. Noteworthy, the high risk-taking behavior in Dlx-DOR mice using elevated plus maze and novelty suppressed feeding tests is consistent with high motor impulsivity observed for the full KO mice in a signaled nose-poke task (Olmstead, Ouagazzal et al. 2009; Befort, Mahoney et al. 2011). Together, the data strongly suggest that DORs expressed in GABAergic neurons of the forebrain, which are absent in both full and Dlx-DOR KO mice, concomitantly increase anxiety and limit risk-taking behavior. This activity likely operates through the regulation of decision-making processes, which are mediated at the level of corticostriatal circuitry tightly connecting the amygdala (Callaway, Hakan et al. 1991; Stuber, Sparta et al. 2011). In the future, genetic studies will further confirm the main anxiolytic role of DORs at the level of amygdala. Also, more behavioral testing of Dlx-DOR mutant mice using decision-making paradigms will strengthen characterization of this novel anxiogenic/inhibitory function of DORs, operating at the level of forebrain inhibitory circuitry.

In conclusion, our study reveals dual roles for DORs in anxiety-related emotional responses. A picture emerges where DORs tonically reduce levels of anxiety under basal conditions (Filliol, Ghozland et al. 2000), but also enhance anxiety-related responses and inhibit behavior under more stressful circumstances (this study). The conditional genetic approach demonstrates that these somehow opposing DOR activities operate at distinct brain sites, and our data demonstrates that DORs expressed in forebrain GABAergic neurons are essential to anxiogenic processes within the cognition/emotion circuitry. Finally, the study also emphasizes usefulness of conditional genetic approaches in the identification of distinct, and sometimes antagonistic receptor mechanisms at integrated level, as previously demonstrated for cannabinoid CB1 (Monory, Massa et al. 2006) and corticotrophin-releasing hormone receptor 1(Refojo, Schweizer et al. 2011).

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**Disclosure/conflict of interest**

The authors report no biomedical financial interests or potential conflicts of interest.

**References**

Befort, K., D. Filliol, et al. (2008). "Mu-opioid receptor activation induces transcriptional plasticity in the central extended amygdala." Eur J Neurosci **27**(11): 2973-2984.

Befort, K., M. K. Mahoney, et al. (2011). "Effects of delta opioid receptors activation on a response inhibition task in rats." Psychopharmacology (Berl) **214**(4): 967-976.

Buot, A. and J. Yelnik (2012). "Functional anatomy of the basal ganglia: limbic aspects." Rev Neurol (Paris) **168**(8-9): 569-575.

Callaway, C. W., R. L. Hakan, et al. (1991). "Distribution of amygdala input to the nucleus accumbens septi: an electrophysiological investigation." J Neural Transm Gen Sect **83**(3): 215-225.

Christie, M. J., R. J. Summers, et al. (1987). "Excitatory amino acid projections to the nucleus accumbens septi in the rat: a retrograde transport study utilizing D[3H]aspartate and [3H]GABA." Neuroscience **22**(2): 425-439.

Chu Sin Chung, P. and B. L. Kieffer (2013). "Delta opioid receptors in brain function and diseases." Pharmacol Ther. **140**(1): 112-120.

Dragunow, M. and R. Faull (1989). "The use of c-fos as a metabolic marker in neuronal pathway tracing." J Neurosci Methods **29**(3): 261-265.

Durieux, P. F., S. N. Schiffmann, et al. (2012). "Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions." EMBO J **31**(3): 640-653.

Erbs, E., L. Faget, et al. (2012). "Distribution of delta opioid receptor-expressing neurons in the mouse hippocampus." Neuroscience **221**: 203-213.

File, S. E., A. S. Lippa, et al. (2004). "Animal tests of anxiety." Curr Protoc Neurosci **Chapter 8**: Unit 8 3.

Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.

Franklin, K. B. J. and G. Paxinos (1997). The Mouse Brain in Stereotaxic Coordinated. San Diego, CA, Academic Press.

Gaveriaux-Ruff, C. (2013). "Opiate-Induced Analgesia: Contributions from Mu, Delta and Kappa Opioid Receptors Mouse Mutants." Curr Pharm Des.

Gaveriaux-Ruff, C. and B. L. Kieffer (2011). "Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches." Behav Pharmacol **22**(5-6): 405-414.

Gaveriaux-Ruff, C., C. Nozaki, et al. (2011). "Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia." Pain **152**(6): 1238-1248.

Goody, R. J., S. M. Oakley, et al. (2002). "Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice." Brain Res **945**(1): 9-19.
Jutkiewicz, E. M., S. T. Kaminsky, et al. (2005). "Differential behavioral tolerance to the delta-opioid agonist SNC80 ([(+)-4-[(alphaR)-alpha-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-me thoxyphenyl)methyl]-N,N-diethylbenzamide) in Sprague-Dawley rats." J Pharmacol Exp Ther **315**(1): 414-422.
Kelly, J. P., A. S. Wrynn, et al. (1997). "The olfactory bulbectomized rat as a model of depression: an update." Pharmacol Ther **74**(3): 299-316.
Kitchen, I., F. M. Leslie, et al. (1995). "Development of delta-opioid receptor subtypes and the regulatory role of weaning: radioligand binding, autoradiography and in situ hybridization studies." The Journal of Pharmacology and Experimental Therapeutics **275**(3): 1597-1607.
Kitchen, I., S. J. Slowe, et al. (1997). "Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene." Brain Res **778**(1): 73-88.
Kitchen, I., S. J. Slowe, et al. (1997). "Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene." Brain Research **778**(1): 73-88.
Le Merrer, J., J. A. Becker, et al. (2009). "Reward processing by the opioid system in the brain." Physiol Rev **89**(4): 1379-1412.
Le Merrer, J., L. Faget, et al. (2012). "Cues predicting drug or food reward restore morphine-induced place conditioning in mice lacking delta opioid receptors." Psychopharmacology (Berl) **223**(1): 99-106.
Le Merrer, J., S. Gavello-Baudy, et al. (2007). "Morphine self-administration into the lateral septum depends on dopaminergic mechanisms: Evidence from pharmacology and Fos neuroimaging." Behav Brain Res **180**(2): 203-217.
Le Merrer, J., X. Rezai, et al. (2013). "Impaired Hippocampus-Dependent and Facilitated Striatum-Dependent Behaviors in Mice Lacking the Delta Opioid Receptor." Neuropsychopharmacology.
Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.
Lutz, P. E. and B. L. Kieffer (2012). "Opioid receptors: distinct roles in mood disorders." Trends Neurosci.
Margolis, E. B., H. L. Fields, et al. (2008). "Delta-opioid receptor expression in the ventral tegmental area protects against elevated alcohol consumption." J Neurosci **28**(48): 12672-12681.
Monory, K., F. Massa, et al. (2006). "The endocannabinoid system controls key epileptogenic circuits in the hippocampus." Neuron **51**(4): 455-466.
Narita, M., C. Kaneko, et al. (2006). "Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala." Neuropsychopharmacology **31**(4): 739-750.
Narita, M., N. Kuzumaki, et al. (2006). "Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction." J Neurochem **97**(5): 1369-1378.
Nozaki, C., B. Le Bourdonnec, et al. (2012). "delta-Opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion, and receptor internalization." J Pharmacol Exp Ther **342**(3): 799-807.

Olmstead, M. C., A. M. Ouagazzal, et al. (2009). "Mu and delta opioid receptors oppositely regulate motor impulsivity in the signaled nose poke task." PLoS ONE **4**(2): e4410.
Peckys, D. and G. B. Landwehrmeyer (1999). "Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study." Neuroscience **88**(4): 1093-1135.
Peng, J., S. Sarkar, et al. (2012). "Opioid receptor expression in human brain and peripheral tissues using absolute quantitative real-time RT-PCR." Drug Alcohol Depend **124**(3): 223-228.
Perrine, S. A., B. A. Hoshaw, et al. (2006). "Delta opioid receptor ligands modulate anxiety-like behaviors in the rat." Br J Pharmacol **147**(8): 864-872.
Poole, D. P., J. C. Pelayo, et al. (2011). "Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice." Gastroenterology **141**(3): 982-991 e981-988.
Porsolt, R. D., G. Anton, et al. (1978). "Behavioural despair in rats: a new model sensitive to antidepressant treatments." Eur J Pharmacol **47**(4): 379-391.
Pradhan, A. A., J. A. Becker, et al. (2009). "In vivo delta opioid receptor internalization controls behavioral effects of agonists." PLoS One **4**(5): e5425.
Pradhan, A. A., K. Befort, et al. (2011). "The delta opioid receptor: an evolving target for the treatment of brain disorders." Trends Pharmacol Sci **32**(10): 581-590.
Ramos, A. (2008). "Animal models of anxiety: do I need multiple tests?" Trends Pharmacol Sci **29**(10): 493-498.
Randall-Thompson, J. F., K. A. Pescatore, et al. (2010). "A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors." Psychopharmacology (Berl) **212**(4): 585-595.
Rezai, X., L. Faget, et al. (2012). "Mouse delta opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells." Cell Mol Neurobiol **32**(4): 509-516.
Roberts, A. J., L. H. Gold, et al. (2001). "Increased ethanol self-administration in delta-opioid receptor knockout mice." Alcohol Clin Exp Res **25**(9): 1249-1256.
Saitoh, A., Y. Kimura, et al. (2004). "Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents." J Pharmacol Sci **95**(3): 374-380.
Saitoh, A., A. Sugiyama, et al. (2011). "The novel delta opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions." Behav Brain Res **223**(2): 271-279.
Saitoh, A., Y. Yoshikawa, et al. (2005). "Role of delta-opioid receptor subtypes in anxiety-related behaviors in the elevated plus-maze in rats." Psychopharmacology (Berl) **182**(3): 327-334.
Sauriyal, D. S., A. S. Jaggi, et al. (2011). "Extending pharmacological spectrum of opioids beyond analgesia: multifunctional aspects in different pathophysiological states." Neuropeptides **45**(3): 175-188.
Scherrer, G., N. Imamachi, et al. (2009). "Dissociation of the opioid receptor mechanisms that control mechanical and heat pain." Cell **137**(6): 1148-1159.
Scherrer, G., P. Tryoen-Toth, et al. (2006). "Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo." Proc Natl Acad Sci U S A **103**(25): 9691-9696.
Simonin, F., K. Befort, et al. (1994). "The human delta opioid receptor: genomic organization, cDNA cloning, functional expression and distribution in human brain." Molecular Pharmacology **46**: 1015-1021.

Slowe, S. J., F. Simonin, et al. (1999). "Quantitative autoradiography of mu-,delta- and kappa1 opioid receptors in kappa-opioid receptor knockout mice." Brain Res **818**(2): 335-345.
Smith, J. S., J. K. Zubieta, et al. (1999). "Quantification of delta-opioid receptors in human brain with N1'-([11C]methyl) naltrindole and positron emission tomography." J Cereb Blood Flow Metab **19**(9): 956-966.
Solati, J., M. R. Zarrindast, et al. (2010). "Dorsal hippocampal opioidergic system modulates anxiety-like behaviors in adult male Wistar rats." Psychiatry Clin Neurosci **64**(6): 634-641.
Steru, L., R. Chermat, et al. (1985). "The tail suspension test: a new method for screening antidepressants in mice." Psychopharmacology (Berl) **85**(3): 367-370.
Stuber, G. D., D. R. Sparta, et al. (2011). "Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking." Nature **475**(7356): 377-380.
Stumm, R. K., C. Zhou, et al. (2004). "Neuronal types expressing mu- and delta-opioid receptor mRNA in the rat hippocampal formation." J Comp Neurol **469**(1): 107-118.
Vergura, R., G. Balboni, et al. (2008). "Anxiolytic- and antidepressant-like activities of H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512), a novel selective delta opioid receptor agonist." Peptides **29**(1): 93-103.

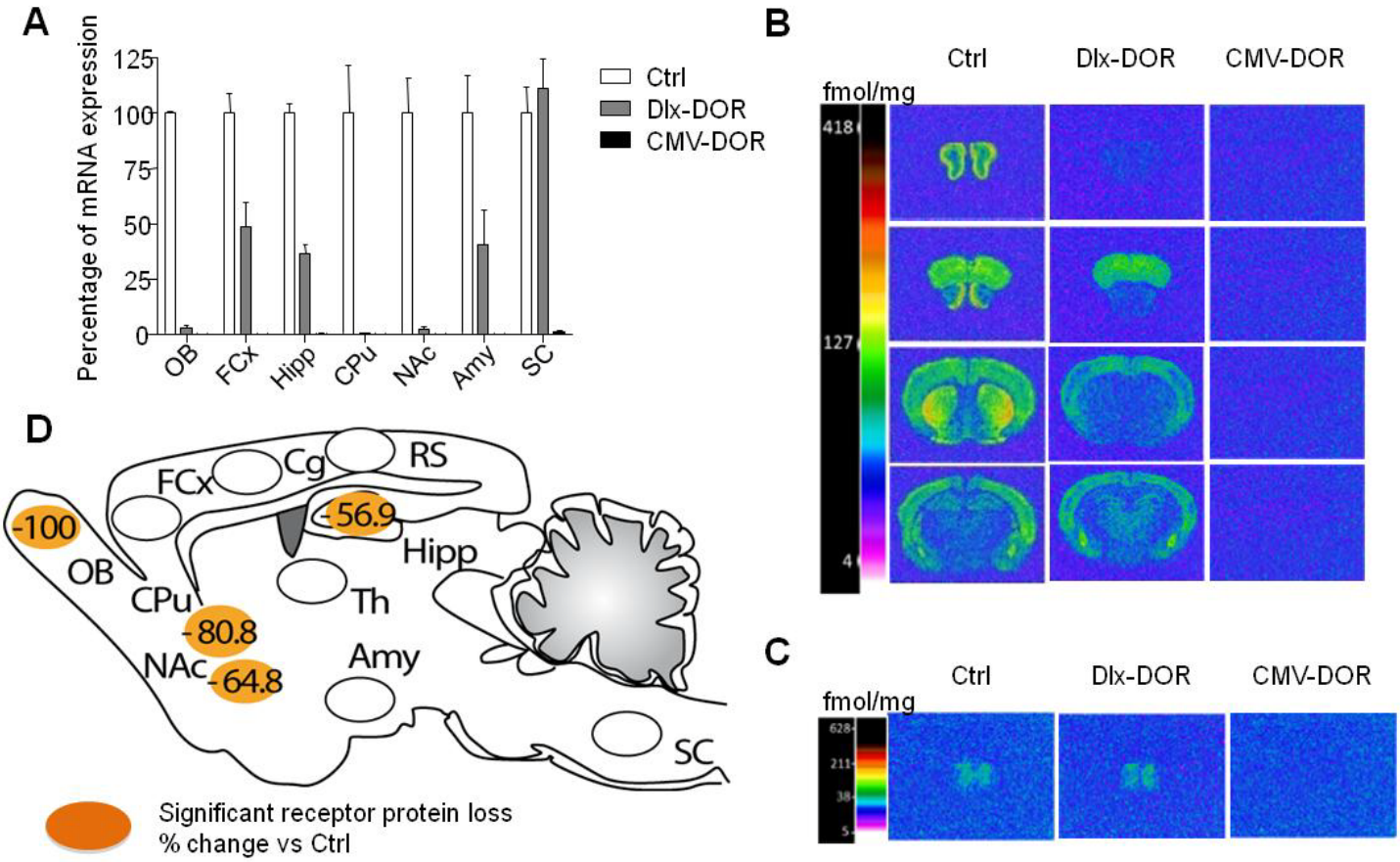



Fig. 1: **Anatomical characterization of Dlx-DOR mice. (A)** Quantitative RT-PCR. DOR mRNA levels were measured in samples from Ctl (control, white bars), Dlx-DOR (conditional mutant, gray bars) and CMV-DOR (constitutive mutant, black bars) mice (n=3-4/group). Data were normalized in comparison with the housekeeping gene 36B4. Expression levels of mutants are expressed as percent change compared to control levels. The DOR transcript was undetectable in OB, CPu and NAc, and partially decreased in PFC, Hipp and AMG of Dlx-MOR mice. **(B-C)** Quantitative DOR ligand binding autoradiography. Brain sections were labeled with [$^3$H] deltorphin-1 and all sections were processed in parallel throughout binding and development of autoradiograms. Representative autoradiograms from brain **(B)** and spinal cord **(C)** sections are shown for the three genotypes. The color bar code shows a pseudo-colour interpretation of relative densities from black and white images calibrated in fmol/mg tissue. Non-specific binding was homogenous and at background levels. **(D)** Shematic representation of DOR expression pattern of Dlx-DOR mice compared to control Ctrl mice. Regions highlighted in orange correspond to brain areas showing significant reduction of DOR expression, and numbers represent percent change of DOR expression in conditional mutant mice from Table 1. Abbreviations: Amy, amygdala; Cg, cingulated cortex; CPu, caudate-putamen nucleus; FCx, frontal cortex; Hipp, hippocampus; NAc, nucleus accumbens; OB, olfactory bulb; RS, retrosplenial cortex; Sc, spinal cord.

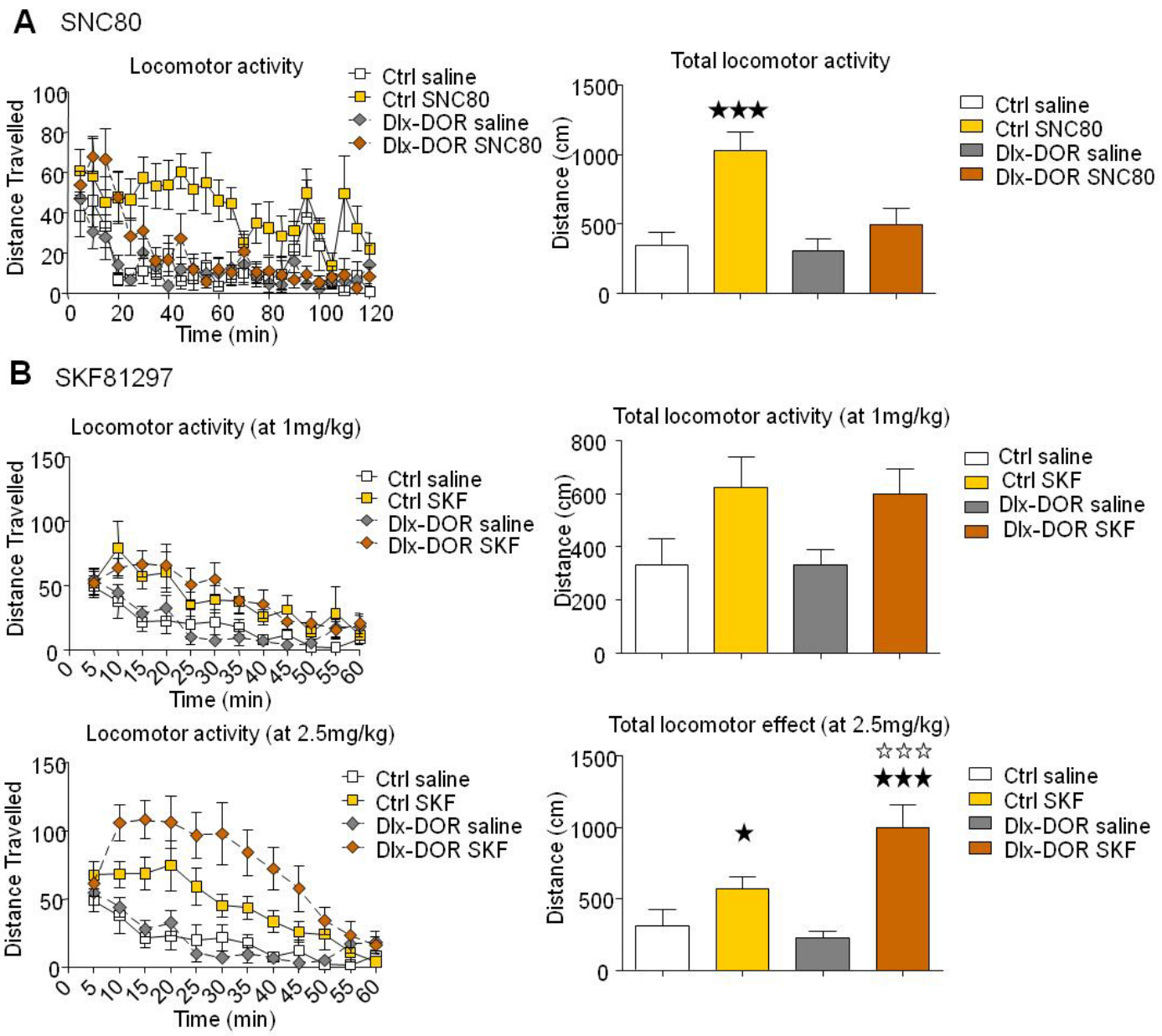



Fig. 2: **DOR and D1-mediated locomotor activity.** Ctrl and Dlx-DOR mice were tested in actimetry boxes for responses to **(A)** DOR agonist SNC80 or **(B)** the dopamine D1 agonist SKF81297. **(A)** Left panel shows locomotor activity in Ctrl and Dlx-DOR mice treated by intraperitoneal injection of SNC80 (10 mg/kg) or saline over a 2H session, and right panel shows total activity. Activity was significantly increased in SNC80 Ctrl mice only (n= 8-10 per genotype and treatment). **(B)** In a second cohort, SKF-81297 was administered subcutaneously (at dose 1 or 2.5 mg/kg). Both Ctrl and Dlx-DOR mice showed increased locomotor activity as compared to vehicle-treated mice, and this effect was significantly stronger in Dlx-DOR mice at the high dose (left, time course; right total activity; n = 9-11 mice per genotype and treatment). Filled and open stars indicate significant treatment or genotype effect, respectively. One star, $P<0.05$; three stars, $P<0.001$ (one-way ANOVA).

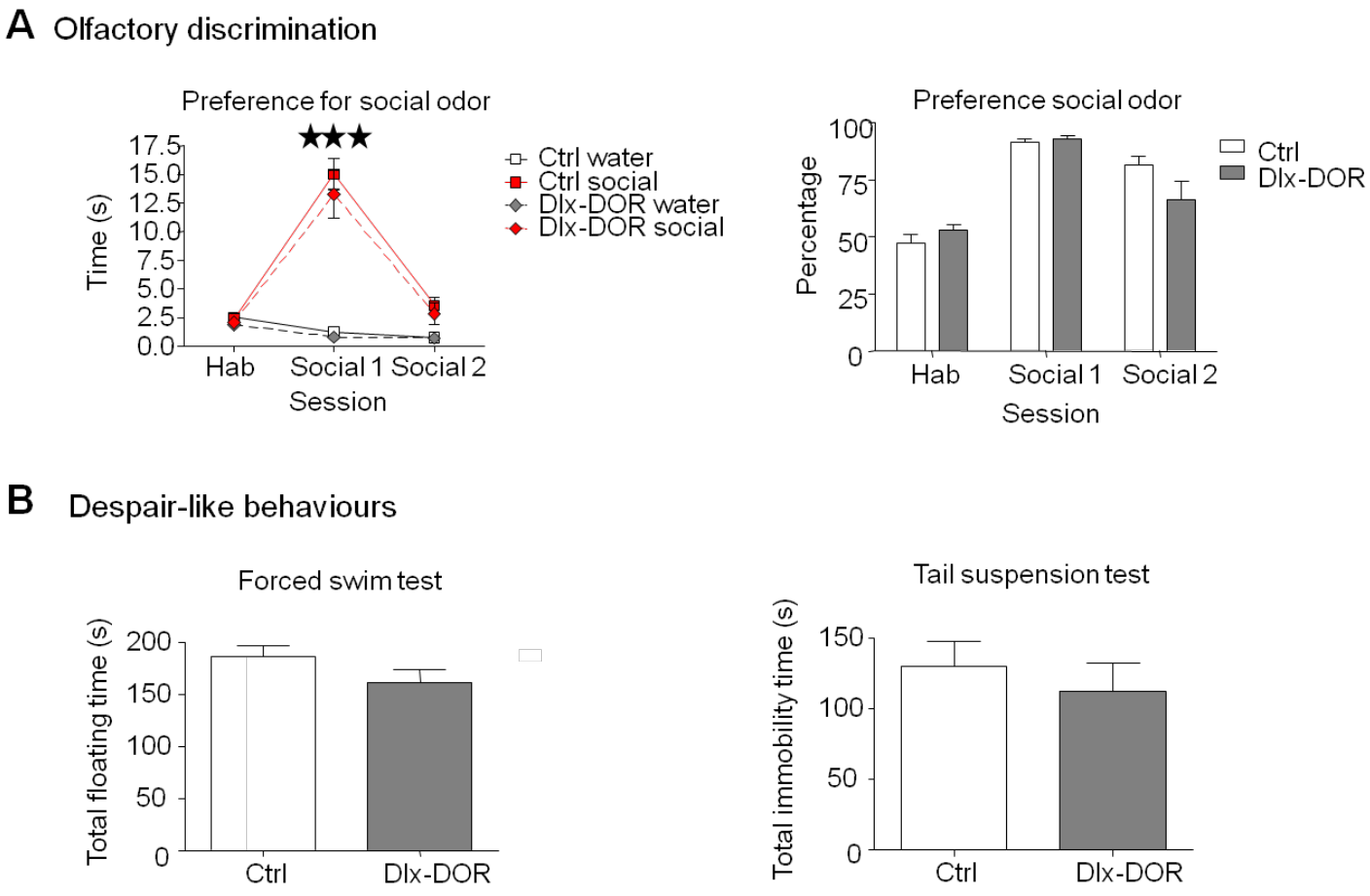



Fig. 3: **Olfactory discrimination skills and despair behavior**. Ctrl and Dlx-DOR mice were tested for olfactory discriminative skills. Hab, habituation session with two neutral odors presented; Social 1, first exposure to social versus neutral odor (water); Social 2, exposure to same odors as Social 1 but in inverted positions **(A)** Time spent sniffing the social and neutral odor (left panel) and preference for the social odor (right). Dlx-DOR and Ctrl mice showed comparable increased exploration time for the social odor at first exposure and showed similar preference above chance level when exposed to the social odor (n= 11-16 per genotype). **(B)** Forced swim test (left) and tail suspension test (right). A slight decrease in immobility time was observed in Dlx-DOR mice (gray bars) compared with Ctrl (white bars), however no significant difference was found across genotypes (n= 16 per genotype). For all the tests, filled stars indicate significant differences between treatments. Three stars, $P<0.001$ (one-way ANOVA).

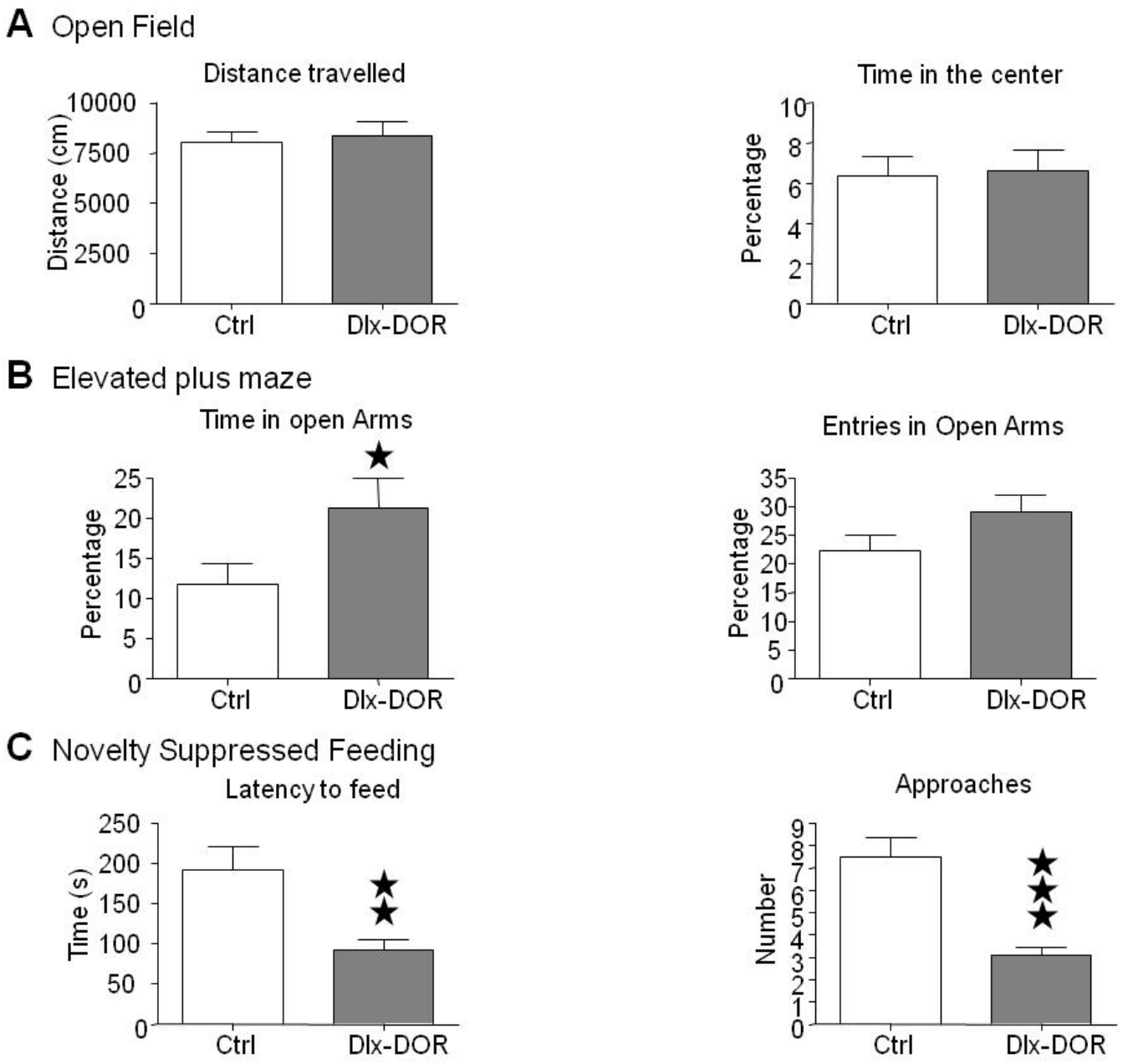



Fig. 4: **Anxiety-related behaviors.** Three tests using increasing stress conditions were used, namely the open field, the elevated plus maze and the novelty suppressed feeding test, in that order. **(A)** Open Field. Distance travelled (left) and time spent in center (right) did not differ across genotypes. **(B)** Elevated plus-maze. Dlx-DOR showed increased time in open arms (left) and a trend to more entries in those arms (right) compared to Ctrl mice. General activity was similar (total visits) between the two groups (data not shown), reflecting no change in spontaneous locomotor activity. **(C)** Novelty suppressed feeding. Latency to feed was decreased (left), and accordingly number of approaches was decreased also (right) in Dlx-DOR mice compared with Ctrl mice. n=16 per genotype, and filled stars represent significant differences compare to Ctrl mice. One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$ (Student *t*-test)

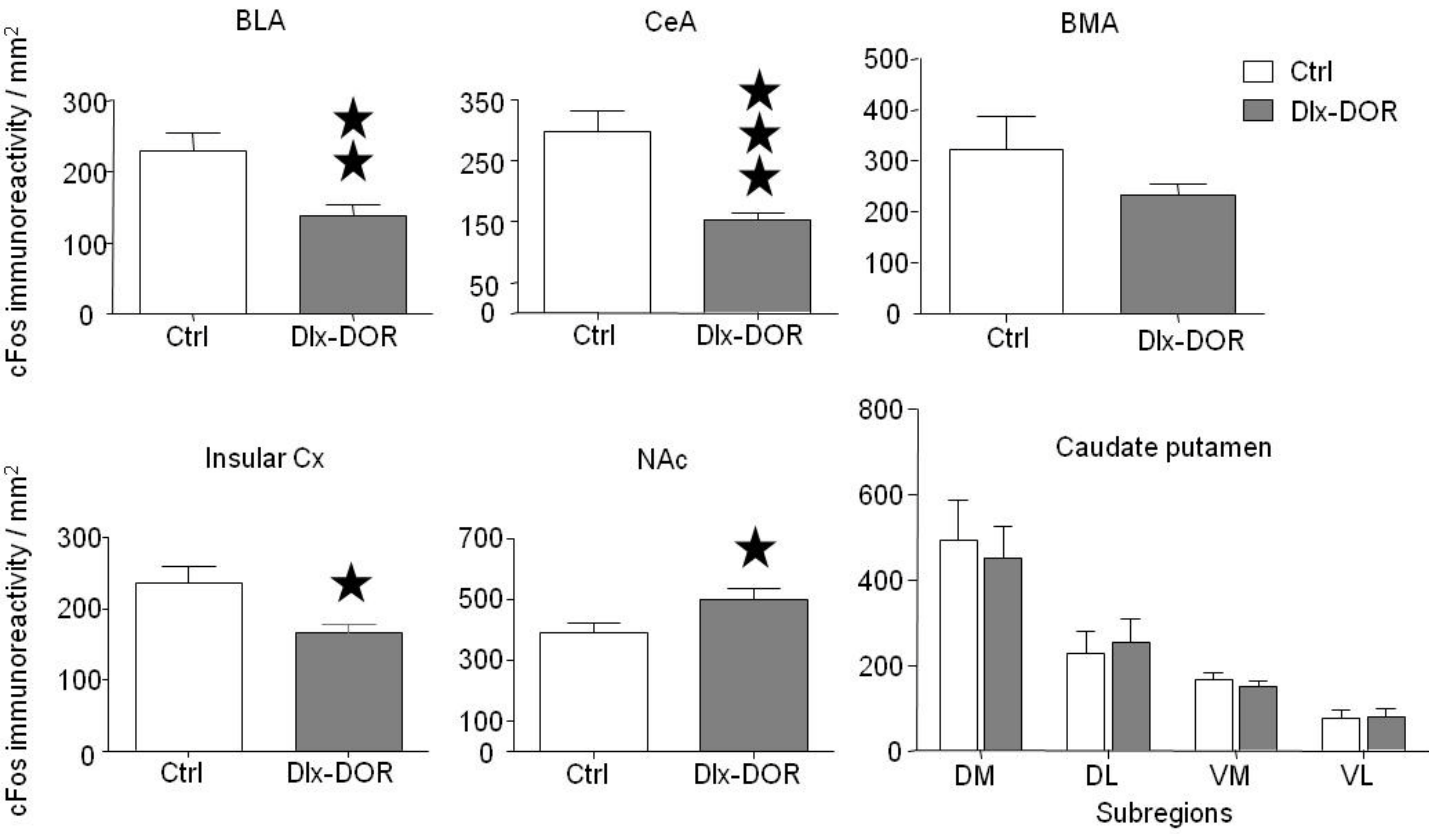



Fig. 5: **Neural activity after novelty suppressed feeding.** Dlx-DOR and Ctrl mice were subjected to the novelty suppressed feeding task, and tested for c-fos immunoreactivity 2 hours after end of the test. Quantification is expressed as number of c-fos positive cells per $mm^2$. There is a significant reduction of c-fos positive cells in the BLA, CeA and insular cortex from Dlx-DOR compared to Ctrl mice. Conversely, c-fos immunoreactivity is significantly increased in the NAc of Dlx-DOR mice. Number of c-fos positive cells is comparable in BMA and caudate putamen of Dlx-DOR and Ctrl mice. Abbreviations: BLA, basolateral nucleus of the amygdala; BMA, basomedial nucleus of the amygdala; CeA, central nucleus of the amygdala; NAc, nucleus accumbens. n = 6-9 animals per genotype / 4-12 sections per regions / 2 counts per sections. One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$ (Student *t*-test).

**Table. 1: Quantitative autoradiography of brain δ-opioidreceptor binding in wild-type (+/+) and conditional homozygous (-/-) mutant mice.**

| Region | | Bregma co-ordinates | [$^3$H]DELT-1 specific binding (fmol/mg tissue) Ctrl (*n* = 3) | Dlx-DOR (*n* = 4) | % change |
|---|---|---|---|---|---|
| **Cortical areas** | | | | | |
| Motor | | 2.10 | | | |
| *Superficial layers* | MtCx(Sl) | | 105.6 ± 16.4 | 103.1 ± 11.1 | -2.3 |
| *Deep layers* | MtCx(Dl) | | 92.1 ± 13.3 | 94.6 ± 11.8 | 2.6 |
| Orbital | | 2.10 | | | |
| *Superficial layers* | OrCx(Sl) | | 61.8 ± 15.0 | 69.3 ± 7.7 | 12.0 |
| *Deep layers* | OrCx(Dl) | | 79.7 ± 12.1 | 83.9 ± 14.0 | 5.3 |
| Frontal | | 1.98 | | | |
| *Superficial layers* | FrCx(Sl) | | 112.7 ± 16.5 | 100.2 ± 12.4 | -11.1 |
| *Deep layers* | FrCx(Dl) | | 83.2 ± 9.8 | 94.6 ± 10.5 | 13.7 |
| Cingulate | | 1.10 | | | |
| *Superficial layers* | CgCx(Sl) | | 89.5 ± 15.4 | 87.3 ± 14.3 | -2.5 |
| *Deep layers* | CgCx(Dl) | | 97.4 ± 14.8 | 95.8 ± 15.1 | -1.7 |
| Frontal-Parietal | | 1.10 | | | |
| *Superficial layers* | FrPCx(Sl) | | 93.3 ± 14.3 | 97.9 ± 13.5 | 4.9 |
| *Deep layers* | FrPCx(Dl) | | 93.6 ± 14.3 | 93.1 ± 15.8 | -0.5 |
| Insular | | 1.10 | | | |
| *Superficial layers* | InCx(Sl) | | 120.6 ± 16.0 | 112.4 ± 20.6 | -6.8 |
| *Deep layers* | InCx(Dl) | | 124.1 ± 19.2 | 115.5 ± 18.0 | -6.9 |
| Rostral somatosensory | | 1.10 | | | |
| *Superficial layers* | SsRCx(Sl) | | 101.1 ± 8.6 | 84.6 ± 13.9 | -16.3 |
| *Deep layers* | SsRCx(Dl) | | 81.6 ± 9.7 | 78.9 ± 12.4 | -3.4 |
| Parietal | | -1.46 | | | |
| *Superficial layers* | PtACx(Sl) | | 124.8 ± 14.9 | 92.5 ± 14.8 | -25.9 |
| *Deep layers* | PtACx(Dl) | | 97.0 ± 18.5 | 87.1 ± 11.0 | -10.2 |
| Caudal somatosensory | | -2.06 | | | |
| *Superficial layers* | SsCCx(Sl) | | 118.7 ± 9.5 | 86.9 ± 11.6 | -26.8 |
| *Deep layers* | SsCCx(Dl) | | 89.9 ± 9.3 | 92.3 ± 15.2 | 2.7 |
| Retrosplenial | | -2.06 | | | |
| *Superficial layers* | RSCx(Sl) | | 70.2 ± 7.1 | 56.7 ± 7.0 | -19.2 |
| *Deep layers* | RSCx(Dl) | | 78.7 ± 11.6 | 79.3 ± 9.0 | 0.8 |
| Temporal | | -2.06 | | | |
| *Superficial layers* | TeACx(Sl) | | 118.0 ± 13.3 | 85.1 ± 15.2 | -27.9 |
| *Deep layers* | TeACx(Dl) | | 117.5 ± 14.7 | 96.3 ± 11.7 | -18.1 |
| Perirhinal | PRhCx | -2.06 | 120.5 ± 19.6 | 106.9 ± 11.1 | -11.3 |
| Auditory | | -2.54 | | | |
| Superficial layers | AuCx(Sl) | | 104.9 ± 11.8 | 76.2 ± 10.5 | -27.4 |
| Deep layers | AuCx(DL) | | 96.0 ± 10.7 | 78.6 ± 10.2 | -18.2 |
| Visual | | -3.52 | | | |

| | | | | | |
|---|---|---|---|---|---|
| Superficial layers | ViCx(Sl) | | 104.3 ± 9.4 | 89.6 ± 7.5 | -14.1 |
| Deep layers | ViCx(Dl) | | 92.9 ± 11.7 | 71.3 ± 10.2 | -23.2 |
| Entorhinal | EntCx | -3.64 | 54.2 ± 8.6 | 52.0 ± 7.4 | -4.1 |
| **Non-cortical areas** | | | | | |
| Olfactory bulb | | 3.56 | | | |
| External plexiform Layer | EPl | | 200.5 ± 27.1 | 0.0 ± 0.0 *** | -100.0 |
| Internal granular layer | IGl | | 84.3 ± 14.0 | 0.0 ± 0.0 *** | -100.0 |
| Nucleus accumbens | | 1.18 | | | |
| Core | AcbC | | 60.0 ± 11.7 | 24.4 ± 9.2 | -59.3 |
| Shell | AcbSh | | 68.2 ± 16.0 | 20.2 ± 8.3 ** | -70.3 |
| Caudate putamen | | 1.10 | | | |
| Medial | CPuL | | 77.3 ± 21.5 | 22.1 ± 7.0 *** | -78.8 |
| Lateral | CPuM | | 128.9 ± 34.6 | 33.2 ± 9.2 *** | -82.9 |
| Tubercle | Tu | 1.10 | 168.4 ± 42.7 | 16.4 ± 6.4 *** | -80.3 |
| Septum | | 0.74 | | | |
| Medial | MS | | 29.7 ± 6.7 | 18.1 ± 7.8 | -39.0 |
| Lateral | LS | | 37.0 ± 8.7 | 22.4 ± 9.2 | -39.4 |
| Vertical limb of the diagonal band | VDB | 0.74 | 16.9 ± 4.2 | 19.9 ± 6.3 | 17.8 |
| Globus pallidus | GP | -0.22 | 44.8 ± 12.1 | 15.5 ± 6.4 | -65.3 |
| Preoptic area | PoA | -0.22 | 12.6 ± 3.7 | 12.8 ± 5.0 | 1.4 |
| Thalamus | Th | -1.46 | 17.2 ± 2.9 | 21.0 ± 6.3 | 22.4 |
| Amygdala | | -1.46 | | | |
| Basolateral | BLA | | 77.5 ± 17.4 | 82.6 ± 21.0 | 6.5 |
| Basomedial | BMA | | 76.7 ± 20.8 | 81.2 ± 13.7 | 5.8 |
| Medial | CeM | | 43.2 ± 12.1 | 47.7 ± 15.1 | 10.4 |
| Hypothalamus | Hyp | -1.46 | 16.3 ± 3.8 | 17.3 ± 5.0 | 6.1 |
| Hipppcampus | | | | | |
| CA1 | CA1 | -2.06 | 47.6 ± 7.3 | 20.3 ± 2.9 | -57.3 |
| CA2/3 | CA2/3 | -2.06 | 52.1 ± 8.6 | 17.3 ± 3.2 * | -66.8 |
| Dentate gyrus | DG | -2.06 | 59.0 ± 8.9 | 28.5 ± 5.6 | -51.7 |
| Dorsal | dHip | -3.80 | 47.4 ± 7.4 | 22.7 ± 4.7 | -52.1 |
| Presubiculum | Prs | -3.64 | 53.0 ± 13.3 | 31.1 ± 8.9 | -41.4 |
| **Spinal Cord** | | | | | |
| Cervical | C( | | | | |
| Whole section | | | 16.0 ± 9.5 | 21.1 ± 7.2 | 30.0 |
| Superficial layers (lamina I and II) | | | 24.8 ± 9.3 | 29.4 ± 8.8 | 18.6 |
| Laminas III-IV | | | 17.9 ± 6.9 | 21.6 ± 6.3 | 21.2 |
| Lamina X | | | 16.8 ± 8.5 | 18.9 ± 7.5 | 12.4 |
| Ventral horn (laminas VII -IX) | | | 18.8 ± 6.9 | 22.1 ± 6.7 | 17 |

Table 1: **Quantification of specific [3H] deltorphin-1 binding.** Values represent mean ± SEM fmol/mg of tissue equivalent in brain regions of Ctrl and Dlx-DOR mice.

Bregma coordinates are taken from the mouse brain atlas of Franklin and Paxinos (Franklin and Paxinos 1997). Specific binding was calculated after the subtraction of non-specific from total [$^3$H] deltorphin-1 binding. Percent change in binding indicates change in Dlx-DOR compared to Ctrl mice. N indicates number of animals per group. No [$^3$H] deltorphin-1 binding could be detected in full knockout brains, data not shown. Two-way ANOVA revealed significant effect of Genotype, Region and Genotype x Region, all $p < 0.001$. Post-hoc t-test comparisons revealed significant within-region differences compared to WT: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

**Table 2. Basal locomotor activity in Ctrl, Dlx-DOR and CMV-DOR mice.**

| Genotype | Total locomotor activity | During light period (8h) | During night period (12h) | Number of food pellet distributed |
|---|---|---|---|---|
| Ctrl | 4850.43 ± 1282.05 | 2246.29 ± 583.2 | 2604.14 ±707.94 | 288 ± 10.59 |
| Dlx-DOR | 5306.80 ± 851.83 | 2208.6 ± 353.72 | 3098.20 ± 514.76 | 303.3 ± 35.43 |
| CMV-DOR | 6606.33 ± 2013.84 | 2476 ± 703.48 | 4130.33 ± 1320.1 | 209.5 ± 41.96 |

Table 2: **Basal locomotor activity in Ctrl, Dlx-DOR and CMV-DOR mice.** Total locomotor activity was automatically recorded during 20h (from 3 P.M. to 11 A.M.) in actimetry boxes. Values represent the number of infrared beams crossed for the whole session, the light period (8h) or the dark period (12h). There were no statistical differences. CMV-DOR mice showed a trend toward an increased locomotor activity as compare to Ctrl and Dlx-DOR mice. Number of food pellet distributed during the session was measured and any differences appeared. n= 8 per genotype. Statistical analysis was performed using repeated 2-way analysis of variance with Bonferroni *post hoc* tests (One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$).

**Table. 3: c-fos immunoreactivity in Ctrl and Dlx-DOR mice following NSF.**

| Region | | Bregma coordinates | c-fos positive cells / $mm^2$ | | Stats |
|---|---|---|---|---|---|
| | | | Ctrl | Dlx-DOR | |
| Nucleus accumbens | NAc | 1.18 | 391.0 ± 38.4 | 497.3 ± 43.2 | $p < 0.05$ |
| Cingulate cortex | Cg Cx | 1.10 | 986.9 ± 60.3 | 870.2 ± 85.4 | $p > 0.05$ |
| Insular cortex | Ins Cx | 1.10 | 237.1 ± 22.2 | 165.4 ± 11.9 | $p < 0.05$ |
| Striatum | | 1.10 | | | |
| *Dorsomedial* | DM | | 492.2 ± 95.1 | 448.9 ± 77.5 | $p > 0.05$ |
| *Dorsolateral* | DL | | 228.1 ± 50.2 | 253.1 ± 55.3 | $p > 0.05$ |
| *Ventromedial* | VM | | 168.0 ± 14.6 | 151.9 ± 9.9 | $p > 0.05$ |
| *Ventrolateral* | VL | | 74.6 ± 21.6 | 79.4 ± 19.3 | $p > 0.05$ |
| Amygdala | | -1.46 | | | |
| *Basolateral nucleus* | BLA | | 228.3 ± 26.4 | 138.7 ± 14.1 | $p < 0.01$ |
| *Basomedial nucleus* | BMA | | 323.0 ± 65.9 | 232.6 ± 21.4 | $p > 0.05$ |
| *Central nucleus* | CeA | | 298.6 ± 33.6 | 153.0 ± 12.0 | $p < 0.001$ |
| Ventral Tegmental Area | VTA | -2.54 | 274.17 ± 28.32 | 352.38 ± 33.25 | $p > 0.05$ |

Table 3: **Quantification of c-fos immunoreactivity after NSF.** Ctrl and Dlx-DOR mice were sacrificed 90 min after the NSF test. c-fos protein labeling is obtained by immunocytochemistry on brain sections. The quantification is performed on images acquired on the Hamamatsu scanner and expressed in number of c-fos positive cells per $mm^2$. The level of c-fos positive cells in the Cg Cx, four subregions of the striatum, BMA and VTA is similar in the two groups. However, the quantification reveals a significant reduction of c-fos positive cells in the BLA, CeA and insular cortex on sections from Dlx-DOR in comparison with the Ctrl mice, whereas a significant increase is found in the NAc. n = 6-9 animals per genotype / 4-12 sections per regions / 2 counts per sections.

CCTGGCCAGCCAGTTCACAATCT (Oprd1 forward) and GGTTAGCCTTCTGAGGGCTGGG (Oprd1 reverse).

**Quantitative real time-PCR.**

The olfactory bulb (OB), prefrontal cortex (PFC), caudate-putamen nucleus (CPu), nucleus accumbens (NAcc), amygdala (AMG) were bilaterally punched and ventral (vHipp), dorsal hippocampus (dHipp) and spinal cord (SC) were dissected from 4 animals per genotype, and RNA samples processed for quantitative real time PCR, as detailed previously (Befort, Filliol et al. 2008). Briefly, total RNA was extracted by TriZol (Invitrogen, Cergy Pontoise, France). Quality and quantity of the RNA was evaluated by a ND-1000 Nanodrop spectrophotometer and gel electrophoresis. Total RNA (1μg) from each bilaterally pooled brain regions was reverse transcribed in a final volume of 20μl real-time PCR was performed on cDNA in triplicate on a Light-Cycler-480 instrument (Roche). Primer sequences were GACGGCCAGGTCATCACTAT (β-actin forward), CCACCGATCCACACAGAGTA (β-actin reverse), TGAGATTCGGGATATGCTGTTG (36B4 forward), TTCAATGGTGCCTCTGGAGAT (36B4 reverse), GCTCGTCATGTTTGGCATC (Oprd1 forward) and AAGTACTTGGCGCTCTGGAA (Oprd1 reverse). Relative expression ratios were normalized to level of the 36B4 reference gene, and the $2^{-\Delta\Delta C_t}$ method was used to evaluate differential expression levels (Livak and Schmittgen 2001).

**Autoradiographic Binding Assay**

Following decapitation, intact brains were removed, snap frozen at -20°C in isopentane and then stored at -80°C until sectioned. Brains were sectioned in a cryostat (Zeiss Hyrax C 25, Carl Zeiss MicroImaging GmbH, Germany), with an internal temperature of -21°C. Spinal cords were mounted in OCT medium before sectioning.20 µm coronal sections were cut at 300 µm intervals, from rostral to caudal levels and thaw-mounted onto gelatine coated ice-cold microscope slides and processed for autoradiography. Adjacent sections were cut from wild-type, Dlx-DOR and full CMV-DOR brains (n = 3) for determination of total binding for DOR using [$^3$H] deltorphin-1. Sections were stored at -20°C for radioligand binding.

On the day of the experiment, sections were thawed and processed according to established protocols (Kitchen, Leslie et al. 1995; 1997), with minor modifications. Sections for analysis were derived from four to six brains from each of the six treatment groups (n=3-4 per group). Multiple, adjacent sections from all groups were processed together in a paired binding protocol.

For binding, slides were pre-incubated for 30 min in 50 mM Tris-HCl pre-incubation buffer, containing 0.9% w/v NaOH, pH 7.4 at room temperature. The slides were then incubated in 50 mMTris-HCl buffer, pH 7.4 at room temperature in the presence of 7 nM [$^3$H]deltorphin-1 for 60 min. Non-specific binding (NSB) was determined in adjacent sections in the presence of 10 µM naloxone. Incubation was

terminated by rapid rinses (3 x 5 min) in ice-cold 50 mM Tris-HCl buffer, pH 7.4 at room temperature and distilled water (3 x 5 min). Slides were then rapidly cool-air dried.

Film exposure and development. Following binding, sections were rapidly dried under cold air for 2 hours, and dried for up to 7 days using anhydrous calcium sulphate (BDH Chemicals, Poole, UK). Adjacent total and non-specific labeled sections were apposed to Kodak BioMax MR-1 film alongside autoradiographic microscale standards of known concentration. [$^{3}$H]-bound sections were exposed to film with $^{3}$H microscale standards for a period of 10 weeks for opioid receptors.

For development, films were covered with an aqueous solution of 50 % v/v Kodak D19 developer for 1 min. The reaction was stopped by 1 min rinse in distilled water containing a drop of glacial acetic acid. Images were fixed by submersion in Kodak rapid fix solution for 5 min. Films were then rinsed in distilled water and dried overnight in a fume cupboard.

Quantitative analysis. Films were analyzed by video-based densiometry using an MCID image analyzer (Imaging Research, Canada) as previously described by Kitchen et al. (1997). In brief, fmol/mg tissue equivalents for receptor binding were derived from [$^{3}$H] microscale standards, and the relationship between tissue radioactivity and optical density was calculated using MCID software, with appropriate adjustments to allow for radioactive decay of both the standards and the radioligands. Specific receptor binding was derived by subtraction of NSB from total binding. NSB was homogenous across each film.

For each region quantified measures were taken from both left and right hemispheres, therefore receptor binding represents a duplicate determination for each brain region and the *n* values listed refer to the number of animals analyzed. The following structures were analyzed by sampling 5 – 20 times with a box tool: cortex (8 x 8 mm), olfactory tubercle (6 x 6 mm) and hippocampus (5 x 5 mm). All other regions were analyzed by free-hand drawing. Brain structures were identified by reference to the mouse atlas of Franklin and Paxinos (1997).

**Agonist-Stimulated [$^{35}$S]-GTPγS Binding Assays**

Brain areas obtained by mechanical punches on 1 mm thickness sections (OB, Hipp and CPu) and spinal cord (SC) from Ctrl, Dlx-DOR and CMV-DOR mice were processed for membrane preparation as described (Pradhan, Becker et al. 2009). Samples were incubated with and without the DOR agonist ARM390 ($10^{-4}$ to $10^{-11}$ M) for 1h at 25°C in assay buffer containing 30 μM GDP and 0.1 nM [$^{35}$S]GTPγS. Non specific binding was defined as binding in the presence of 10 μM GTPγS, and basal binding indicates binding in the absence of agonist (Figure S1 and Table S1).

**Behavioral Assays**

The behavioral tests were performed as previously described (Goeldner, Lutz et al. ; Filliol, Ghozland et al. 2000; Le Merrer, Rezai et al. 2013).

**Olfactory discrimination**

This test has been adapted from a previously described procedure (Yang and Crawley 2009) and is used to assess olfactory discriminative ability. A cohort of Ctrl and Dlx-DOR mice was assessed for water versus social odor discrimination, and a second cohort for water versus non-social odors (lemon) discrimination. Odor exploration was measured in a clean cage with fresh bedding. Odors were presented on two cotton-tipped wooden applicators. Animals were first habituated to the novel cage for 3 min, and then tested during 3 consecutive sessions of 3 min each with 2 min inter-trial interval (ITI). During each session, two applicators are presented: ("Hab" session) water-water, ("Odor 1" session) water-odor and ("Odor 2" session) odor-water. The position of the odor for the sessions ("Odor 1") and ("Odor 2") was randomized. Two stopwatches are used to record the cumulative time spent sniffing each tip.

**Depressive-like behavioral tests**

*Forced swim test (FST)*. This test allows induction of a depressed state by forcing mice to swim in a narrow cylinder from which they cannot escape. Each mouse was placed in a Plexiglas cylinder containing water to a depth of 15 cm (21 °C–23 °C) as previously described (Porsolt, Anton et al. 1978). After a brief period of vigorous activity, the mice adopt a characteristic immobile posture that is reversed by the administration of compounds with antidepressant activity. Each animal was submitted to a forced swim session of 6 min, and the total duration of immobility, swimming and climbing behaviors were measured.

*Tail Suspension test (TS)*. This test allows assessment of depressive-like behaviors and was performed as previously described (Steru, Chermat et al. 1985). Mice were suspended 50cm above the floor by adhesive tape placed approximately 1cm from the tip of the tail. The total duration of immobility and the latency for the first immobility period of at least 2s are automatically recorded during a 6-min period as previously described.

**Locomotion**

*Locomotor activity boxes.* Mice were placed individually in actimetry boxes consisting of a plastic square area (25 x 25 x 25 cm, 100 lux). The distance covered by the mouse was recorded by a videotracking system for periods of 5 min, over 24h, with water and food pellet ad libitum.

*DOR or dopamine D1/D3 receptor agonists-induced hyperlocomotion*. Animals were tested in actimetry boxes as described above. Mice freely explored the box during 2h (habituation session), then received an injection of saline or SNC80 (10

mg/kg, intraperitoneal) or D1/D3 dopamine receptor agonist SKF-81297 (1 or 2.5 mg/kg, subcutaneous injection) and were placed back in the same boxes for further recording (90 min).

**Anxiety-related tests**

*Open Field (OF).* The apparatus is composed of a black ground square (45 x 45 cm) limited by transparent Plexiglas walls (18 cm) and under indirect illumination (50 lux). Test was performed as previously described (Filliol, Ghozland et al. 2000). Movements are detected by infrared beams and sensors so that distance (cm) and time spent (s) in periphery and center parts of the apparatus were automatically recorded (Viewpoint software) each period of 5 min. Sessions lasted for 30min starting with the mouse is positioned in a corner.

*Elevated Plus-Maze test (EPM).* The EPM consisted of four arms (30 x 5 cm) in black Plexiglas set in cross from a neutral central square (5 x 5 cm) (Imetronic). Two opposite arms were delimited by vertical walls (closed arms) and the two other opposite arms had unprotected edges (3 mm) (open arms). The maze is elevated 60cm above the ground and place in indirect light (50 lux). Test was performed as previously described (Pellow, Chopin et al. 1985; Filliol, Ghozland et al. 2000). Movements are detected by infrared beams and sensors so that locomotor activity, time spent, number of entries and number of attempts to enter in open or closed arms were automatically recorded (Viewpoint software). Sessions lasted for 5min starting with the mouse in the central square.

*Novelty Suppressed Feeding (NSF)*. The NSF is a conflict test based on opposite behaviors: the motivation to obtain the food pellet versus the natural avoidance of an aversive environment. The NSF was carried out as previously described (Santarelli, Gobbi et al. 2001). The testing box consisted of an open field box (50 x 50 x 35 cm) with 5 cm of fresh sawdust on the floor. Two or three food pellets of ordinary lab chow were placed on a white paper positioned at the center of the apparatus. After 24 h of food deprivation (no water deprivation), mice were placed in a corner of the testing apparatus. Sessions lasted for a maximum of 15 min. We count the number of approaches to the food pellets and the latency to feed that was scored as the time when the mice began biting the food. Immediately after that, the mice were transferred to their home cage for 5 min, and food intake amount over this time was measured (home-cage food intake).

**c-Fos immunohistochemistry**

Animals were deeply anesthetized with an overdose of ketamine (1 g/kg) and xylazine (100 mg/kg) solution (10 ml/kg, intraperitoneal) 90 min after the beginning of the behavioral test (NSF), and perfused transcardially with 50 ml of 0.1 M phosphate buffer (PB, pH 7.4) followed by 50 ml of cold 4% paraformaldehyde prepared in 0.1 M PB. Brains were dissected, post-fixed for 48 hours in the same fixative and cryoprotected in 30 % sucrose/PB overnight at 4°C. Frozen brains were stored at -80°C until 50 μm coronal sections were cut on a cryostat.

Immunohistochemistry was performed on free-floating sections using a standard avidin-biotin (ABC) peroxidase method (Elite Vectastain Kit, Vector Laboratories, Burlingame, CA, USA) as previously (Le Merrer, Gavello-Baudy et al. 2007). The peroxidase was detected with diaminobenzidine (Sigma-Aldrich, Saint-Quentin, France) as chromogen. The primary antibody was a rabbit polyclonal antibody (Ab-5, Calbiochem®, Merck, Darmstadt, Germany, 1:2,000) rose against a synthetic peptide derived from amino acid sequences 4-17 of the Fos protein. The secondary antibody was a biotinylated goat anti-rabbit IgG (Jackson Immunoresearch, West Baltimore Pike, PA, USA, 1:2,000).

Slides were acquired using a Hamamatsu Nanozoomer 2-HT whole slide scanner (Hamamatsu Photonics, Hamamatsu, Japan) at 20x magnification. Frames focused on each structure of interest were acquired using NDP View software, and Fos-positive nuclei were counted using ImageJ software (NIH). Data were expressed as the number of Fos-positive nuclei per $mm^2$. The number of Fos-immunoreactive neurons in each brain region was assessed bilaterally using 6 to 12 sections for each animal (6 to 8 mice per genotype). Fos immunostaining was evaluated in 8 cerebral regions (the basolateral, central and basomedial nuclei of the amygdala; the caudate-putamen nucleus; the cingulate cortex; the insular cortex; the nucleus accumbens core and shell; the ventral tegmental area) according to the mouse brain atlas (Franklin and Paxinos 1997). Brain regions of interest were selected as involved in anxiety and reward/approach processes.

**Drugs**

The SNC80 (Tocris Bioscience, Bristol, UK) was prepared as previously described (Pradhan, Becker et al. 2009). The powder was dissolved in NaCl 0.9 % at a concentration of 10 mg/kg. The solutions were prepared before the experiments.

The dopamine D1 receptor agonist SKF-81297 (2,3,4,5-tetrahydro-6-chloro-7,8-dihydroxy-phenyl-1H-3-benzazepine; Tocris Bioscience, Bristol, UK) was prepared as previously described (Le Merrer, Rezai et al. 2013). The SKF-81297 was dissolved in NaCl 0.9 % at concentrations of 1 or 2.5 mg/kg.

Both compounds were administered intraperitoneally before the experiments in a volume of 10 ml/kg.

**Statistical analyses**

Statistical differences were determined by analysis of variance (ANOVA) (StatView 5, SAS Institute Inc., Cary, North Carolina) followed by Bonferroni/Dunn post hoc analysis. The F values and experimental degrees of freedom are included in the Results Section. For experiments with two groups, a Student t test was used. The level of statistical significance was set at $p < 0.05$. For the behavioral tests during which data were obtained on several periods during the same session (locomotor tests, the Open Field test and despair-like behavior paradigms), the analysis of variance repeated measures was used.

**Supplemental References**


Befort, K., D. Filliol, et al. (2008). "Mu-opioid receptor activation induces transcriptional plasticity in the central extended amygdala." Eur J Neurosci **27**(11): 2973-2984.

Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.

Franklin, K. B. J. and G. Paxinos (1997). The Mouse Brain in Stereotaxic Coordinated. San Diego, CA, Academic Press.

Gaveriaux-Ruff, C., C. Nozaki, et al. (2011). "Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia." Pain **152**(6): 1238-1248.

Goeldner, C., P. E. Lutz, et al. "Impaired emotional-like behavior and serotonergic function during protracted abstinence from chronic morphine." Biol Psychiatry **69**(3): 236-244.

Kitchen, I., F. M. Leslie, et al. (1995). "Development of delta-opioid receptor subtypes and the regulatory role of weaning: radioligand binding, autoradiography and in situ hybridization studies." The Journal of Pharmacology and Experimental Therapeutics **275**(3): 1597-1607.

Kitchen, I., S. J. Slowe, et al. (1997). "Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene." Brain Research **778**(1): 73-88.

Le Merrer, J., S. Gavello-Baudy, et al. (2007). "Morphine self-administration into the lateral septum depends on dopaminergic mechanisms: Evidence from pharmacology and Fos neuroimaging." Behav Brain Res **180**(2): 203-217.

Le Merrer, J., X. Rezai, et al. (2013). "Impaired Hippocampus-Dependent and Facilitated Striatum-Dependent Behaviors in Mice Lacking the Delta Opioid Receptor." Neuropsychopharmacology.

Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.

Metzger, D. and P. Chambon (2001). "Site- and time-specific gene targeting in the mouse." Methods **24**(1): 71-80.

Monory, K., F. Massa, et al. (2006). "The endocannabinoid system controls key epileptogenic circuits in the hippocampus." Neuron **51**(4): 455-466.

Pellow, S., P. Chopin, et al. (1985). "Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat." J Neurosci Methods **14**(3): 149-167.

Porsolt, R. D., G. Anton, et al. (1978). "Behavioural despair in rats: a new model sensitive to antidepressant treatments." Eur J Pharmacol **47**(4): 379-391.

Pradhan, A. A., J. A. Becker, et al. (2009). "In vivo delta opioid receptor internalization controls behavioral effects of agonists." PLoS One **4**(5): e5425.

Santarelli, L., G. Gobbi, et al. (2001). "Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function." Proc Natl Acad Sci U S A **98**(4): 1912-1917.

Steru, L., R. Chermat, et al. (1985). "The tail suspension test: a new method for screening antidepressants in mice." Psychopharmacology (Berl) **85**(3): 367-370.

Yang, M. and J. N. Crawley (2009). "Simple behavioral assessment of mouse olfaction." Curr Protoc Neurosci **Chapter 8**: Unit 8 24.

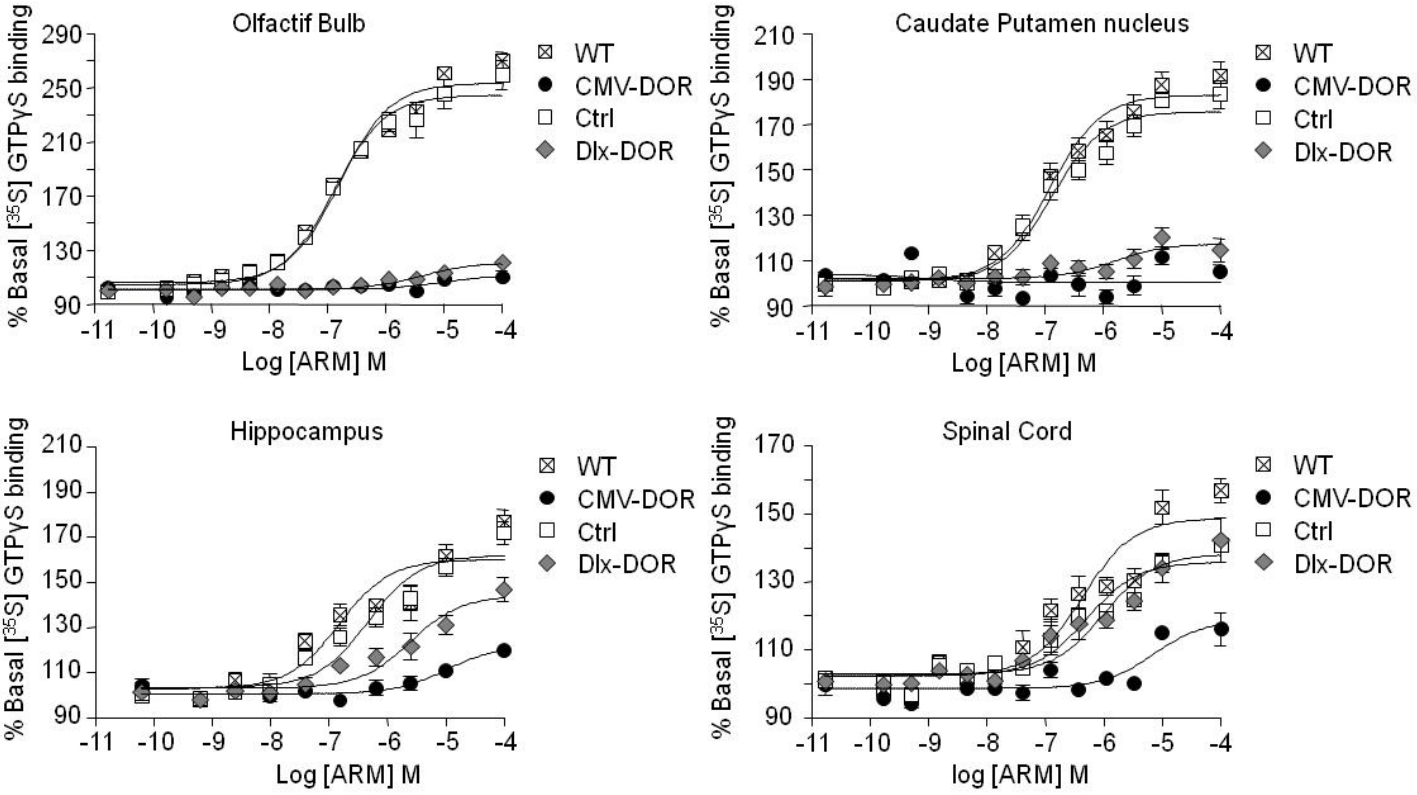



Fig. S1: **Agonist-stimulated [$^{35}$S]-GTPγS binding assays in mouse brain membrane**. The WT and Oprd$^{fl/fl}$ mice (Ctrl) expressed similar activation in all regions. The DOR agonist ARM390 induced a significant decreased level of G-protein activation in the olfactory bulbs and caudate putamen nucleus from Dlx-DOR mice compared to their control Ctrl littermates mice. There was a partial reduction in the hippocampus and no change in the spinal cord. The CMV-DOR mice samples showed no detectable G-protein activation in any regions. Abbreviations: AMG, amygdala; CPu, caudate-putamen nucleus; Hipp, hippocampus; NAc, nucleus accumbens; OB, olfactory bulb; PFC, prefrontal cortex; SC, spinal cord.

**Table S1. Agonist-Stimulated [$^{35}$S]-GTPγS Binding Assays**

**$EC_{50}$ ($10^{-7}$M)**

| Region | WT | Ctrl | Dlx-DOR | CMV-DOR |
|---|---|---|---|---|
| Olfactory bulb | 1.61 ± 0.16 | 1.38 ± 0.33 | 60.9 ± 24.8 | N/A |
| Hippocampus | 5.79 ± 3.7 | 6.47 ± 1.72 | 30.2 ± 11.6 | N/A |
| Caudate-putamen | 1.3 ± 0.2 | 2.4 ± 1.5 | 9.3 ± 4.9 | N/A |
| Spinal Cord | 4.3 ± 2.1 | 6.2 ± 2.3 | 17.9 ± 8.4 | 69.3 ± 34.7 |

**$E_{max}$ (%)**

| Region | WT | Ctrl | Dlx-DOR | CMV-DOR |
|---|---|---|---|---|
| Olfactory bulb | 253.6 ± 3.5 | 245.3 ± 10.2 | 121.7 ± 2.8 | 107.8 ± 2.9 |
| Hippocampus | 164.5 ± 7.9 | 163.1 ± 3.6 | 144.0 ± 4.2 | 119.1 ± 6.2 |
| Caudate-putamen | 183.0 ± 5.7 | 177.8 ± 5.6 | 116.5 ± 4.9 | 107.5 ± 4.4 |
| Spinal Cord | 150.6 ± 4.3 | 136.0 ± 1.1 | 140.4 ± 6.6 | 116.3 ± 3.9 |

Table. S1: **$EC_{50}$ and $E_{max}$ in the agonist-stimulated [$^{35}$S]-GTPγS binding assays**. $EC_{50}$ ($10^{-7}$M) represent the agonist concentration required to have 50% of the maximal activation. $E_{max}$ (%) correspond to the maximal percentage of activation. Similar $EC_{50}$ and $E_{max}$ were measured between the WT and Ctrl mice all regions. The CMV-DOR mice samples showed $E_{max}$ values close to 100%, reflecting no detectable G-protein activation in any regions. The decrease of $E_{max}$ values in the olfactory bulb and caudate-putamen from Dlx-DOR mice samples showed that the G-protein activation was strongly reduced. This reduction was also partially observed in the hippocampus of Dlx-DOR mice sample, whereas it remains at comparable level in the spinal cord.

# Supplementary experiments

## Introduction

We previously demonstrated that specific excision of DOR on forebrain GABAergic neurons alters behavioural responses in the elevated plus-maze and novelty suppressed feeding, whereas no phenotype is detected in the open field test. This difference emphasizes that diverse physiological processes are involved in these behavioural paradigms modelling anxiety-related responses. Here, we pursued experiments to further investigate emotional responses in Dlx-DOR mice.

The exposure to predator odor is another paradigm used to assess anxiety-related behaviors, with high ethological significance (Staples 2010). Interestingly, a recent study showed that exposure to 2, 4, 5-trimethylthiazoline, a predator odor increases Enk mRNA especially in the paraventricular nucleus of the hypothalamus, suggesting that the DOR/Enk system may be involved in physiological responses triggered by aversive odors (Asok, Ayers et al. 2013). Dlx-DOR mice previously demonstrated intact discriminative olfactory skills toward appetitive odours. In the present study, we therefore addressed the reactivity of Dlx-DOR mice when facing an aversive odour stimulus.

The reduced anxiety/high risk taking phenotype was detected in EPM, as well as the NSF representing a conflict test. Therefore, we secondly explored whether increased stress conditions in the elevated plus-maze may affect differentially Dlx-DOR mice behaviour. Furthermore, we addressed emotional responses of our mutant in another paradigm used to assess anxiety-related behaviours, the light-dark box test (LD).

Finally, evidence indicates that the social interaction test is a relevant model to address anxiolytic effects of DOR agonists. Indeed, pharmacological activation of DOR by the DOR agonist AZD2327 induced twice more social interactions than baseline conditions in rats (Hudzik, Maciag et al. 2011). Additionally, SNC80 enhanced social interactions engaged by adolescent mice (Terranova and Laviola

2001). We thus investigated whether the lack of DOR in forebrain GABAergic neurons may lead to altered social interactions.

## Material and methods

### Animals

Experiments were performed on animals aged between 6 and 18 weeks, housed 2-4 per cage under standard laboratory conditions (12h dark/light cycle light on at 7am). Food and water were available ad libitum. All mice were generated at Institut Clinique de la Souris-Institut de Genetique et Biologie Moleculaire et Cellulaire. Independent cohorts of $Oprd^{fl/fl}$ (Ctrl), conditional knockout (Dlx-DOR) and full knockout mice (CMV-DOR) were tested in the different behavioral paradigms. Mice were habituated to their new experimental environment and handled for 1 week before starting the experiments. All behavioral testing was performed with the observer blinded to the genotype and/or treatment of the animals. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).

### The aversive effect of predator odor

The predator odor is a synthetic olfactory stimulus naturally present in fox anal secretions, the 2, 4, 5-trimethylthiazoline (TMT) (Pherotech, Canada). TMT was diluted with an agitator at 0.1 % in 100 % ethanol. For odorant exposure, synthetic predator odor (a filter paper soaked with 10 µl of a solution containing 0.1 % of TMT) was placed in a drilled circular plastic box. A new filter paper was used for each test and each animal. As a control, mice were exposed to the vehicle ethanol. Mice were exposed to predator odor in an open field apparatus (45 x 45 x 18 cm) under a light intensity of 45 lux. Odorant was placed at the limit between the center and the periphery. Distance travelled, time spent sniffing the odor, and number of rearing and grooming were manually recorded during 10 min.

### Light/Dark Box test

The light/dark box is composed of two rectangular compartment (27 x 21 x 14 cm) separated by a tunnel (7 x 10 cm) (Imetronic). One is constituted of black floor and walls dimly lit (5 lux), whereas the other is made of a white floor and walls intensely lit (500 lux). The apparatus is equipped with infrared beams and sensors. Viewpoint software collects information and gives the exact mouse position in live. Test was performed as previously described (File, Lippa et al. 2004). Locomotor activity, time spent and number of entries in each compartment are automatically recorded. Sessions last for 5min and started when mouse is positioned in dark compartment, back facing the tunnel.

**Social Interaction**

The Dlx-DOR, CMV-DOR and Ctrl mice were tested in an open-field apparatus (4 equal square arenas of 50x50 cm separated by 35 cm-high opaque grey Plexiglas walls). Each tested animal is placed simultaneously with an unfamiliar wild-type mouse (C57BL6/J) in the arena under indirect light intensity of 20 lux. The test was performed as described (File, Lippa et al. 2004). The test lasted for 10 min and the session was recorded by a camera system above the apparatus. The time spent sniffing, following or in physical contact with the interacted mouse was scored as social behaviors. The self-grooming duration was also measured as an index of individual self-centered behavior.

**Elevated Plus-Maze test under high light intensity**

The EPM was conducted in a similar apparatus (Imetronic, four arms of 30 x 5 cm set in cross from a neutral central square of 5 x 5 cm) and experimental conditions (5 min of test, parameters measured: locomotor activity, time spent, number of entries and number of attempts to enter in open or closed arms) as previously described (see Manuscript 1), except for the light intensity. The maze was elevated 60cm above the ground and placed in indirect light (100 lux). Movements were detected by infrared beams and sensors. Viewpoint software collects information and gives the exact mouse position in live.

**Statistical analyses**

Statistical differences were determined by analysis of variance (ANOVA) (StatView 5, SAS Institute Inc., Cary, North Carolina) followed by Bonferroni/Dunn

**Aversive odor test**

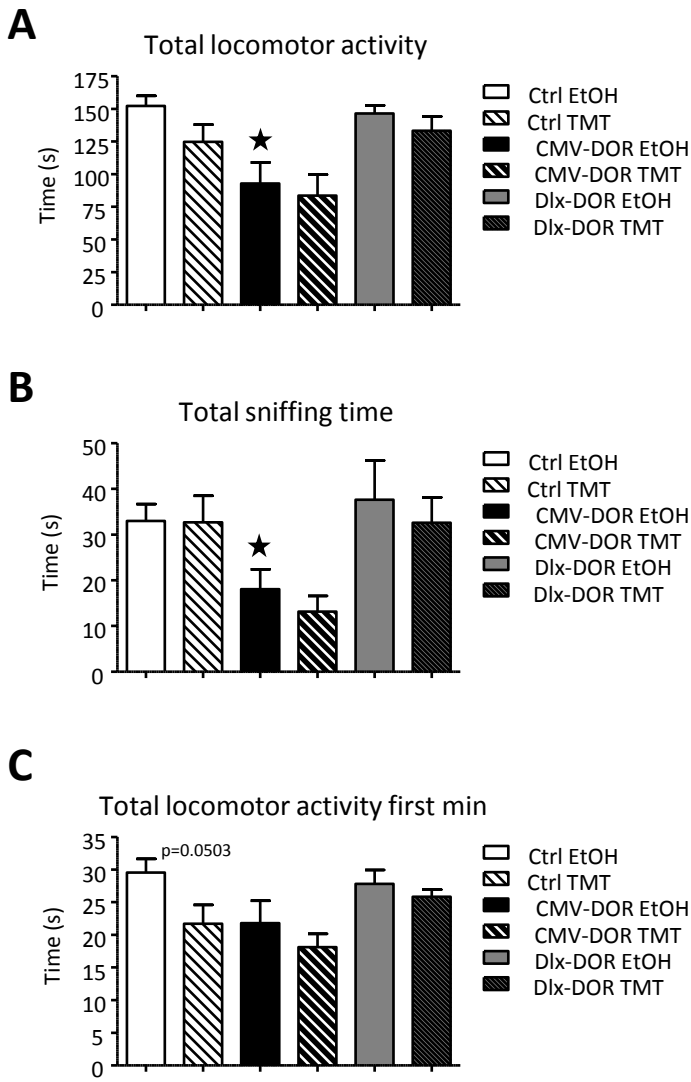



Fig. 1.1: **Aversive effect of predator odor**. Ctrl, Dlx-DOR and CMV-DOR mice were tested for suppressed exploratory activity in an open field apparatus upon exposure to the predator odor TMT. **(A)** Total locomotor activity and **(B)** Sniffing time. Dlx-DOR and Ctrl mice showed comparable exploration and sniffing time when exposed to the vehicle or TMT. CMV-DOR mice displayed decreased values for both parameters compared to Ctrl mice when confronted to both vehicle and predator odor, reflecting their high levels of anxiety. **(C)** Locomotor activity during the first minute. A slight decrease in exploration time was observed in Ctrl mice, however no significant difference was found across genotypes (n= 5-8 per genotype and odor). For all the tests, filled stars indicate significant differences between genotype. One star, $P<0.05$ (one-way ANOVA).

post hoc analysis. The F values and experimental degrees of freedom were included in Results. For experiments with two groups, a Student t test was used. The level of statistical significance was set at $p < 0.05$. For the statistical analysis along the sessions divided in bin-periods, the analysis of variance repeated measures was used.

## Results

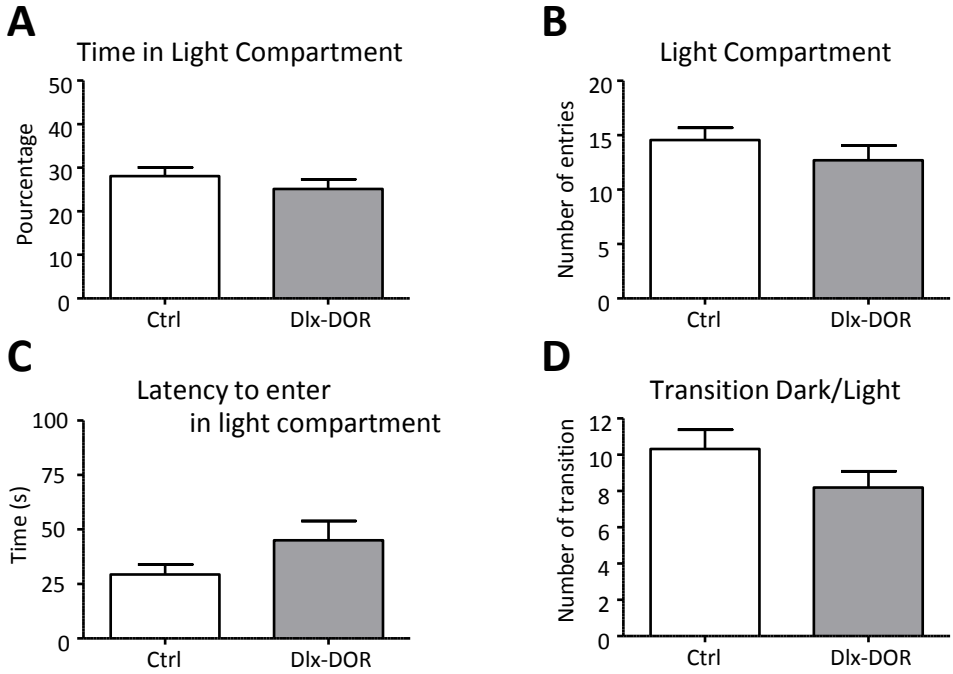
### Dlx-DOR and Ctrl react similarly to predator odor

We previously showed that Dlx-DOR mice display normal capacities to detect and discriminate a neutral from an aversive odor, suggesting that DOR expressed on forebrain GABAergic neurons are not involved in the olfactory detection. The olfactory sense is critical in rodents behaviors and the olfactory bulbectomy is long been established as a model of despair-like behaviors (Song and Leonard 2005). We therefore examined whether the loss of DORs in the olfactory bulb from Dlx-DOR mice could lead to an alteration of reactivity towards an aversive stimulus.

Dlx-DOR, CMV-DOR and Ctrl mice were submitted to the predator odor. Locomotor activity (Fig 1.1A) and sniffing (Fig 1.1B) durations were monitored as indexes of emotional reactivity towards the aversive odor. During exposure to the vehicle odor, Dlx-DOR and Ctrl mice showed a similar global exploration and sniffing time, whereas the CMV-DOR mice expressed a strong decrease of both parameters, thus reflecting the increased anxiety-related behavior previously described in total DOR knockout mice.

When animal were exposed to the predator odor, all genotypes showed similar global locomotor activity and sniffing durations. The ANOVA performed on global locomotor activity revealed a genotype effect ($F_{(2, 36)} = 10.41$; $p<0.001$) but no treatment effect ($F_{(1, 36)} = 2.46$; $p>0.05$) or genotype x treatment interaction ($F_{(2, 36)} = 0.29$; $p>0.05$). *Post hoc* analysis showed that CMV-DOR exhibited significant increase of anxiety-related behaviors in comparison with the Ctrl mice, regardless the treatment ($p<0.01$, Bonferroni/Dunn test). Similar results were obtained for the time spent sniffing (ANOVA: genotype effect $F_{(2, 36)} = 8.07$; $p<0.01$; treatment $F_{(1, 36)} =$

## Light / Dark box test




## Social Interaction test

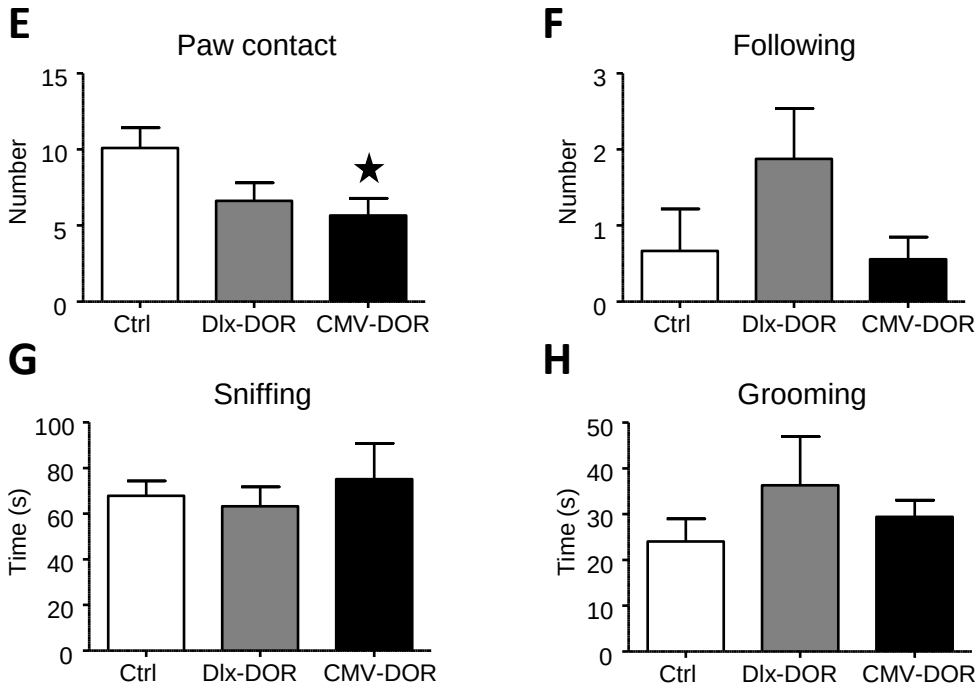



Fig. 1.2: **Anxiety-related behaviors.** Light-dark box and social interaction were used to further explore emotional responses in Dlx-DOR. **(A-D)** Light-dark box. Time spent **(A)** and entries in lit compartment **(B)** did not differ across genotypes. Latency to enter in lit compartment as well as dark/light transitions were also comparable. (n= 16-17 per genotype). **(E-H)** Social Interaction. **(E)** Paw contact, **(F)** following and **(G)** sniffing time correspond to social behaviors. **(H)** Grooming represent an individual self-centered behavior. No significant difference was found between Dlx-DOR and Ctrl mice. CMV-DOR showed significant decrease of paw contact. (n= 8-9 per genotype). For all the tests, filled stars indicate significant differences compare to Ctrl mice. One star, $P<0.05$ (one-way ANOVA).

0.62; p>0.05; genotype x treatment interaction $F_{(2, 36)}$ = 0.13; p>0.05). Our results suggest that DORs are not involved in the detection and processing of olfactory aversive stimuli. Alternatively, experimental conditions may not be optimal for the detection of aversive odor, and a small genotype effect may be undetectable.

Nevertheless, the analysis of the first 1min period (Fig 1.1C) revealed that predator odor modifies locomotor activity, as reflected by the statistical analysis (ANOVA: genotype $F_{(2, 36)}$ = 4.55; p<0.05; treatment effect $F_{(1, 36)}$ = 5.13; p<0.05; genotype x treatment interaction $F_{(2, 36)}$ = 0.77; p>0.05). *Post hoc* analysis showed that predator odor TMT significantly reduces locomotor activity in comparison with the vehicle EtOH (p<0.05, Bonferroni/Dunn test) and this effect was almost significant in Ctrl mice (p=0.0503, Student *t*-test). This result suggests that DOR may contribute to an increased sensitivity to aversive predator odor, and that DORs expressed on forebrain GABAergic neurons may contribute to this effect. Further experiments with refined experimental conditions and increased number of animals would be necessary in order to draw conclusions about the contribution of DORs in this process.

**Dlx-DOR and Ctrl mice exhibit similar anxiety-related behaviors in the Light/Dark Box test**

We investigated the anxiety-related behavior of the Dlx-DOR mice in the light-dark box test. Traditional parameters measured in this task are the percentage of time spent in light compartment, latency for the first entry in the light compartment, number of entries in the dark compartment and the number of Dark/Light transitions (Fig 1.2) (Bourin and Hascoet 2003).

The Dlx-DOR mice expressed anxiety levels similar to Ctrl mice. The time spent in the lit compartment (Fig 1.2A), which is used as an index of anxiety state, was comparable between genotypes (p>0.05, Student *t*-test). Similarly, the number of entries in the light compartment (Fig 1.2B) remained equivalent between the two groups (p>0.05, Student *t*-test). Dlx-DOR mice tended to exhibit enhanced latency to first entry in the light compartment, but statistical analysis revealed no significant differences (p>0.05, Student *t*-test, Fig 1.2C). The number of Dark/Light transitions

(Fig 1.2D), as an indicator of activity-exploration, was also statistically similar between the two groups ($p>0.05$, Student *t*-test). Our results suggest that under classical experimental conditions, selective deletion of DORs in forebrain GABAergic neurons does not affect the anxiety-related behavior measured in the light/dark box test.

**Dlx-DOR and Ctrl mice show comparable anxiety-related behaviors in the social interaction test**

We previously reported that Dlx-DOR and Ctrl mice are able to discriminate between social and neutral odors. In the present study, we initiated the evaluation of social behaviors in Ctrl, Dlx-DOR and CMV-DOR mice (Figure 1.2).

CMV-DOR mice displayed decrease social interactions, as reflected by the significant decrease of paw contact ($p<0.05$, One-way ANOVA, see Figure 1.2E). Dlx-DOR mice displayed comparable social interactions (see Figure 1.2E, F and G) and self-centered behavior (Figure 1.2H) in comparison with the Ctrl mice, as reflected by the similar number of paw contact and following as well as the equivalent time spent sniffing the interacted animal ($p>0.05$, One-way ANOVA). Our data indicate that enhanced anxiety-related behavior described in constitutive DOR KO mice can be detected in the social interaction test, as reflected by reduced social behaviors. Moreover, this study suggests that lack of DOR on forebrain GABAergic neurons does not impact social interactions.

**Dlx-DOR exhibit a trend toward increased risk-taking in the EPM with higher stressful conditions**

Using the elevated plus-maze, we previously reported reduced anxiety in Dlx-DOR mice, revealed by increased time spent in the open arms. This anxiety-related phenotype was not detected in all behavioral paradigms, since Dlx-DOR and Ctrl littermates expressed similar anxiety levels in the open field (Manuscript 1) and light-dark box tests. We hypothesized that the hypoanxiety phenotype could only be observed in a precise scale of stress intensity, therefore depending on experimental paradigm and conditions.

**Elevated plus maze test (100lux)**

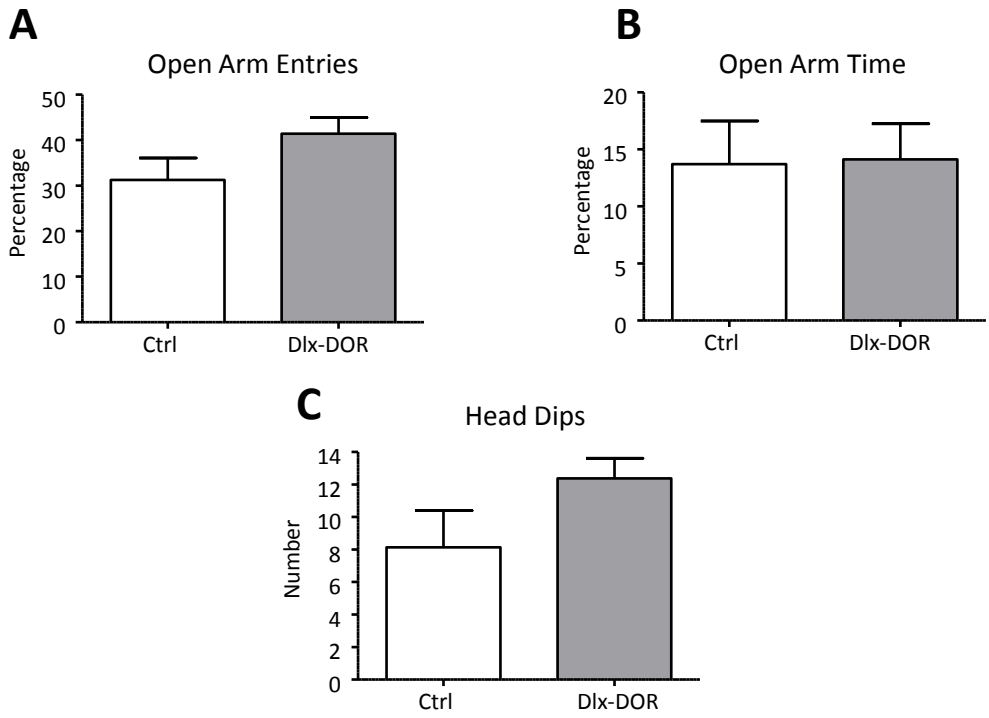



Fig. 1.3: **Elevated plus maze test under strong light intensity (100lux). (A)** Dlx-DOR showed a trend to increase percentage of entries and **(B)** comparable time spent in the open arms in comparison with Ctrl mice. **(C)** Tendency for increased number of head dips in Dlx-DOR mice. General activity was similar (total visits) between the two groups (data not shown), reflecting no change in spontaneous locomotor activity. n= 7-8 per genotype.

Here, we assessed whether the Dlx-DOR mice phenotype could also be observed, or even increased under more anxiogenic conditions, i. e. higher light intensity (see discussion manuscript 1). Under these experimental conditions, Dlx-DOR mice expressed similar anxiety levels in comparison with Ctrl mice, as reflected by the similar percentage of entries ($p>0.05$, Student *t*-test, Fig 1.3A) and time spent in the open arms ($p>0.05$, Student *t*-test, Fig 1.3B). Interestingly, Dlx-DOR mice tended to perform more head dips, an index of risk-taking behavior, in comparison to Ctrl mice (12.38 ± 1.24 in Dlx-DOR mice vs. 8.14 ± 2.24 in Ctrl mice, Fig 1.3C). In these experimental conditions, therefore, mutant mice showed behavior reflecting enhanced risk-taking behaviors although statistical analysis did not show significance difference between Dlx-DOR and Ctrl mice. Together, our data demonstrate that enhanced stressful conditions attenuate the emotional phenotype previously observed in Dlx-DOR mice. Further experiments using larger number of animals would be necessary to confirm these results.

## Discussion

In this set of experiments, our further evaluation of behavioral reactivity to odors does not detect any phenotype in Dlx-DOR mice exposed to a predator odor. However, behavioral analysis of mutant mice provides additional evidence for the low anxiety/fear-high risk-taking phenotype of Dlx-DOR mice described in manuscript 1, but also further documents that this phenotype depends on experimental paradigm and conditions.

Anxiogenic effects of predator odors are classically measured either in anxiety models after a pre-exposure to TMT (Hacquemand, Choffat et al. 2013) or in avoidance tests with two compartments and one containing the aversive odor (Kobayakawa, Kobayakawa et al. 2007). In both situations, animals are exposed to predator odor in a confined area, generally the size of standard homecage. Under our experimental conditions, the predator odor TMT used at classical concentrations (Hacquemand, Choffat et al. 2013) failed to significantly alter mouse behavior (vehicle versus TMT) which ever genotype is considered. Although a slight effect was observed during the first minute of the test in Ctrl mice, statistical analysis showed no

TMT effect. Refined experimental conditions therefore should be used in order to properly detect the aversive effect of TMT, perhaps through exposure under more confined conditions. Interestingly however, constitutive DOR knockout (CMV-DOR) mice showed decreased locomotor activity and sniffing exploration time in both vehicle and TMT conditions. These parameters likely reflect anxiety-related behaviors, and our observation is in-line with the well-established high anxiety phenotype in these mice (Filliol, Ghozland et al. 2000).

In the light-dark box test, Dlx-DOR mice showed similar level of anxiety-related behavior than Ctrl mice, characterized by comparable time spent exploring the lit compartment. The absence of detectable emotional phenotype in this test is consistent with the lack of phenotype in the open field, which is performed under comparable under mild stress conditions (manuscript 1). As hypothesized previously, lower levels of anxiety in Dlx-DOR mice may only be detectable under more anxiogenic conditions (elevated plus maze and novelty suppressed feeding).

The social interaction test is a paradigm largely used to evaluate anxiety, in which tested animals are directly confronted to a potential aggressor (File and Seth 2003; File, Lippa et al. 2004). Pharmacological activation of DOR promotes social interactions, indicating DOR-mediated modulation of social behaviors possibly related to emotional control (Terranova and Laviola 2001; Hudzik, Maciag et al. 2011). Decreased interactions in CMV-DOR mice indicates that genetic inactivation of DOR inhibits social interactions, and parallels high anxiety-related behavior previously reported in constitutive knockout mice (Filliol, Ghozland et al. 2000). Dlx-DOR mice otherwise displayed unchanged emotional responses in the open field apparatus and expressed comparable social behaviors, as compared to Ctrl mice. The reduced anxiety/high risk taking phenotype could not be detected in the social interaction test under our experimental conditions. Because environmental stress conditions may influence anxiolytic versus anxiogenic DOR function in the brain (see discussion manuscript 1), it may be interesting to assess Dlx-DOR mice in a social defeat or resident-intruder test.

We previously demonstrated that Dlx-DOR mice exhibit decrease anxiety-related behaviors in the elevated plus maze test, as reflected by decrease time spent in the open arms. Here, Dlx-DOR mice were further tested in the elevated plus maze with increased light intensity to assess whether the phenotype previously observed might be detected or altered under more stressful conditions. Mutant mice tended to enter more in open arms in comparison to control Ctrl mice. In addition, Dlx-DOR mice tended to perform more head dips, a relevant parameter for risk-taking behaviors (Hoshino, Uga et al. 2004; Walf and Frye 2007). Overall however, their behavior in the maze did not significantly differ from control animals, supporting the notion that altered risk-taking and anxiety behavior is a subtle phenotype, which is detectable only under specific stress intensity conditions. Increasing the number of animals should confirm the risk-taking behavior of mutant mice in the intensely lit elevated plus maze.

Finally, other paradigms may be used to specifically assess low fear-high risk-taking behavior in Dlx-DOR mice. Risk-taking behavior is a relevant indicator of emotional reactivity and can be defined as the tendency to engage potentially dangerous behaviors (Marques, Olsson et al. 2008; Ishii, Ohara et al. 2012). Although risky behaviors and emotional state are closely related, altered decision-making towards rewards is another feature of risk-taking behavior. Drug addiction is known to alter decision-making processes and promote risky behaviors (Schultz 2011). Therefore, the study of decision-making for food reward in experimental conditions that overcome the anxiogenic effect of the environment would be an additional index of risk-taking behaviors. A suitable approach therefore could be the probabilistic selection task in which animals choose between two amounts of food reward reinforced with different probability (Parker, Wanat et al. 2011).

These additional experiments have not been included in the main manuscript (manuscript 1), because data are either too preliminary, or do not provide any useful additional information. All the data however concur to support the intriguing low anxiety/high risk-taking phenotype of Dlx-Cre mice, and reveal the unforeseen role of DORs in regulating inhibitory forebrain circuits towards protective behaviors under threatening situations.

Asok, A., L. W. Ayers, et al. (2013). "Immediate early gene and neuropeptide expression following exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT)." Behav Brain Res **248**: 85-93.
Bourin, M. and M. Hascoet (2003). "The mouse light/dark box test." Eur J Pharmacol **463**(1-3): 55-65.
File, S. E., A. S. Lippa, et al. (2004). "Animal tests of anxiety." Curr Protoc Neurosci **Chapter 8**: Unit 8 3.
File, S. E. and P. Seth (2003). "A review of 25 years of the social interaction test." Eur J Pharmacol **463**(1-3): 35-53.
Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.
Hacquemand, R., N. Choffat, et al. (2013). "Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice." Behav Brain Res **238**: 227-231.
Hoshino, K., D. A. Uga, et al. (2004). "The compulsive-like aspect of the head dipping emission in rats with chronic electrolytic lesion in the area of the median raphe nucleus." Braz J Med Biol Res **37**(2): 245-250.
Hudzik, T. J., C. Maciag, et al. (2011). "Preclinical pharmacology of AZD2327: a highly selective agonist of the delta-opioid receptor." J Pharmacol Exp Ther **338**(1): 195-204.
Ishii, H., S. Ohara, et al. (2012). "Inactivating anterior insular cortex reduces risk taking." J Neurosci **32**(45): 16031-16039.
Kobayakawa, K., R. Kobayakawa, et al. (2007). "Innate versus learned odour processing in the mouse olfactory bulb." Nature **450**(7169): 503-508.
Marques, J. M., I. A. Olsson, et al. (2008). "Evaluation of exploration and risk assessment in pre-weaning mice using the novel cage test." Physiol Behav **93**(1-2): 139-147.
Parker, J. G., M. J. Wanat, et al. (2011). "Attenuating GABA(A) receptor signaling in dopamine neurons selectively enhances reward learning and alters risk preference in mice." J Neurosci **31**(47): 17103-17112.
Schultz, W. (2011). "Potential vulnerabilities of neuronal reward, risk, and decision mechanisms to addictive drugs." Neuron **69**(4): 603-617.
Song, C. and B. E. Leonard (2005). "The olfactory bulbectomised rat as a model of depression." Neurosci Biobehav Rev **29**(4-5): 627-647.
Staples, L. G. (2010). "Predator odor avoidance as a rodent model of anxiety: learning-mediated consequences beyond the initial exposure." Neurobiol Learn Mem **94**(4): 435-445.
Terranova, M. L. and G. Laviola (2001). "delta-Opioid modulation of social interactions in juvenile mice weaned at different ages." Physiol Behav **73**(3): 393-400.
Walf, A. A. and C. A. Frye (2007). "The use of the elevated plus maze as an assay of anxiety-related behavior in rodents." Nat Protoc **2**(2): 322-328.

# Second Part

# Delta opioid receptors expressed on forebrain GABAergic neurons

# Contribution in other physiological processes

## Introduction

Taking advantage of the Dlx5/6-Cre driver mouse we were able to generate conditional knockout of delta opioid receptor on forebrain GABAergic neurons. Delta opioid receptors were fully removed from the olfactory bulb, caudate-putamen and nucleus accumbens. Our mutant mice also showed a partial deletion of the receptor in the hippocampus.

Delta opioid receptors agonists were reported to play a central role in several physiological processes such as pain control, learning and memory, motor control and hedonic homeostasis (Gaveriaux-Ruff and Kieffer 2011; Chu Sin Chung and Kieffer 2013). However, the development of delta drug for therapeutic purposes encountered issues mainly due to the pro-convulsing effect of delta opioid receptor agonists. On the other hand, the contribution of inhibitory tone in epileptic seizures emphasizes the critical role of GABA system in this phenomenon (Mann and Mody 2008). Consequently, it would be pertinent to determine whether delta opioid receptor expressed on GABAergic neurons may contribute to the pro-convulsing effect of delta agonists.

Delta opioid receptor control over learning and memory processes was previously reported in drug-context association (Le Merrer, Faget et al. 2012). Additionally, genetic and pharmacological inactivation of delta opioid receptors alters spatial memory performances (Le Merrer, Rezai et al. 2013).

Therefore, we decided to use the conditional knockout mouse model developed previously to address the contribution of DOR expressed on forebrain GABAergic neurons on memory abilities.

# Manuscript 2

## Delta opioid receptors expressed in forebrain GABAergic neurons are responsible for SNC80-induced seizures

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### Abstract

The delta opioid receptor (DOR) has raised much interest for the development of new therapeutic drugs, particularly to treat patients suffering from mood disorders and chronic pain. Unfortunately, the prototypal DOR agonist SNC80 induces mild epileptic seizures in rodents. Although recently developed agonists do not seem to show convulsant properties, mechanisms and neuronal circuits that support DOR-mediated epileptic seizures remain to be clarified. DORs are expressed throughout the nervous system. In this study we tested the hypothesis that SNC80-evoked seizures stem from DOR activity at the level of forebrain GABAergic transmission, whose inhibition is known to facilitate the development of epileptic seizures. We generated a conditional DOR knockout mouse line, targeting the receptor gene specifically in GABAergic neurons of the forebrain (Dlx-DOR). We measured effects of SNC80 (4.5, 9, 13.5 and 32 mg/kg), ARM390 (10, 30 and 60 mg/kg) or ADL5859 (30, 100 and 300 mg/kg) administration on electroencephalograms (EEGs) recorded in Dlx-DOR mice and their control littermates (Ctrl mice). SNC80 produced dose-dependent seizure events in Ctrl mice, but these effects were not detected in Dlx-DOR mice. As expected, ARM390 and ADL5859 did not trigger any detectable change in mice from both genotypes. These results demonstrate for the first time that SNC80-induced DOR activation induces epileptic seizures via direct inhibition of GABAergic forebrain neurons, and supports the notion of differential activities between first and second-generation DOR agonists.

*Keywords*: Delta opioid receptor; Conditional Knockout; Epileptic seizures; delta agonist; biased agonism; in vivo

Abbreviations : DOR, delta opioid receptor ; EEG, electroencephalogramm

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## Introduction

Delta opioid receptors (DOR) emerged during the last decade as a major player for the modulation of chronic pain, the control of emotional processes and regulation of some aspects of addiction including impulsivity. Preclinical studies, using both genetic and pharmacological approaches, have emphasized the beneficial contribution of DOR agonists to reduce chronic pain (Gaveriaux-Ruff and Kieffer 2011; Pradhan, Befort et al. 2011) and anxiety/depressive-like behaviors (Chu Sin Chung and Kieffer 2013). More recently, DOR agonists have entered clinical trials in order to treat mood disorders (Hudzik, Maciag et al. 2011).

The development of new delta drugs encountered untoward effects of DOR agonists, in particular their convulsive properties. The first non-peptidic agonists BW373U86 and SNC80 were described to mediate brief and non-lethal convulsions in rodents (Comer, Hoenicke et al. 1993; Broom, Nitsche et al. 2002). In addition, pro-convulsive effects of SNC80 were also reported in rhesus monkeys (Danielsson, Gasior et al. 2006). New agonists were developed with less or no adverse effects on epileptic thresholds, such as ADL5859, ADL5747 (Le Bourdonnec, Windh et al. 2008; Le Bourdonnec, Windh et al. 2009) or KNT-127 (Saitoh, Sugiyama et al. 2011) for example. Mechanisms underlying differential DOR agonists effects on behavioral responses may engage distinct intracellular processes, a concept referred as to biased agonism or functional selectivity, and those involved in DOR agonist-dependent convulsant activity remain to be clarified (Pradhan, Befort et al. 2011).

Genetic and pharmacological studies have demonstrated that BW373U86 and SNC80-induced seizures are mediated by DORs (Broom, Nitsche et al. 2002; Jutkiewicz, Rice et al. 2005; Jutkiewicz, Baladi et al. 2006). At present however, the precise neuroanatomical site, as well as neurotransmitter systems involved in SNC80-induced epileptic seizures are unknown. The contribution of GABAergic systems in the onset and spreading of absence seizures has been long established, and for example, progressive decrease of GABAergic phasic inhibition in the hippocampus was shown in a rat model of spontaneous seizures (Crunelli, Cope et al. 2011). DORs are broadly expressed in the nervous system (Le Merrer, Becker et al. 2009). In the forebrain, a main site for the control of epileptic seizures (Lalonde and Strazielle 2012), DORs are expressed in cortex and hippocampus with demonstrated expression in GABAergic neurons for hippocampus (Erbs, Faget et al. 2012; Rezai, Faget et al. 2012).

Here we tested the hypothesis that DORs expressed in GABAergic neurons of the forebrain are responsible for SNC80-induced seizures. To this aim, we used a conditional knockout mouse line (Dlx-DOR mice, Chu Sin Chung et al., in preparation) with a specific DOR gene deletion in these neurons. In these mice, DOR binding is significantly decreased at the level of hippocampus, and intact in the

cortex. We tested effects of SNC80 (high proconvulsant activity), as well as ARM-390 and Adolor-5859 (low proconvulsant activity) in Dlx-DOR and control littermates. As expected, ARM-390 and Adolor-5859 had no effect in any mouse line. Remarkably SNC80-induced modifications of electroencephalogram recordings (EEGs) were abolished in Dlx-DOR mice, demonstrating for the first time that SNC80-evoked convulsions arise from direct inhibition of forebrain GABAergic neurons.

**Methods and Materials**

Conditional knockout (Dlx-DOR) mice were obtained by crossing mice with a floxed DOR gene ($Oprd^{fl/fl}$, (Gaveriaux-Ruff, Nozaki et al. 2011) with Dlx5/6-Cre driver mice (Monory, Massa et al. 2006). Total knockout (CMV-DOR) mice were produced by crossing $Oprd^{fl/fl}$ mice with CMV-Cre driver mice (ubiquitous Cre expression). All mice were bred on a mixed genetic background (C57BL6/J x SV129Pas) and $Oprd^{fl/fl}$ mice used as controls (Ctrl). The DOR pattern of expression in Ctrl and Dlx-DOR mice was described previously (Chu Sin Chung et al., in preparation) and is summarized in Figure 1. All mice used in the present study were created and produced at the Institut Clinique de la Souris-Institut de Genetique et Biologie Moleculaire et Cellulaire, and genotyped as described (Gaveriaux-Ruff, Nozaki et al. 2011) (Chu Sin Chung et al., in preparation). Two independent cohorts were assessed and each was composed of 8 mice per genotype.

Only male mice were used in all experiments, aged 2-6 months, maintained in standard conditions (12h dark/light cycle light on at 7am) with food and water ad libitum, except during the EEG recording sessions. Mice were habituated to their new experimental environment and handled for 1 week before starting the experiments. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).

One week after their arrival in the animal facility, mice were anesthetized with an intraperitoneal injection of a ketamine 1% / xylazine 0.5% solution (Kétamine 1g/kg; Xylazine 100mg/kg) and went through a surgery on a stereotaxic apparatus. Four tungstene electrodes were positioned on the skull, one over the frontal and one over the hippocampus on each side, as previously described (Pitman, Rasmusson et al. 2012; Smith and Rudolph 2012). A fifth electrode was positioned at a caudal level, over the cerebellum, and served as a reference. The animals were allowed to recover for 24h individually housed, and after for one week in their normal environment.

During the test, mice were equipped with five single-contact electrodes. Mice were individually tested in a Plexiglas cylinder and EEG traces were continuously recorded during 3 h. EEG recordings were performed on freely moving animals. Basal EEG trace was monitored for 2 h, then the animal received the drug and EEG traces were monitored for 1 h. Cohort 1 received SNC80 at 4.5, 9, 13.5 and 32

mg/kg. SNC80 (Tocris Bioscience, Bristol, UK) was dissolved in saline and injected intraperitoneally. Cohort 2 received ARM390 at 10, 30 and 60 mg/kg followed by ADL5859 at 30, 100 and 300 mg/kg. ARM390 (AstraZeneca, Montreal, Canada) was administered orally by gavage, as described (Pradhan, Becker et al. 2009). ADL5859 (Adolor Corporation, Exton, PA) was dissolved in distilled water and administered by gavage orally as described previously (Le Bourdonnec, Windh et al. 2008; Le Bourdonnec, Windh et al. 2009). A period of one week between each dose and two weeks between different compounds were applied in order to allow a sufficient washout period. The behavior of animals was observed during the whole recording sessions. The different seizure events were quantified though analysis of EEG recordings. Seizure patterns were the following: (1) myoclonies; (2) isolated or repeated clonic seizures; and (3) tonico-clonic seizures leading to status epilepticus. Bilateral spike-and-wave discharges (SWS) were scored as a reminiscence of absence seizures. A representative trace is shown in Figure 2. Latency for first occurrence of each event, and number of events were scored (Figure 3). We also determined percentage of mice that expressed each seizure events (Figure 4).

## Results

### SNC80-induced seizures are abolished in Dlx-DOR mice

The non-peptidic DOR agonist SNC80 is described as a pro-convulsant drug in rats (Broom, Nitsche et al. 2002; Jutkiewicz, Baladi et al. 2006). We examined the effects of SNC80 administration on the latency to first seizure and total duration of seizures in Ctrl, Dlx-DOR and CMV-DOR mice (Figure 3). At the low dose (4.5 mg/kg), SNC80 did not evoke detectable change in any of the three groups. At the 9 mg/kg SNC80 dose, EEG recordings were modified in Ctrl mice, but this effect was not significant (latency before seizure, $F_{(2, 20)} = 2.782$; $p>0.05$; duration of seizure, $F_{(2, 20)} = 2.52$; $p>0.05$, two-way ANOVA). At higher doses (13.5 and 32 mg/kg), SNC80 produced seizures in Ctrl mice, reflected by dose-dependent decrease of latency before the first seizure (Figure 3A) as well as increase of seizure duration (Figure 3B). Two-way ANOVA indicated a statistically significant genotype effect at 13.5 mg/kg on both latency before first seizure ($F_{(2, 20)} = 5.205$; $p<0.05$) and duration of seizure ($F_{(2, 20)} = 4.175$; $p<0.05$). Post hoc analysis confirmed that SNC80, administered at 13.5 mg/kg, induced significant decrease of the latency to first seizure ($p<0.05$, Bonferroni/Dunn test) and enhanced duration of seizures ($p<0.05$, Bonferroni/Dunn test) in Ctrl mice compared to the two other genotypes. At the highest dose (32 mg/kg), two-way ANOVA revealed a strongly significant effect of SNC80 on both latency before seizure ($F_{(2, 20)} = 17.217$; $p<0.001$) and duration of seizure ($F_{(2, 20)} = 14.708$; $p<0.001$). Similarly, post hoc analysis revealed that SNC80 administered at 32 mg/kg induced a significant decrease of latency ($p<0.001$, Bonferroni/Dunn test) and increase in duration ($p<0.001$, Bonferroni/Dunn test) in the Ctrl mice, as compared to the other genotypes.

No sign of seizure could be detected in any of the two mutant lines (Figure 3). Thus, total mutant mice (CMV-DOR) were insensitive to the DOR agonist, as previously described (Broom, Nitsche et al. 2002), confirming that SNC80-induced seizures are specifically mediated by DORs. Remarkably, Dlx-DOR mice were equally insensitive to SNC80, demonstrating that DORs expressed in forebrain GABAergic neurons are essential for this effect.

We further examined types of seizure events produced by SNC80, including spike-and-wave discharges (SWS), clonies and myoclonies (Figure 4) and determined the proportion of mice exhibiting the different seizure events in the three genotypes (Figure 4 A-D). In Ctrl mice, SNC80 at 13.5mg/kg induced a significant increased in percentage of mice that showed clonic seizures (Figure 4C) (genotype effect, $F_{(2, 101)} = 5.217$; $p<0.05$; Bonferonni Post-hoc analysis, Ctrl vs. Dlx-DOR mice $p<0.05$; Ctrl vs. CMV-DOR mice $p<0.05$) and 32 mg/kg (genotype effect, $F_{(2, 101)} = 17.391$; $p<0.001$; Bonferonni Post-hoc analysis, Ctrl vs. Dlx-DOR mice $p<.0001$; Ctrl vs. CMV-DOR mice $p<0.001$). Further, SNC80 also induced myoclonic seizures in all mice from the Ctrl group, and at three doses (9, 13.5 and 32 mg/kg) (Figure 4C-D). Two-way ANOVA revealed that the percentage of mice expressing myoclonic seizures was affected by the genotype ($F_{(2, 101)} = 50.04$; $p<0.001$). Post-hoc analysis confirmed that a significant proportion of Ctrl mice showed SNC80-induced myoclonies at 9 mg/kg ($p<0.001$, Bonferroni/Dunn test), 13.5 mg/kg ($p<0.001$, Bonferroni/Dunn test) and 32 mg/kg ($p<0.001$, Bonferroni/Dunn test), as compared to Dlx-DOR and CMV-DOR mice. Finally, no tonico-clonic seizures were detected in Ctrl mice showed (data not shown), in line with the notion that epileptogenic effects of SNC80 are mild (Jutkiewicz, Baladi et al. 2006).

In CMV-DOR mice, SNC80 produced no changes on spike-and-wave discharges, myoclonic, clonic and tonico-clonic seizures. This again is consistent with previous studies showing lack of convulsant effects of SNC80 upon behavioral observation of DOR knockout mice (Broom, Nitsche et al. 2002). Dlx-DOR mice injected with SNC80 showed few SWS discharges at the highest doses (Figure 4A), and no sign of seizure was detected, including clonic, myoclonic and tonic-clonic seizures (Figure 4 B-D). The scoring of seizure events, therefore, further confirms that DORs expressed in forebrain GABAergic neurons are necessary for convulsing SNC80 effects

**ARM-390 and Adolor-5859 show no convulsant properties**

Several studies have reported that second-generation delta drugs do not show convulsant properties (Le Bourdonnec, Windh et al. 2008; Pradhan, Becker et al. 2009; Nozaki, Le Bourdonnec et al. 2012). To verify this, we also tested effects of DOR agonists of this category (ARM-390 and ADL-5859) in our experimental system. As expected, we found that neither ARM-390 nor ADL5859 modified EEG traces in any the three groups of mice (data not shown).

**Discussion**

In the present study, we confirm pro-convulsive effects of the non-peptidic delta agonist SNC80 in normal mice (Ctrl). This pharmacological activity of SNC80 was previously demonstrated upon behavioral observation (Jutkiewicz, Rice et al. 2005) and, to our knowledge, this is the first report of EEG modifications in mice after SNC80 administration. Classical pentylenetetrazole treatment produces strong and long lasting crises, and may eventually lead to the animal death (Loscher, Honack et al. 1991), however SNC80 seizures are reported to be mild (Jutkiewicz, Baladi et al. 2006). In accordance, pro-convulsing effects of SNC80 were brief and mild since no strong tonico-clonic seizures could be observed along the study.

The GABAergic system is known as a critical neurotransmitter system involved in epileptic seizures (Lalonde and Strazielle 2012). Here, conditional knockout mice characterized by a genetic deletion of DOR in forebrain GABAergic neurons, especially in hippocampus, striatum and olfactory bulb (see Manuscript 1, Chu Sin Chung et al., in preparation), did not respond to SNC80 under conditions were EEG recordings are strongly modified in Ctrl mice. This clear-cut observation demonstrates that the subset of receptors expressed in forebrain GABAergic neurons indeed mediate convulsing effects SNC80. Further, mutant mice were obtained by using a Dlx5/6-Cre driver mouse line. *Dlx* genes are required for GABAergic interneurons development and in particular parvalbumin positive interneurons (Wang, Dye et al. 2010). Our results, therefore, suggest that SNC80-stimulated DORs may exert their pro-convulsive effect via parvalbumin-positive GABAergic neurons of the forebrain.

DORs are strongly expressed on GABAergic neurons (Rezai, Faget et al. 2012) and their inhibitory activity on these neurons normally leads to increase local network excitability. The pro-convulsant effect of SNC80, therefore, likely results from enhanced excitation of forebrain networks.

In Dlx-DOR mice, receptors are deleted in GABAergic neurons from olfactory bulb (100% deletion), striatum (65-81% deletion) and hippocampus (57% deletion). Although receptors responsible for epileptogenic effects of SNC80 remain to be precisely determined, it is unlikely that DORs mediate seizure events via olfactory bulb networks. In contrast, both striatal and hippocampal circuitry have been involved in epileptic events (Lalonde and Strazielle 2012), and our data therefore suggest that SNC80 convulsant activity operate at the level of DORs in striato-hippocampal networks.

DORs are also expressed in other neuronal populations than GABAergic neurons, as suggested par partial receptor deletion upon Cre-mediated recombination in Dlx-positive neurons. DORs may therefore be present in some glutamatergic neurons, where their activity (anti-convulsant) would counteract DOR

activity at GABAergic cells (pro-convulsant). Opposing activities of distinct DOR populations in epileptogenic circuits may explain the mild convulsant effects of SNC80, as compared to those of pentylenetetrazole, which directly and specifically block GABA receptors (Huang, Bell-Horner et al. 2001). An interesting future experiment would be to test whether SNC80 has protective effects over pentylenetetrazole-induced convulsions in Dlx-DOR mice, which lack pro-convulsive DORs but retain anti-convulsant receptors.

Our analysis shows EEG modifications following administration of SNC80, but not ARM390 or ADL5849 compounds. These findings are in line with previous findings. ARM390 and ADL-5849 (Adolor) were developed for clinical purposes and produce no convulsions or EEG disturbances in the rat (Le Bourdonnec, Windh et al. 2008; Le Bourdonnec, Windh et al. 2009). To our knowledge, our results indicate for the first time the absence of detectable effect of ARM-390 compound (AstraZeneca) on epileptic seizures. An interesting correlate is the lack of trafficking effects of both compounds that, in contrast to SNC80, do not trigger receptor endocytosis in vivo (Pradhan, Becker et al. 2009; Nozaki, Le Bourdonnec et al. 2012). It is likely that active forms of DOR bound to ARM390 or ADL5849 differ from SNC80-bound receptors, engaging distinct intracellular signaling pathways within epilepsy-associated circuits that do not trigger seizure events. This is another example of biased agonism at DOR in vivo (Pradhan, Smith et al. 2012), and the identification of differentially recruited effector pathways require further investigation.

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**Disclosure/conflict of interest**

The authors report no biomedical financial interests or potential conflicts of interest.

Fig. 1: **Anatomical distribution of delta opioid receptors in Ctrl and Dlx-DOR mice**. **(A)** Sagittal sections in Ctrl mice at the top, in Dlx-DOR mice at the bottom; **(B)** Coronal sections at 2 different anterio-posterior levels (bregma 0.98mm; bregma -1.46mm) in Ctrl on (left side) and Dlx-DOR mice (right side). Quantification of DOR

expression levels in fmole/mg of tissue. In Ctrl mice, DORs are particularly abundant in the OB, cortical regions (FCx, Cg, MCx, PCx and InsCx), amygdala and striatum (CPu and NAc). DORs are also expressed at moderate levels in the Hipp, RS, and at much lower level in Hyp, Th and SC. Orange circles represent brain regions showing detectable change of DOR expression in Dlx-DOR as compared to Ctrl mice. DORs are fully removed in the OB; strongly in the CPu and NAc; and partially in the Hipp of Dlx-DOR mice. Abbreviations: Amy, Amygdala; Cg, Cingulate cortex; CPu, Caudate Putamen; FCx, Frontal cortex; Hipp, Hippocampus; Hyp, Hypothalamus; InsCx, Insular cortex; MCx, Motor cortex; NAc, Nucleus Accumbens; OB, Olfactory Bulb; PCx, Parietal cortex; RS, Retrosplenial; SC, Spinal Cord; Th, Thalamus.

Fig. 2: **EEG recordings from Dlx-DOR (top) and Ctrl mouse (bottom) after SNC80 administration.** A representation EEG recording session is shown (extracted from Cartool software) and seizures parameters measured are indicated. Recording starts 2min after SNC80 injection (32 mg/kg, s.c.). On the Ctrl mouse trace, a spike-and-wave discharge (SWS) is observed at the beginning of the session, followed by 18 myoclonic events which in turn lead to clonic seizure. No characteristic events are observed on Dlx-DOR mouse trace.

Fig. 3: **Epileptic seizures induced by SNC80. (A)** Latency before the first seizure event and **(B)** duration of the seizure are represented. Highest doses (9, 13.5 and 32 mg/kg) of SNC80 decreased latency before seizure and increased duration of seizures in Ctrl (black bars) mice, whereas no detectable change occurred in Dlx-DOR and CMV-DOR mice. n= 8 per genotype. All data are presented as means ± S.E.M. Data were analyzed using StatView 5.0 software (SAS Institute, Cary, NC). Drug pharmacokinetics was analyzed by using repeated-measures ANOVA followed by Student's t test for individual time points when appropriate. The analysis of pharmacological effect was performed by using two-way ANOVA for drug and genotype effects followed by Bonferroni *post hoc* analysis to determine statistically significant differences (One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$).

Fig. 4: **SNC80-induced EEG patterns.** Graphs represent the percentage of Ctrl, Dlx-DOR and CMV-DOR mice that showed **(A)** SWS, **(B)** myoclonic and **(C)** clonic seizures on EEG records. Highest doses (9, 13.5 and 32 mg/kg) of SNC80 lead to increased percentage of Ctrl mice that exhibited myoclonic and clonic seizures. No detectable change occurred on EEG recordings for Dlx-DOR and CMV-DOR mice. **(D)** The number of myoclonies per period of 20 min was measured. n= 8 per genotype. All data are presented as means ± S.E.M (One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$).

**References**

Broom, D. C., J. F. Nitsche, et al. (2002). "Comparison of receptor mechanisms and efficacy requirements for delta-agonist-induced convulsive activity and antinociception in mice." J Pharmacol Exp Ther **303**(2): 723-729.

Chu Sin Chung, P. and B. L. Kieffer (2013). "Delta opioid receptors in brain function and diseases." Pharmacol Ther. **140**(1): 112-120.

Comer, S. D., E. M. Hoenicke, et al. (1993). "Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice." J Pharmacol Exp Ther **267**(2): 888-895.

Danielsson, I., M. Gasior, et al. (2006). "Electroencephalographic and convulsant effects of the delta opioid agonist SNC80 in rhesus monkeys." Pharmacol Biochem Behav **85**(2): 428-434.

Erbs, E., L. Faget, et al. (2012). "Distribution of delta opioid receptor-expressing neurons in the mouse hippocampus." Neuroscience **221**: 203-213.

Gaveriaux-Ruff, C. and B. L. Kieffer (2011). "Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches." Behav Pharmacol **22**(5-6): 405-414.

Gaveriaux-Ruff, C., C. Nozaki, et al. (2011). "Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia." Pain **152**(6): 1238-1248.

Huang, R. Q., C. L. Bell-Horner, et al. (2001). "Pentylenetetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and site of action." J Pharmacol Exp Ther **298**(3): 986-995.

Hudzik, T. J., C. Maciag, et al. (2011). "Preclinical pharmacology of AZD2327: a highly selective agonist of the delta-opioid receptor." J Pharmacol Exp Ther **338**(1): 195-204.

Jutkiewicz, E. M., M. G. Baladi, et al. (2006). "The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid agonists in rats: comparison with pentylenetetrazol." J Pharmacol Exp Ther **317**(3): 1337-1348.

Jutkiewicz, E. M., K. C. Rice, et al. (2005). "Separation of the convulsions and antidepressant-like effects produced by the delta-opioid agonist SNC80 in rats." Psychopharmacology (Berl) **182**(4): 588-596.

Lalonde, R. and C. Strazielle (2012). "Brain regions and genes affecting myoclonus in animals." Neurosci Res.

Le Bourdonnec, B., R. T. Windh, et al. (2008). "Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: discovery of N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide (ADL5859)." J Med Chem **51**(19): 5893-5896.

Le Bourdonnec, B., R. T. Windh, et al. (2009). "Spirocyclic delta opioid receptor agonists for the treatment of pain: discovery of N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4'-piperidine]-4-yl) benzamide (ADL5747)." J Med Chem **52**(18): 5685-5702.

Le Merrer, J., J. A. Becker, et al. (2009). "Reward processing by the opioid system in the brain." Physiol Rev **89**(4): 1379-1412.

Loscher, W., D. Honack, et al. (1991). "The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. III. Pentylenetetrazole seizure models." Epilepsy Res **8**(3): 171-189.

Monory, K., F. Massa, et al. (2006). "The endocannabinoid system controls key epileptogenic circuits in the hippocampus." Neuron **51**(4): 455-466.

Nozaki, C., B. Le Bourdonnec, et al. (2012). "delta-Opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion, and receptor internalization." J Pharmacol Exp Ther **342**(3): 799-807.

Pitman, R. K., A. M. Rasmusson, et al. (2012). "Biological studies of post-traumatic stress disorder." Nat Rev Neurosci **13**(11): 769-787.

Pradhan, A. A., J. A. Becker, et al. (2009). "In vivo delta opioid receptor internalization controls behavioral effects of agonists." PLoS One **4**(5): e5425.

Pradhan, A. A., K. Befort, et al. (2011). "The delta opioid receptor: an evolving target for the treatment of brain disorders." Trends Pharmacol Sci **32**(10): 581-590.

Pradhan, A. A., M. L. Smith, et al. (2012). "Ligand-directed signalling within the opioid receptor family." Br J Pharmacol **167**(5): 960-969.

Rezai, X., L. Faget, et al. (2012). "Mouse delta opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells." Cell Mol Neurobiol **32**(4): 509-516.

Saitoh, A., A. Sugiyama, et al. (2011). "The novel delta opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions." Behav Brain Res **223**(2): 271-279.

Smith, K. S. and U. Rudolph (2012). "Anxiety and depression: mouse genetics and pharmacological approaches to the role of GABA(A) receptor subtypes." Neuropharmacology **62**(1): 54-62.

Wang, Y., C. A. Dye, et al. (2010). "Dlx5 and Dlx6 regulate the development of parvalbumin-expressing cortical interneurons." J Neurosci **30**(15): 5334-5345.

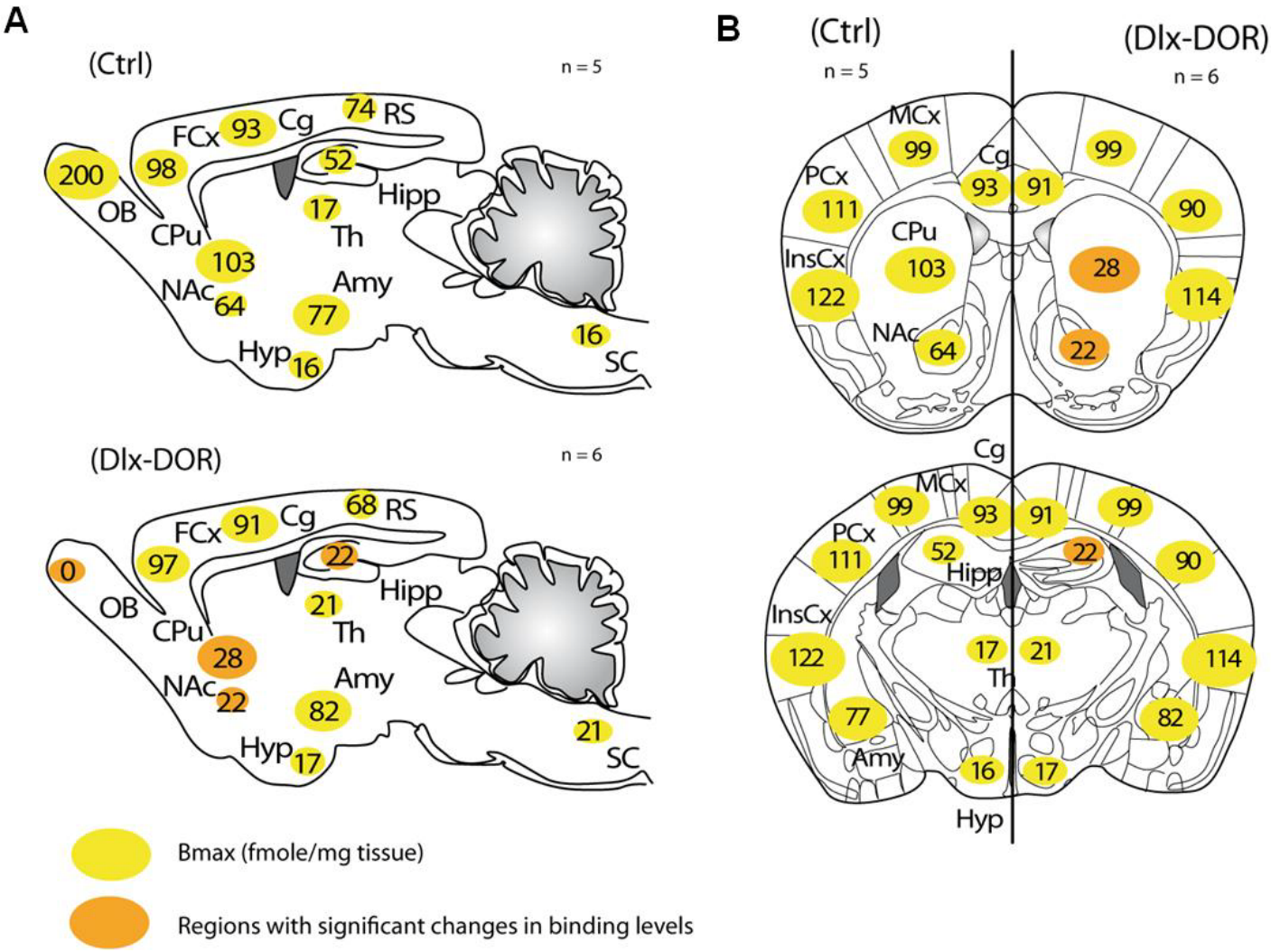



Fig. 1: **Anatomical distribution of delta opioid receptors in Ctrl and Dlx-DOR mice**. **(A)** Sagittal sections in Ctrl mice at the top, in Dlx-DOR mice at the bottom; **(B)** Coronal sections at 2 different anterio-posterior levels (bregma 0.98mm; bregma -1.46mm) in Ctrl on (left side) and Dlx-DOR mice (right side). Quantification of DOR expression levels in fmole/mg of tissue. In Ctrl mice, DORs are particularly abundant in the OB, cortical regions (FCx, Cg, MCx, PCx and InsCx), amygdala and striatum (CPu and NAc). DORs are also expressed at moderate levels in the Hipp, RS, and at much lower level in Hyp, Th and SC. Orange circles represent brain regions showing detectable change of DOR expression in Dlx-DOR as compared to Ctrl mice. DORs are fully removed in the OB; strongly in the CPu and NAc; and partially in the Hipp of Dlx-DOR mice. Abbreviations: Amy, Amygdala; Cg, Cingulate cortex; CPu, Caudate Putamen; FCx, Frontal cortex; Hipp, Hippocampus; Hyp, Hypothalamus; InsCx, Insular cortex; MCx, Motor cortex; NAc, Nucleus Accumbens; OB, Olfactory Bulb; PCx, Parietal cortex; RS, Retrosplenial; SC, Spinal Cord; Th, Thalamus.

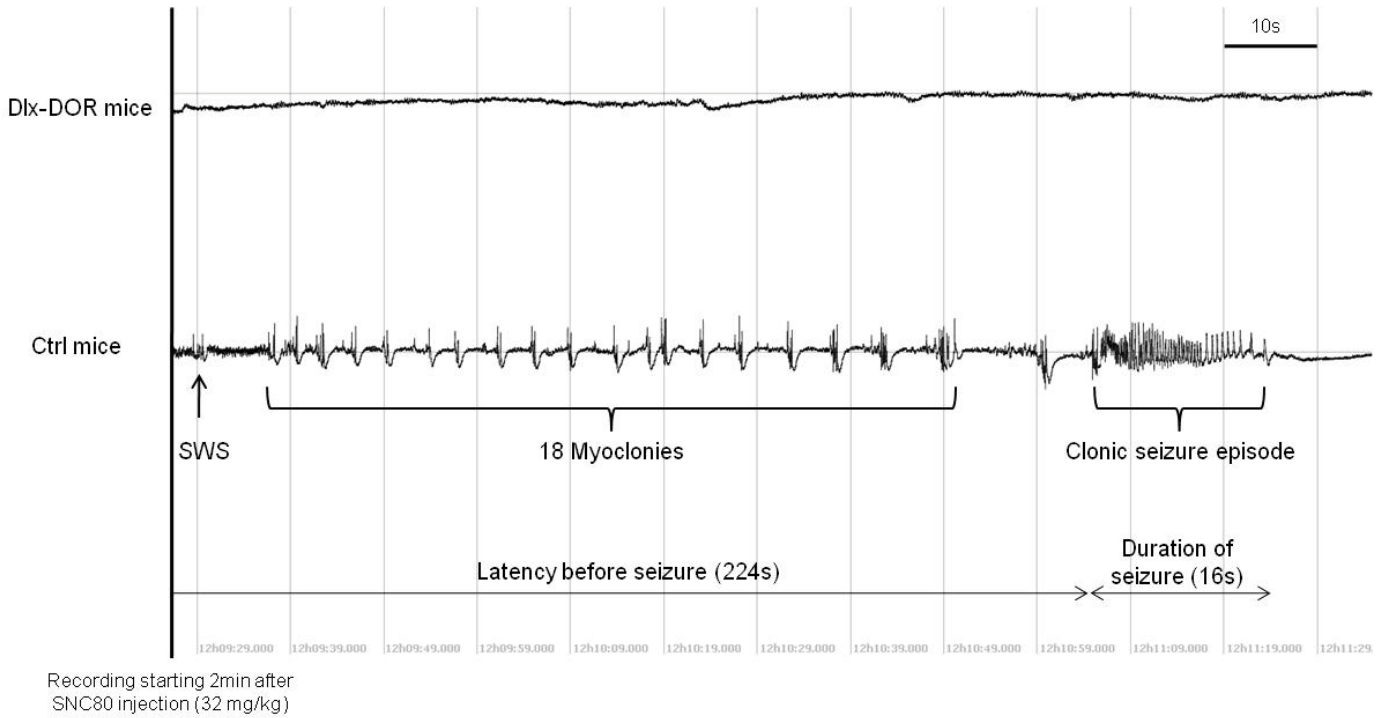



Fig. 2: **EEG recordings from Dlx-DOR (top) and Ctrl mouse (bottom) after SNC80 administration.** A representation EEG recording session is shown (extracted from Cartool software) and seizures parameters measured are indicated. Recording starts 2min after SNC80 injection (32 mg/kg, s.c.). On the Ctrl mouse trace, a spike-and-wave discharge (SWS) is observed at the beginning of the session, followed by 18 myoclonic events which in turn lead to clonic seizure. No characteristic events are observed on Dlx-DOR mouse trace.

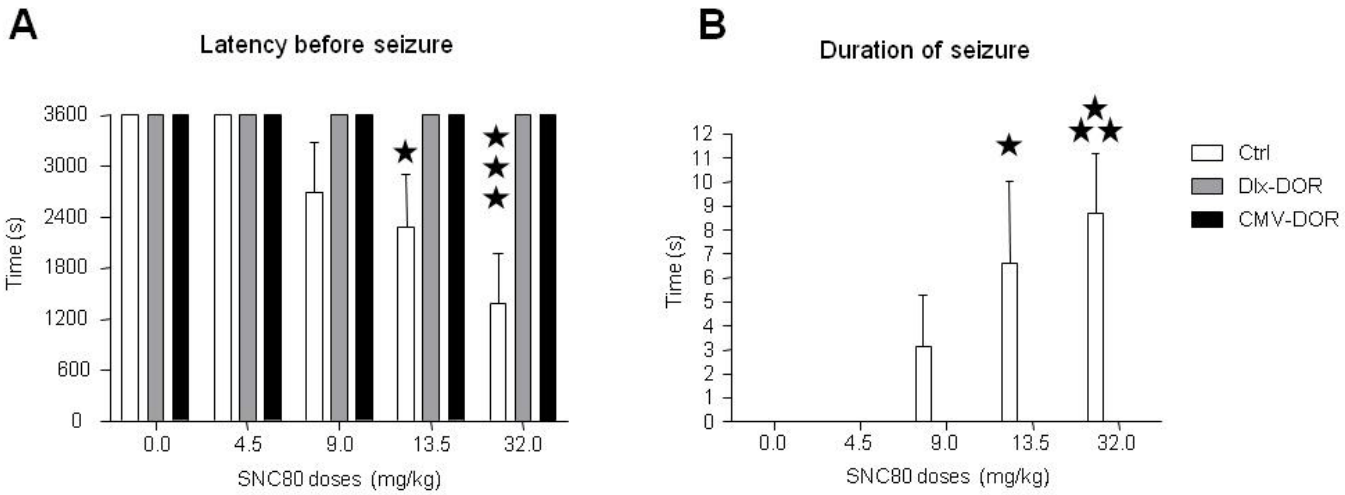



Fig. 3: **Epileptic seizures induced by SNC80. (A)** Latency before the first seizure event and **(B)** duration of the seizure are represented. Highest doses (9, 13.5 and 32 mg/kg) of SNC80 decreased latency before seizure and increased duration of seizures in Ctrl (black bars) mice, whereas no detectable change occurred in Dlx-DOR and CMV-DOR mice. n= 8 per genotype. All data are presented as means ± S.E.M. Data were analyzed using StatView 5.0 software (SAS Institute, Cary, NC). Drug pharmacokinetics was analyzed by using repeated-measures ANOVA followed by Student's t test for individual time points when appropriate. The analysis of pharmacological effect was performed by using two-way ANOVA for drug and genotype effects followed by Bonferroni *post hoc* analysis to determine statistically significant differences (One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$).

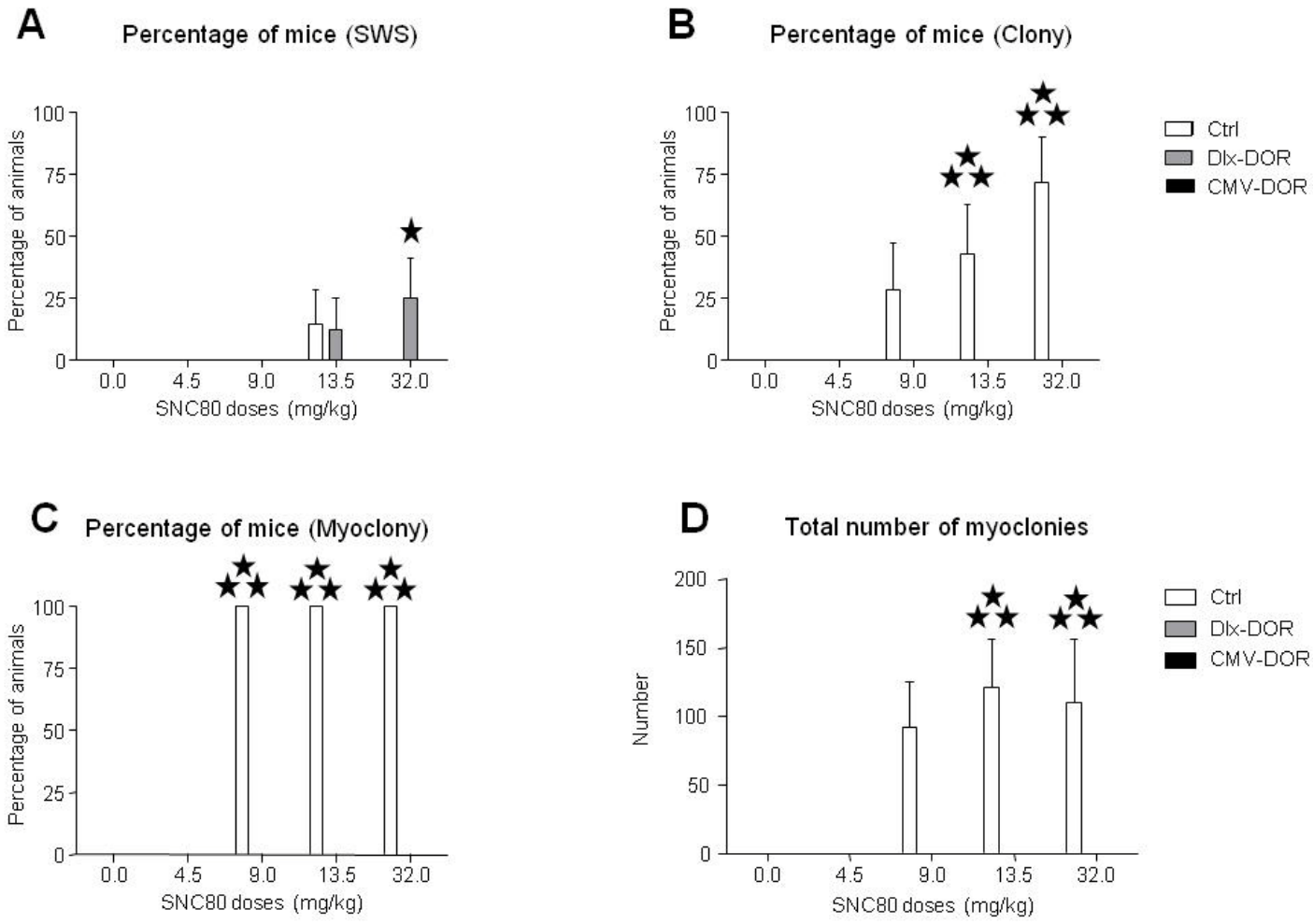



Fig. 4: **SNC80-induced EEG patterns.** Graphs represent the percentage of Ctrl, Dlx-DOR and CMV-DOR mice that showed **(A)** SWS, **(B)** myoclonic and **(C)** clonic seizures on EEG records. Highest doses (9, 13.5 and 32 mg/kg) of SNC80 lead to increased percentage of Ctrl mice that exhibited myoclonic and clonic seizures. No detectable change occurred on EEG recordings for Dlx-DOR and CMV-DOR mice. **(D)** The number of myoclonies per period of 20 min was measured. n= 8 per genotype. All data are presented as means ± S.E.M (One star, $P<0.05$; two stars, $P<0.01$; three stars, $P<0.001$).

# Supplementary experiments

## Introduction

Beyond modification of anxiety-related behaviors, we demonstrated that the lack of DOR on forebrain GABAergic neurons abolishes SNC80-induced epileptic seizures. The conditional deletion also modifies SNC80- and D1R agonist-induced locomotor stimulation (manuscript 1). We therefore continued investigating whether the specific excision of DOR from forebrain GABAergic neurons may alter other behavioral processes; in particular those that we know are altered in constitutive knockout mice.

We previously found that Dlx-DOR mice showed comparable locomotor activity to Ctrl mice under basal non-stressful conditions (see Manuscript 1). In contrast, mutant mice expressed facilitated D1/D3 agonist-induced locomotor stimulation and we hypothesized that this effect could be mostly attributed to massive deletion of DOR in the striatum. The dorsal striatum plays a critical role in the regulation of several physiological responses, especially the regulation of motor activity and coordination (Kreitzer and Malenka 2008). Motor skill learning is strongly dependent on dorsal striatum function (Durieux, Schiffmann et al. 2012) and we previously reported enhanced performance on the rotarod for constitutive DOR knockout mice (Le Merrer, Rezai et al. 2013). Therefore, we also investigated performance of Dlx-DOR mice in this test, classically used to assess the motor skill learning abilities.

The anatomical characterization of DOR expression in Dlx-DOR mice also revealed partial deletion in the hippocampus. The hippocampus has been extensively studied for its implication in learning and memory processes (Morris, Garrud et al. 1982; Langston, Stevenson et al. 2010). Spatial memory critically depends on hippocampal activity (Oliveira, Hawk et al. 2010). We have previously shown that genetic and pharmacological inactivation of DOR induces a deficit in the novel object recognition task (Le Merrer, Rezai et al. 2013), a behavioral paradigm classically used to evaluate the ability to discriminate either novel objects or their spatial

location. We also examined whether the deletion of DOR from forebrain GABAergic neurons alters mnemonic capacities in a fear conditioning paradigm.

## Material and methods

### Animals

Experiments were performed on animals aged between 6 and 18 weeks, housed 2-4 per cage under standard laboratory conditions (12h dark/light cycle light on at 7am). Food and water were available ad libitum. All mice were generated at Institut Clinique de la Souris-Institut de Genetique et Biologie Moleculaire et Cellulaire. Independent cohorts of $Oprd^{fl/fl}$ (Ctrl), conditional knockout (Dlx-DOR) and full knockout mice (CMV-DOR) were tested in the different behavioral paradigm. Mice were habituated to their new experimental environment and handled for 1 week before starting the experiments. All behavioral testing was performed with the observer blinded to the genotype and/or treatment of the animals. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).

### Rotarod

Mice were placed on a rotarod apparatus (Bioseb, Valbonne, France) accelerating from 4 to 40 rpm in 5 min. The external perimeter of the rod covered with insulation tubing was 5 cm. Light intensity in the room was 40lux. Test was performed under classical conditions as previously described (Le Merrer, Rezai et al. 2013). Behavioral testing lasted five days. On day 1, mice were habituated to rotation on the rod under a constant speed of 4 rpm, until they were able to stay on the rod more than 180s. From day 2 to day 9, mice were tested for three trials a day (1 min ITI) on consecutive days. Each trial started by placing the mice on the rod and beginning rotation at constant 4 rpm-speed for 60 s. Then the accelerating program was launched, and trial ended for a particular mouse when falling off the rod. Time spent on the rod was automatically recorded.

### Novel Object Recognition

The experiments were conducted in 4 equal square arenas (50x50 cm) separated by 35 cm-high opaque grey Plexiglas walls. Light intensity of the room was set at 15 lx to facilitate exploration and minimize anxiety levels, the floor was a white Plexiglas platform (View Point, Lyon, France), spread with sawdust. The room was equipped with an overhead video camera connected to a computerized interface, allowing visualization and recording of behavioral sessions on a computer screen in the adjacent room.

The experimental paradigm was adapted from (Carey, Lyons et al. 2009), and lasted for 2 days. On day 1, animals were placed in an arena for a 15 min-habituation session with two copies of an unfamiliar object (T-shaped plastic tubing, 1.5x3.5 cm). These objects were not used later for recognition test. On day 2, the recognition test was performed. The test consisted of 3 trials of 10 minutes separated by 2 intertrial intervals of 5 minutes, during which the animals returned to their home cage. On the first trial, or familiarization phase, two copies of an unfamiliar object are presented to mice. On the second trial, or place phase, one of the two copies was displaced to a novel location in the arena. Finally, on the third trial, or object phase, the copy that had not been moved on previous trial was replaced by a novel object. Stimuli objects used in all previous experiments were Lego bricks, plastic rings, dices or marbles (size 1.5-3x2-3 cm). The identity of objects as well as spatial location of these objects was balanced between subjects. The number of visits and the time spent to explore each object were scored manually on video recordings. A visit was counted when the nose of the mouse came in direct contact with an object. A percentage of discrimination was calculated for number of visits and time exploring the objects as following: exploration of displaced or novel object / total exploration * 100. The percentage of discrimination during familiarization phase was arbitrary calculated for the object located in the right up corner of the arena.

**Fear conditioning paradigm**

Experiments were conducted in four operant chambers (28 x 21 x 22 cm, Coulbourn Instruments, Allentown, US), with a Plexiglas door and a metal bar floor linked to a shocker (Coulbourn Instruments). Chambers were dimly lit with a permanent house-light and equipped with a speaker for tone delivery. An infrared activity monitor, placed on the ceiling of each chamber, was used to assess animal

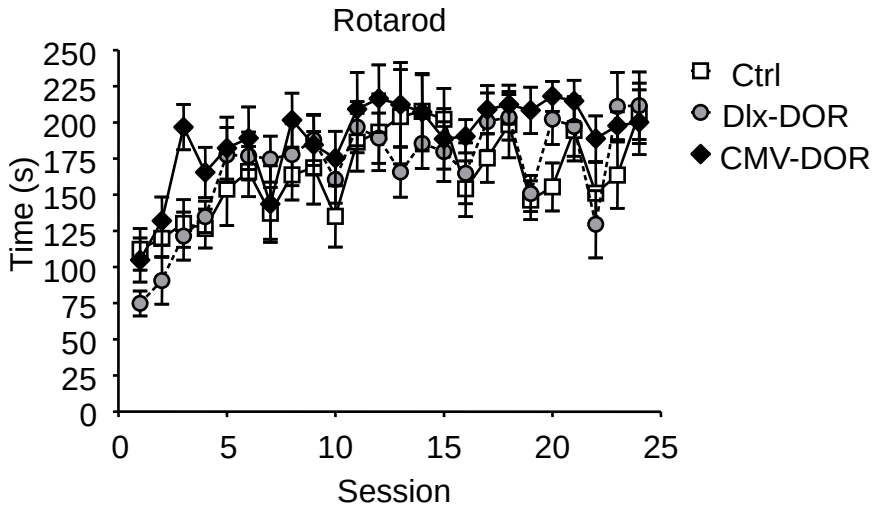



Fig. 2.1: **(A) Motor skill learning**. Ctrl, Dlx-DOR and CMV-DOR mice were tested on the accelerating rotarod. No significant difference was found across genotypes. (n= 7-8 per genotype).

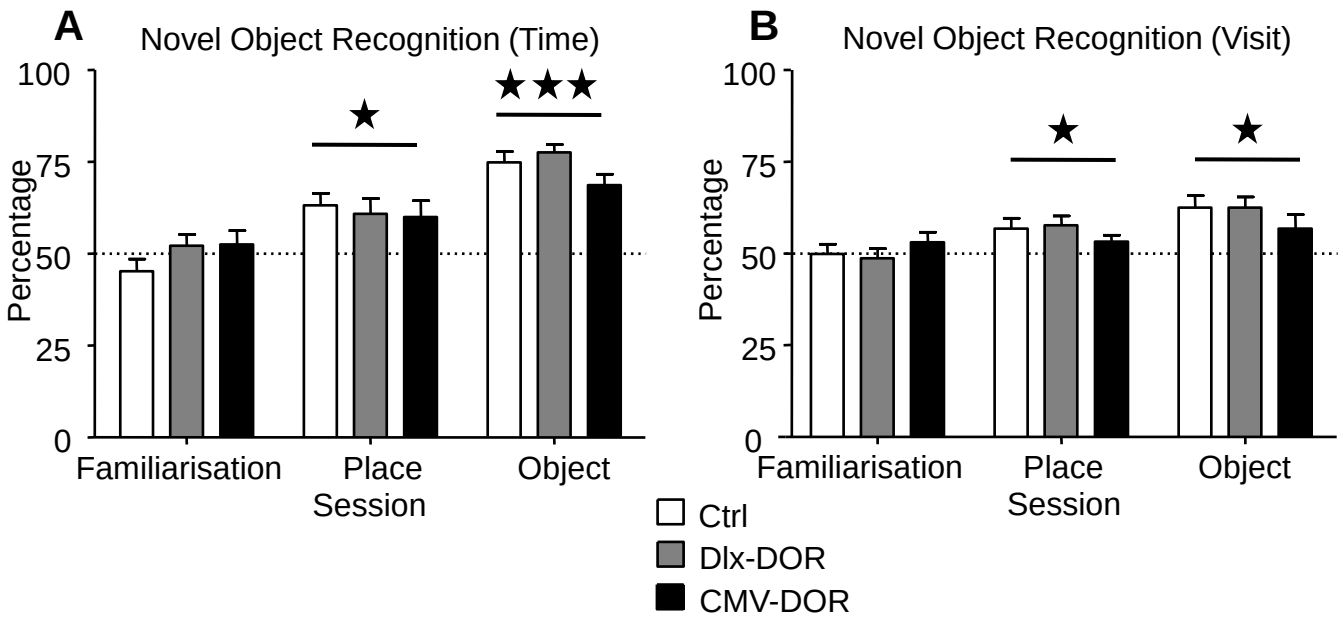



Fig. 2.2: **Spatial memory**. Ctrl, Dlx-DOR and CMV-DOR mice were tested on the novel object recognition test. Familiarization, exposure to two identical object; Place, one object from the familiarization session is moved; Object, unmoved object in place session is replaced by another one. All groups displayed increased **(A)** time exploration and **(B)** number of visit significantly above chance level in the phase and object (n= 14-18 per genotype). For all the tests, filled stars indicate significant differences compare to chance level. One star, $P<0.05$; three stars, $P<0.001$ (two-way ANOVA).

motion. The activity/inactivity behavior was monitored continuously during 100 ms period. Data were expressed in duration of inactivity per 1 s and the total time of inactivity displayed by each subject during training and testing sessions was counted. The procedure was similar as previously described (Goeldner, Reiss et al. 2009). Briefly, animals went through one conditioning session and two testing sessions (contextual and cued fear conditioning). The conditioning session was initiated with a 4-min habituation period followed by a 20 s long tone of 20 KHz/75 dB (conditional stimulus, CS) that was coupled with a 0.4 mA footshock (unconditional stimulus, US) during the last second. A similar CS-US pairing was presented 2 min later and the mice were removed from the apparatus 2 min after the footshock. The following day, mice were exposed again to the conditioning chamber and freezing behavior characterized by episodes of immobility was measured during 2 min to assess contextual fear conditioning. Then, 5 h later cued fear conditioning was assessed in modified chambers.

## Results

### Dlx-DOR mice display normal motor skill learning performances

In our experimental conditions, Dlx-DOR, CMV-DOR and Ctrl mice displayed similar performances on the accelerated rotarod ($p>0.05$, Two-way ANOVA, see Figure 2.1). The three groups reached a maximum of performances already from the second day of experiment ($6^{th}$ session), suggesting that experimental conditions made the test too easy, and that a ceiling effect prevented the possibility to observe improved performances. This may explain why enhanced performance was not detected for CMV-DOR mice, as previously shown for constitutive knockout mice (Le Merrer, Rezai et al. 2013).

### Dlx-DOR mice display normal memory performances in novel object recognition

Since partial deletion of DOR in the hippocampus may affect memory performances, we tested Dlx-DOR mice in the novel object recognition task. Figures 2.2 A and B respectively show percentage of time and approach to both displaced and novel object.

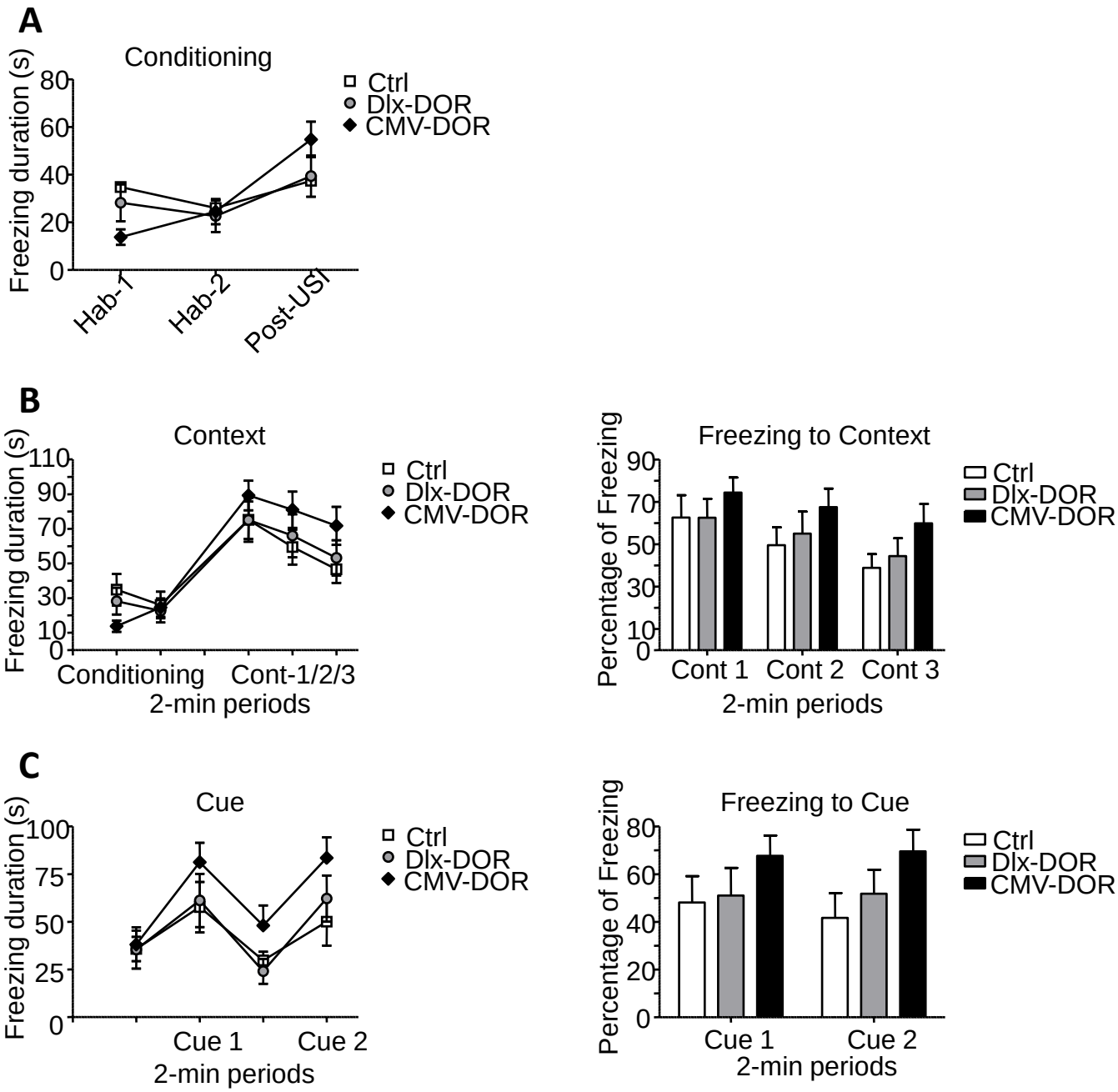



Fig. 2.3: **Fear conditioning test**. Ctrl, Dlx-DOR and CMV-DOR mice were tested for fear conditioning responses. Hab-1 or -2, habituation session in apparatus without shocks; Post-USI, session with footshock; Cont-1, -2 or -3, session with reexposition to the context; Cue-1 or-2, sessions with reexposition to the auditory cue. **(A)** Conditioning session. A slight increase in immobility time was observed in CMV-DOR mice (black diamond) compared with Ctrl (white square), however no significant difference was found across genotypes **(B)** Context session. Freezing duration and percentage did not differ across genotypes when mice were re-exposed to the conditioned context (Cont-1, Cont-2 and Cont-3). **(C)** Cue session. Freezing duration and percentage did not differ across genotypes when mice were re-exposed to the conditioned auditory cue (Cue-1 or Cue-2). (n= 7-10 per genotype).

The familiarization session showed similar exploration of both objects across genotypes. During place and object sessions, the three groups spent a significant increased amount of time to explore the displaced or novel object than the control object ($p<0.001$, Two-way ANOVA). *Post hoc* revealed a significant increase of exploration time and number of visit above chance level for each group in the place ($p<0.05$, Bonferroni/Dunn test) and object sessions ($p<0.01$, Bonferroni/Dunn test) which indicates intact detection of spatial location and novelty. Additionally, memory performances reached comparable levels across genotypes during place and object sessions ($p>0.05$, Two-way ANOVA). Our results suggest that DOR expressed on forebrain GABAergic neurons are not involved in the modulation of learning and memory processes. However, these results should be treated with caution since memory deficit previously reported in constitutive knockout (Le Merrer, Rezai et al. 2013) was not found either in these conditions.

**Fear conditioning responses remain intact in the Dlx-DOR mice**

Previous studies emphasized the role of DOR in drug-context associations (Le Merrer, Plaza-Zabala et al. 2011; Faget, Erbs et al. 2012; Laurent, Leung et al. 2012). Moreover, DOR were described as implicated in memory formation (Robles, Vivas-Mejia et al. 2003). To investigate whether DOR are involved in the acquisition of contextual and cued fear conditioning, we tested Ctrl, Dlx-DOR and CMV-DOR mice in a standard fear conditioning paradigm (Fig. 2.3).

During the training session (Fig. 2.3A), animals received a footshock paired with a tone. The three group displayed similar immobility level before and after footshock, an index of fear response. ANOVA repeated measures on habituation session showed no Genotype effect ($F_{(2, 44)} = 2.07$; $p>0.05$) but significant Time ($F_{(2, 44)} = 16.57$; $p<0.0001$) and Time*Genotype interaction ($F_{(4, 44)} = 4.53$; $p<0.01$) effects. The CMV-DOR mice showed a trend toward higher freezing level after the shock in comparison with Ctrl mice (54.75 ± 7.46s in CMV-DOR mice vs. 37.43 ± 9.04s in Ctrl mice).

For the context session (Fig. 2.3B), animals were re-exposed to the context 24h later and exhibited a similar context-induced fear conditioning, as reflected by increase of freezing. The analysis of variance on repeated measures showed no Genotype effect ($F_{(2, 44)} = 1.08$; $p>0.05$) but a significant Time effect $F_{(2, 44)} = 13.95$; $p<0.0001$) and no Genotype x Time interaction effect ($F_{(2, 44)} = 0.29$; $p>0.05$). The CMV-DOR mice tend to display more contextual fear behavior as compare to Ctrl and Dlx-DOR mice (67.22 ± 7.33% in CMV-DOR mice; 50.32 ± 7.8% in Dlx-DOR mice; 53.94 ± 8.81% in Dlx-DOR mice).

Similarly, when re-exposed to the cue previously associated with shocks (Fig. 2.3C) the three groups showed similar level of freezing. The ANOVA repeated measures revealed a significant Time effect ($F_{(3, 66)} = 18.75$; $p<0.0001$) but no Genotype effect ($F_{(2, 66)} = 1.40$; $p>0.05$) and no Time*Genotype interaction effect ($F_{(6, 66)} = 1.01$; $p>0.05$). Although, the three genotypes showed increased freezing during the tone presentation, the CMV-DOR mice tend to be more immobile in comparison with Ctrl and Dlx-DOR mice (Cue-1: 67.71 ± 8.5% in CMV-DOR mice; 48.1 ± 11.06% in Ctrl mice; 51 ± 11.65% in Dlx-DOR mice). These results suggest that DORs are not involved in the acquisition of contextual and cued fear memory. The tendency for increased freezing to both context and cue in CMV-DOR mice may reflect the well-established high anxiety phenotype in constitutive knockout mice (Filliol, Ghozland et al. 2000).

## Discussion

In the present experiment, we showed that despite a massive deletion of DOR in the olfactory bulb, striatum and hippocampus, Dlx-DOR mice show intact performance in accelerated rotarod, novel object recognition and fear conditioning. These results suggest that DORs expressed on forebrain GABAergic neurons do not affect motor skill learning and memory performances, or alternatively that our experimental conditions did not allow detection of a phenotype that may be mild.

We found that the three genotypes displayed comparable performances on the accelerated rotarod task. Under our experimental conditions, all animals rapidly

reached a maximal level of performances suggesting that experimental apparatus and procedure were not adapted to detect an improvement of motor skill learning. Indeed, our experimental conditions are similar to those described previously as “easy” experimental conditions (Le Merrer, Rezai et al. 2013) and could be more appropriate to see impaired motor skill learning.
Constitutive DOR KO mice were previously described to exhibit improved motor skill learning under more difficult experimental conditions (Le Merrer, Rezai et al. 2013). Therefore, in the future additional experiment will be performed under these conditions to further assess motor skill learning in constitutive knockout CMV-DOR and conditional knockout Dlx-DOR mice.

CMV-DOR mice did not show altered performances in place session of the novel object recognition task, unlike previously reported (Le Merrer, Rezai et al. 2013). This discrepancy could be related to the genetic background. Indeed, constitutive DOR knockout mice which display a deficit in spatial memory in previous study were bred on 50% 129SvPas-50% C57BL/6J background, whereas the CMV-DOR in the present study are bred on 25% 129SvPas-75% C57BL/6J background. Strain differences in learning and memory tasks were previously reported and highlighted the better performances of C57BL/6J mice in spatial memory (Holmes, Wrenn et al. 2002; Patil, Sunyer et al. 2009).

To further explore the contribution of DOR in memory processes, Ctrl, Dlx-DOR and CMV-DOR mice were also tested for fear conditioning in context-shock and cue-shock association conditions. Total knockout (CMV-DOR) mice were never previously tested in fear conditioning paradigm. Mutant mice showed a tendency to increased immobility in both context and cue-induced fear conditioning sessions, suggesting that DOR activity normally impairs acquisition and expression of fear conditioning. Additional experiments, increasing number of animals, may confirm this phenotype.

Conditional Dlx-DOR mice show significantly reduced receptor number in the hippocampus. In the fear conditioning paradigm, processing of contextual informations are mainly associated with the hippocampal function (Sanders and

Fanselow 2003; Chang, Chen et al. 2008), and we anticipated that modifications potentially detected in constitutive CMV-DOR mice would also be detected in Dlx-DOR mice in this test. Conditional mutant mice, however, did not even show a trend to increased freezing, as do total knockout mice. Because associations to conditioned cues also involve the basolateral nucleus of the amygdala (LeDoux 2000), and Dlx-DOR mice show only partial receptor deletion in the hippocampus and intact receptors in basolateral amygdala, it is likely that enough DORs remain functional at the level of hippocampal-amygdala circuits to ensure normal fear conditioning. Other explanations for lack of phenotype, of course, are that (i) a mild phenotype was not detectable under our experimental conditions, (ii) DORs in forebrain GABAergic neurons are not tonically involved in this behavior or (iii) compensatory modifications in both total and conditional knockout hinder DOR influence on fear conditioning. Altogether, additional experiments should be performed to definitely conclude for a role, or lack of role of DORs in fear conditioning.

In conclusion our data show that, despite the dramatic deletion of DOR in olfactory bulb, striatum and hippocampus, many behavioral responses seem intact in Dlx-DOR mice. Refined experimental conditions should definitely established whether DORs expressed in forebrain GABAergic neurons tonically regulate coordination skills and memory performances, which were previously identified as DOR-regulated behavior in total knockout mice. Also, the conditional deletion of DORs in non GABAergic neuron populations may reveal, in the future, a prominent role for DORs expressed in glutamatergic or cholinergic neurons in the control of some forms of cognition.

Carey, A. N., A. M. Lyons, et al. (2009). "Endogenous kappa opioid activation mediates stress-induced deficits in learning and memory." The Journal of neuroscience : the official journal of the Society for Neuroscience **29**(13): 4293-4300.

Chang, S. D., D. Y. Chen, et al. (2008). "Infusion of lidocaine into the dorsal hippocampus before or after the shock training phase impaired conditioned freezing in a two-phase training task of contextual fear conditioning." Neurobiol Learn Mem **89**(2): 95-105.

Durieux, P. F., S. N. Schiffmann, et al. (2012). "Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions." EMBO J **31**(3): 640-653.
Faget, L., E. Erbs, et al. (2012). "In vivo visualization of delta opioid receptors upon physiological activation uncovers a distinct internalization profile." J Neurosci **32**(21): 7301-7310.
Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.
Goeldner, C., D. Reiss, et al. (2009). "Activation of nociceptin opioid peptide (NOP) receptor impairs contextual fear learning in mice through glutamatergic mechanisms." Neurobiol Learn Mem **91**(4): 393-401.
Holmes, A., C. C. Wrenn, et al. (2002). "Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice." Genes Brain Behav **1**(1): 55-69.
Kreitzer, A. C. and R. C. Malenka (2008). "Striatal plasticity and basal ganglia circuit function." Neuron **60**(4): 543-554.
Langston, R. F., C. H. Stevenson, et al. (2010). "The role of hippocampal subregions in memory for stimulus associations." Behav Brain Res **215**(2): 275-291.
Laurent, V., B. Leung, et al. (2012). "mu- and delta-opioid-related processes in the accumbens core and shell differentially mediate the influence of reward-guided and stimulus-guided decisions on choice." J Neurosci **32**(5): 1875-1883.
Le Merrer, J., A. Plaza-Zabala, et al. (2011). "Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement." Biol Psychiatry **69**(7): 700-703.
Le Merrer, J., X. Rezai, et al. (2013). "Impaired Hippocampus-Dependent and Facilitated Striatum-Dependent Behaviors in Mice Lacking the Delta Opioid Receptor." Neuropsychopharmacology.
LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci **23**: 155-184.
Morris, R. G., P. Garrud, et al. (1982). "Place navigation impaired in rats with hippocampal lesions." Nature **297**(5868): 681-683.
Oliveira, A. M., J. D. Hawk, et al. (2010). "Post-training reversible inactivation of the hippocampus enhances novel object recognition memory." Learn Mem **17**(3): 155-160.
Patil, S. S., B. Sunyer, et al. (2009). "Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze." Behav Brain Res **198**(1): 58-68.
Robles, Y., P. E. Vivas-Mejia, et al. (2003). "Hippocampal gene expression profiling in spatial discrimination learning." Neurobiol Learn Mem **80**(1): 80-95.
Sanders, M. J. and M. S. Fanselow (2003). "Pre-training prevents context fear conditioning deficits produced by hippocampal NMDA receptor blockade." Neurobiol Learn Mem **80**(2): 123-129.

# Third Part

# DOR expressed in the basolateral nucleus of the amygdala

## Introduction

As previously mentioned, constitutive knockout mice exhibit enhanced anxiety-related and despair-like behaviours, revealing the mood-enhancing activity of DORs in normal physiology (Filliol, Ghozland et al. 2000). An extensive literature confirmed this function by using pharmacological blockade or activation of DORs with systemic or local administration (Chu Sin Chung and Kieffer 2013). These studies emphasized the anxiolytic function of DOR especially expressed in the cingulate cortex (Narita, Kuzumaki et al. 2006), hippocampus (Solati, Zarrindast et al. 2010) as well as in the amygdala (Narita, Kaneko et al. 2006; Randall-Thompson, Pescatore et al. 2010). In addition, we previously uncovered highly distinct –somehow opposing- emotional responses in Dlx-DOR mice compared to total knockout mice (see Manuscript 1), suggesting that receptors responsible for the anxiolytic DOR effect have remained intact in these mice. These receptors may be expressed in non-GABAergic neurons of cortical areas, hippocampus and/or basolateral nucleus of the amygdala (BLA), or be transported from more posterior regions where Cre recombinase is ineffective. Altogether, current data have not allowed identifying the precise neuronal population responsible for the anxiolytic and antidepressant DOR activity. In third part, we initiated experiments to address the hypothesis of BLA-mediated mechanisms.

The basolateral amygdala (BLA) presents a cortex-like cytoarchitectonic composition. The BLA is essentially composed of glutamatergic projecting neurons (≈ 75%) and of a few local GABAergic interneurons (≈ 25%) (McDonald 1982; McDonald 2003).

The BLA receive massive afferent projections from cortical areas (agranular insular, prelimbic, infralimbic, parietal, piriform, entorhinal, perirhinal and temporal cortex), thalamus (dorsomedial, paraventricular, rhomboidal nuclei), hippocampus (CA1 and subiculum), hypothalamus (ventromedial, lateral, posterior and perifornical areas), basal forebrain nuclei (ventral pallidum, globus pallidus, substantia innominata) and some brainstem nuclei (dorsal raphe, ventral tegmental area, locus coeruleus and parabrachial nucleus) (McDonald 1998; Knapska, Radwanska et al. 2007).

The information processing in the amygdala has been traditionally viewed as a serial model with linear dorso-ventral and latero-medial intra- and inter-nuclear

connections (Rogan and LeDoux 1996; Ehrlich, Humeau et al. 2009). This model suggested that the thalamus, primary sensory areas and associative cortex send multimodal sensory informations to the amygdala, primarily entering through the dorsal and ventral parts of the lateral nucleus of the amygdala. In this model, the lateral nucleus of the amygdala is considered as the main sensory integrative area. Then, information is distributed throughout the entire basolateral nuclei. The glutamatergic projecting neurons of the BLA transfer the information to the CeA nuclei. The CeA represent the main output areas of the amygdala and thus, connect to several brainstem and hypothalamic nuclei to trigger the autonomic and behavioral responses relevant for the environment.

Several studies assessing instrumental and pavlovian aversive conditioning paradigms following lesions of different amygdalar nuclei supported the view of a "parallel" model for information processing (Figure 13) (Killcross, Robbins et al. 1997). Indeed, animals with a lesion of the BLA showed inability to avoid a conditioned aversive stimulus, whereas performances of animals with a lesion of the CeA were not affected. Conversely, the excitotoxic lesion of the CeA, but not of the BLA, decreased the extinction towards conditioned fear stimulus. Additionally, a similar dissociation between the CeA and the BLA has been reported in a task towards appetitive stimulus (Parkinson, Robbins et al. 2000). These studies emphasize that the BLA and CeA may act independently and support the "parallel" model of amygdala function (Balleine and Killcross 2006)

The BLA has been described to be a key player in the regulation of emotions and has been extensively studied in the context of fear conditioning processes (LeDoux 2000; Mamiya, Fukushima et al. 2009). Moreover, radiolabeled binding assays revealed high DOR expression level in the BLA (Goody, Oakley et al. 2002). Consequently, the BLA seems an interesting brain area candidate to support the anxiolytic function of DORs. In this third part, we focused on the BLA and first initiated an experiment of local DOR knockdown using an Adeno-Associated Virus (AAV) technology.

AAV is a virus containing a single-stranded DNA of 4.7kb composed of two inverted terminal repeats sequence, two open reading frames (*rep* and *cap*) required

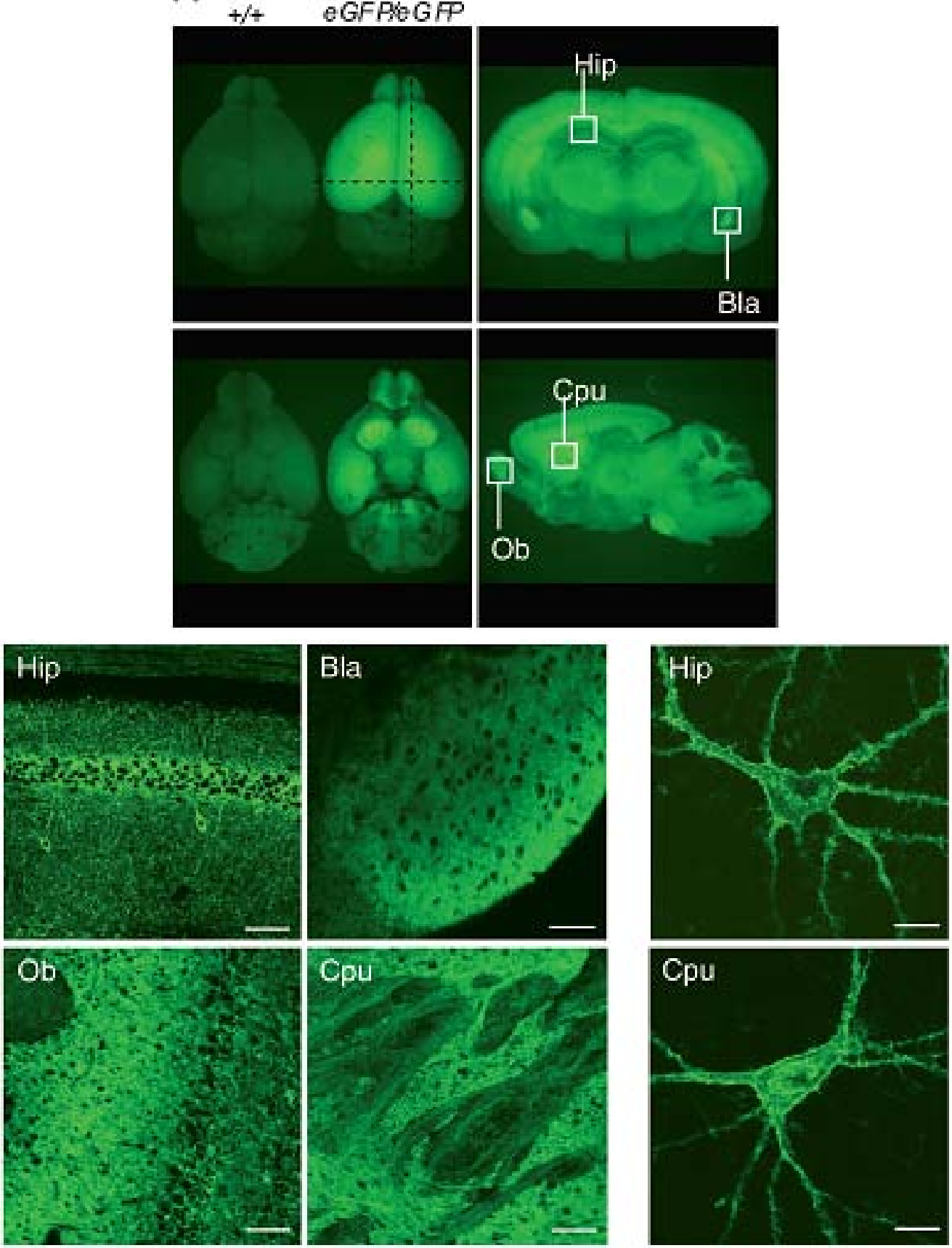
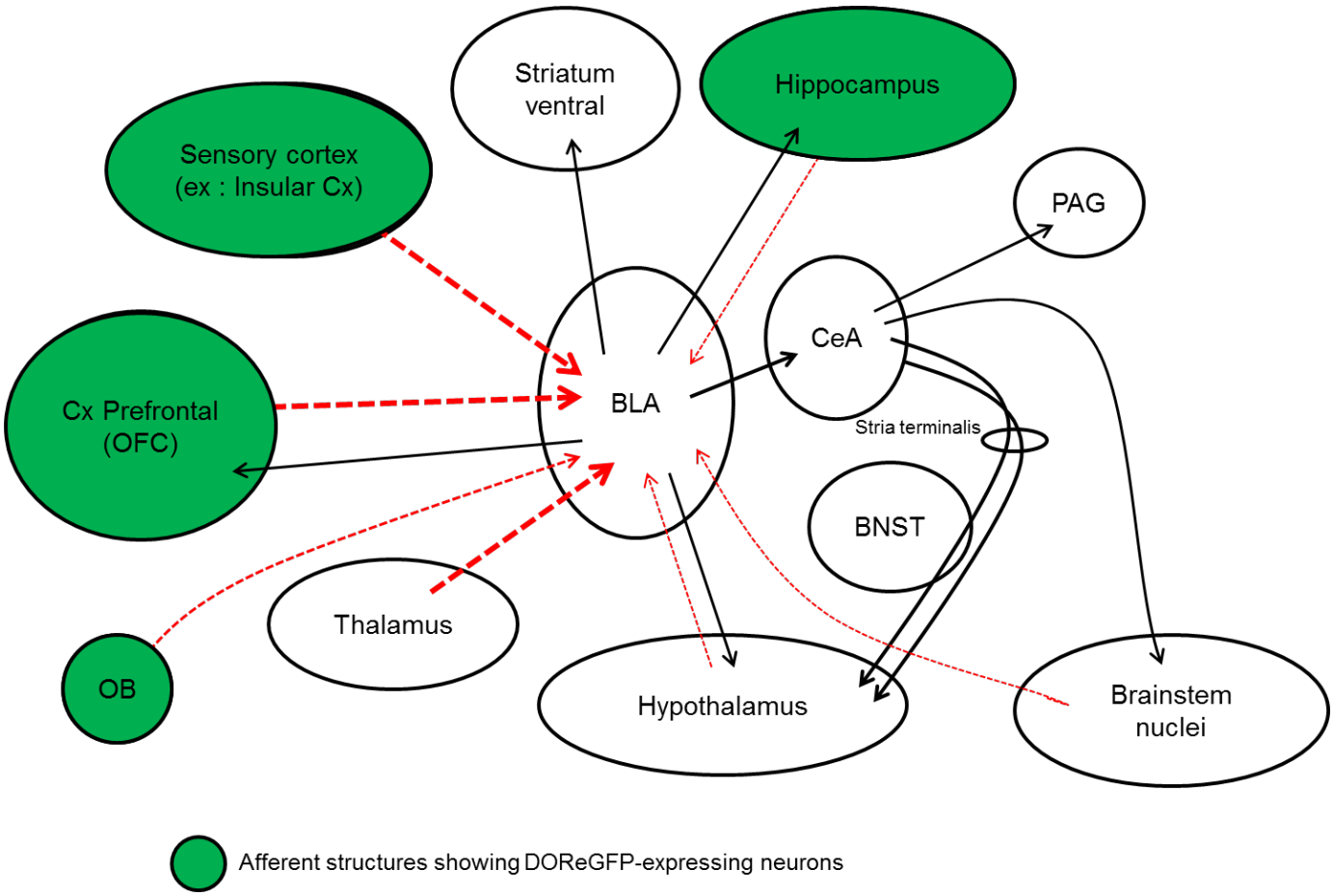



Figure 3.1:
DOR-eGFP knockin mice (Scherrer et al., 2006). The DOR-eGFP is expressed in same regions as the native endogenous DOR, in the olfactory bulb (Ob) hippocampus (Hip), basolateral nucleus of the amygdala (Bla), caudate-putamen nucleus (Cpu). This mouse line will allow to further investigate the cellular localization of DOR expression *in vivo* in various physiological conditions.

for AAV capsids proteins (Goncalves 2005). Recombinant AAV (rAAV) is a technology that allows the expression of sequence of interest in infected cells (Tenenbaum, Chtarto et al. 2004). AAVs present different tropism depending on their capside proteins composition and structures, which define AAV serotype. Currently up to 11 AAV serotypes were described. For instance, serotypes 1, 2 and 5 were described to exhibit a specific neuronal tropism (Burger, Gorbatyuk et al. 2004; Paterna, Feldon et al. 2004). This technology allows an accurate spatial and temporal control over expression of integrated sequence. In the present study, we used a rAAV2 encoding the Cre recombinase and targeted the BLA of DOR floxed mice.

Our laboratory has generated a knock-in mouse model in which DOR is expressed in fusion with the enhanced green fluorescent protein (eGFP) in place of the native endogenous DOR (Figure 3.1) (Scherrer, Tryoen-Toth et al. 2006). The autofluorescent GFP protein has been discovered and originally extracted from the jellyfish *Aequorea Victoria*. This molecule has been shown to be excited at 488 nm (blue) and to emit with a maximum at 510 nm wavelengths (green) (Zhang, Gurtu et al. 1996). The DOR-eGFP knock-in mice exhibit a functional receptor expressed at physiological levels. In these mice, mRNA levels analyzed by real-time PCR assays as well as the receptor activation measured by [$^{35}$S]-GTPγS binding experiments both revealed expression levels and signaling activity similar to wild-type animals. Moreover, classical DOR agonists SNC80, deltorphin-II or met-enkephalin showed similar affinity and selectivity values in mutant and wild-type animals. The use of DOR-eGFP knock-in mice allowed determining the precise neuronal and subcellular localization of DOR (Scherrer, Imamachi et al. 2009; Erbs, Faget et al. 2012; Rezai, Faget et al. 2012). Regarding the subcellular localization, DOR-eGFP is mainly expressed at the cellular surface and receptor activation induces internalization in about 20 minutes both ex-vivo (primary neurons from DOR-eGFP mice) and in vivo. Availability of these mice also allowed demonstrating distinct internalizing properties of DOR agonists (Pradhan, Becker et al. 2009), and that physiological internalization detected in a context-induced paradigm differs from drug-induced internalization (Faget, Erbs et al. 2012). In these mice, strong green fluorescent signal observed in the BLA confirms high DOR expression level in this particular brain structure (Scherrer, Tryoen-Toth et al. 2006).




Abbreviations
BLA, basolateral nuclmeus of the amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; Cx, cortex; OB, olfactory bulb; OFC, orbitofrontal cortex; PAG, periacqueductal gray.

Fig. 3.2: **Schematic representation of basolateral nuclmeus of the amygdala BLA projections.** BLA receive projections mainly from olfactory bulb, prefrontal cortex areas, sensory cortex regions, hippocampus, thalamus, hypothalamus and some brainstem nuclei (red dashed arrows). It project predominantly to central nucleus of the amygdala, prefrontal cortex regions, ventral striatum (ventral caudate-putamen and nucleus accumbens) and hippocampus (black arrows). (Adapted from Mansour et al., 1995; Knapska et al., 2007; Le Merrer et al., 2009).

The fluorescent signal observed at the level of BLA in DOR-eGFP mice is surprisingly homogeneous and, in contrast to hippocampus or cortex, no cell body is visible in brain sections (see Figure 3.1). Therefore animals were treated with SNC80 (10mg/kg, subcutaneous, 20 min), which triggers receptor concentration into endocytic vesicles and allows better detection of brightly stained cell bodies expressing DORs. Only few cell bodies became detectable upon this treatment (G. Scherrer, personal communication), suggesting that most fluorescence observed in the BLA represents presynaptic expression of DOR-eGFP on afferent terminals. Many brain areas project onto BLA. Among these are cortical areas, hippocampus and lateral hypothalamus (Mansour, Fox et al. 1995; Le Merrer, Becker et al. 2009), where DORs are also expressed. The main DOR-eGFP containing regions projecting to BLA are summarized in Figure 3.2.

Here, we therefore hypothesized that DOR located in the BLA are mostly expressed on presynaptic terminals. A consequence would be that most BLA DORs are, in fact, synthesized at a BLA-afferent site elsewhere in the brain. Identifying this site is therefore necessary to genetically knock-down this particular receptor population. We thus initiated retrograde tracing experiments using cholera toxin subunit B tracer loaded into the BLA of DOR-eGFP knockin mice, to identify the DOR-expressing BLA afferent pathways.

Cholera toxin is an oligomeric complex composed of one A subunit and five copies of the B subunit (CTB) (Lencer and Tsai 2003). Although the A subunit is support the enzymatic role, the B subunits form a pentameric ring responsible for tropism and transport of the toxin to cell bodies. Gangliosides GM1 enriched in the lipid rafts are bind by CTB and this triggers toxin endocytosis. CTB bypass cellular retrograde transport mechanism. CTB properties are largely used in neuroanatomical studies for retrograde neurons labeling (Angelucci, Clasca et al. 1996; Brown and Dyck 2005; Conte, Kamishina et al. 2009; Kaufling, Veinante et al. 2009).

## Material and methods

**Animals**

Experiments were performed on animals aged between 10 and 14 weeks, housed 2-4 per cage under standard laboratory conditions (12h dark/light cycle light on at 7am). Food and water were available ad libitum. All mice were generated at Institut Clinique de la Souris-Institut de Genetique et Biologie Moleculaire et Cellulaire. For the knockdown of DOR in the BLA, Oprdfl/fl were stereotaxically injected with AAV2-eGFP (n=10) or AAV2-Cre-eGFP (n=10). In the retrograde tracing experiment, DOR-eGFP knockin mice (n=4) received stereotaxic injection of Cholera Toxin Subunit B (CTB) in the BLA. In both experiments, the mice were individually housed for 3 days of recovery following the surgery. All experimental procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the local ethical committee (Comité d'éthique pour l'expérimentation animale IGBMC-ICS).

**Surgery**

Animals are anesthetized with an intraperitoneal injection of Ketamine 10% / Xylazine 5% solution (Kétamine 1g/kg; Xylazine 100mg/kg). Once deep anesthesia was confirmed, they were positionned on the stereotaxic device by placing blunt earbars into the ears. Ocrygel was applied to protect eyes and local anesthesic (lidocaïne) on the skin. A surgery was performed to make the skull available (sagital incision of the skin). A stainless-steel injector needle (0.18 mm internal diameter) was placed on the mount of the stereotaxic device and used to measure bregma coordinates. Then, according to Franklin and Paxinos Mouse Brain Atlas, coordinates of the target region are added or subtracted to bregma coordinates. A craniotomy was performed using a drill until the meninges were reached. Dura mater was excised with a syringe or a scalpel blade tip. The injected compound was loaded into the injector needle. Slowly lower the injector needle into the brain until the desired depth was reached. Injections of AAV and CTB were performed in similar experimental conditions: a 5 µl microsyringe (SGE Analytical Science, Australia) was mounted to a micro-drive pump (Harvard apparatus, France) and connected by a PE-10 polyethylene tubing (Harvard apparatus, France) to the stainless-steel injector needle and 1.5µl of AAV or CTB were injected at a rate of 0.1µl/min. When injection was done, the injector needle stayed at the injection site for 15min, and then

removed slowly (≈1min for 3mm). The skull surface was rinsed with sterile water and the wound sutured. 0.3-1ml of saline 0.9% was injected subcutaneously and the animal placed on hot plate (37°C) for recovering.
For both experiments, targeted region was the BLA (anteroposterior AP = -1.15mm, dorsoventral DV = +5mm, lateromedial LM = ±3.3mm) (Franklin K. B. J. and Paxinos G., 1997).

**Adeno-associated Virus**

Recombinant adeno-associated virus serotype 2 $rAAV_2$-Cre viral vectors were generated expressing enhanced green fluorescent protein (eGFP) and Cre recombinase under the control of CMV and mU6 promoters respectively. Control vectors encode eGFP alone ($rAAV_2$-eGFP). The $Oprd^{fl/fl}$ mice received bilateral injections of either $rAAV_2$-Cre (n=11) or $rAAV_2$-eGFP (n=9) into the BLA, as previously described (Del Boca, Lutz et al. 2012). The virus administration was performed by volumetric stereotaxic injections using stainless-steel injector needle (0.18 mm internal diameter). Animals were individually housed for 2 days following the surgery and were replaced in the regular housing (4 mice/cage) during 10 weeks. This time course of receptor down-regulation was previously determined using DOR GTPγS binding (data not shown). Twenty-four hours after the last behavioral experiment, all mice were sacrificed and brains analyzed for injection accuracy and viral spread, on epifluorescent microscope. After the histological analysis, 5 AAV-Cre and 2 AAV-eGFP injected mice were excluded from the study due to mis-targeted injections.

**Retrograde tracing experiment**

Experiments were performed in DOR-eGFP mice (25-30g, IGBMC-ICS). CTB conjugate with a biotin molecule was prepared at a concentration of 1mg/ml in neutral phosphate buffer. CTB was administered by volumetric stereotaxic injections.

DOR-eGFP mice were unilaterally injected with CTB-biotin conjugate into the BLA (n=4). Animals receive a subcutaneous injection of SNC80 (10 mg/kg) 20 min before the intracardiac perfusion to trigger DOR-eGFP internalization and simplify the visualization of DOR positive cells. After a survival time of 7 days, animals were sacrificed under anesthesia and brain extracted after paraformaldehyde 4%

intracardiac perfusion (rate = 4ml/min). The brain extracted was kept at 4°C in 4% paraformaldehyde overnight. Brain sections of 30µm were performed using the vibratome system and sections were collected into PB 0.1M.

Tracer labeling was obtained by immunochemistry on floating sections. Sections were washed 3 times into a blocking solution (PB 0.1M + Normal Goat Serum 5% + TritonX100 0.5%) and incubated during 2 hours in a revealing solution (Streptavidin-Alexa Fluor$^{594}$ conjugated 1/2000 + PB 0.1M). Then, sections were mounted serially onto SuperfrostTM glass (Menzel-Glaser) with a mowiol solution containing DAPI (4',6-diamidino-2-phenylindole) (0.5 µg/ml).

**Images acquisition**

Sections were processed with the slide scanner NanoZoomer 2 HT equipped with the fluorescence module L11600-21 (Hamamatsu Photonics, Japan) to acquire images at high resolution of the fluorescent signal. The scanner is equipped with a filter-set optimized for DAPI, fluorescein and tetramethylrhodamine. The number of cells retrogradely labeled, expressing DOR-eGFP or which shows a colocalized signal were counted manually on images acquired with the Nanozoomer 2.0 HT using the NDP viewer system.

In addition, some images were also acquired with the LCS (Leica) software on the confocal microscope (SP2RS, Leica) using 40x (NA: 1.25) and 63x (NA: 1.4) objectives.

**Behavioral experiments**

The Oprd$^{fl/fl}$ mice received a bilateral injection of $AAV_2$-eGFP (n=7) or $AAV_2$-Cre-eGFP (n=6) were tested in a battery of tests in the following order: the light-dark box, elevated plus maze and tail suspension tests. The behavioral experiments were conducted following the same procedure as described above (see Manuscript 1, Supplementary).

**Statistical analyses**

Statistical differences were determined by analysis of variance (ANOVA) (StatView 5, SAS Institute Inc., Cary, North Carolina) followed by Bonferroni/Dunn post hoc analysis. The F values and experimental degrees of freedom are included in

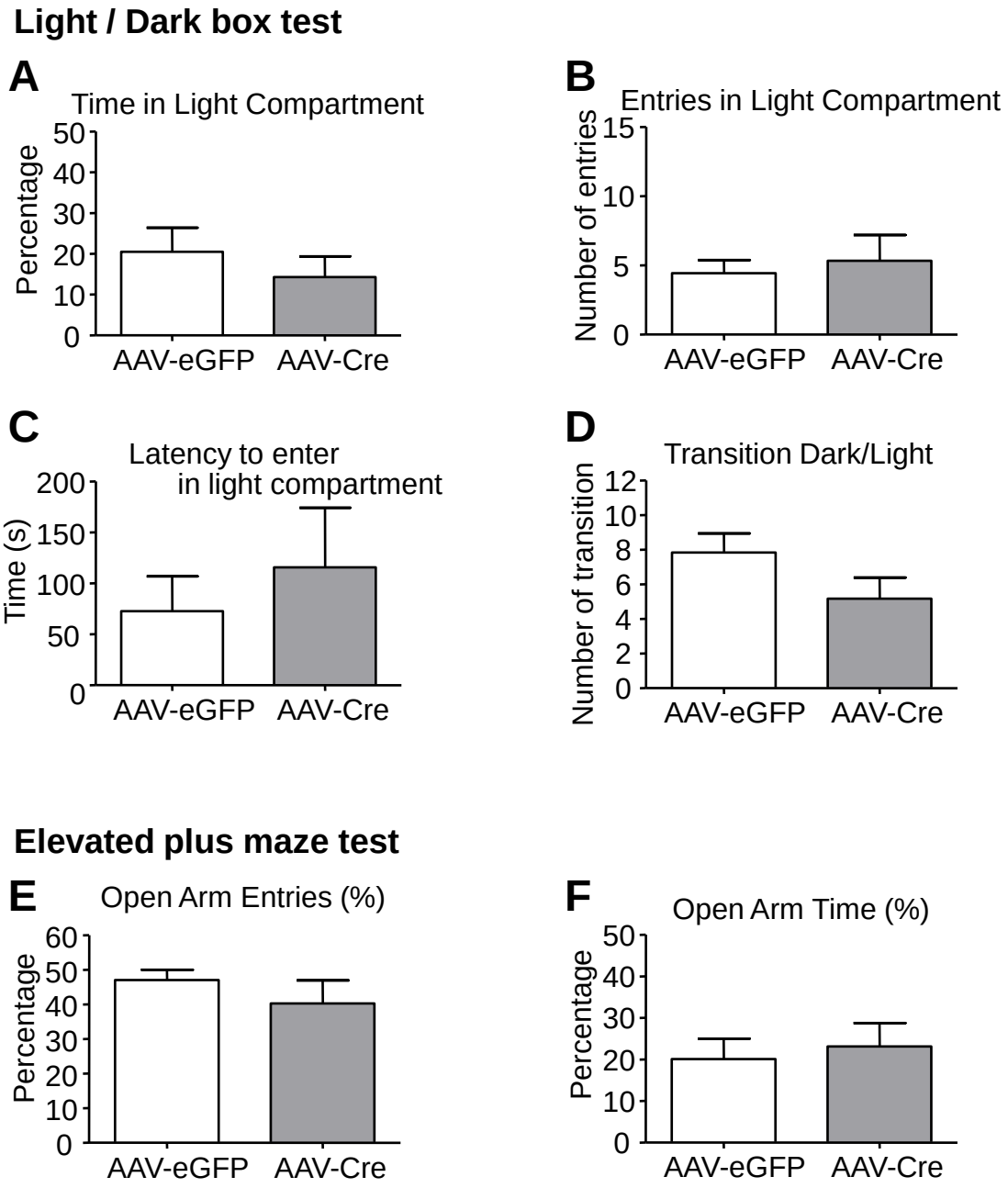



Fig. 3.3: **Anxiety-related behaviors.** Light-dark box and elevated plus maze were used to explore emotional responses in BLA-injected AAV-Cre animals. **(A-D)** Light-dark box. **(A)** Time spent and **(B)** entries in lit compartment did not differ between AAV-Cre and AAV-eGFP injected mice. **(C)** Latency to enter in lit compartment as well as **(D)** dark/light transitions were also comparable. (n= 6-7 per conditions). **(E-G)** Elevated plus-maze. No differences across groups for **(E)** the entries and **(F)** time spent in open arms. General activity was similar (total visits) between the two groups (data not shown), reflecting no change in spontaneous locomotor activity. n= 6-7 per condition.

Results. For experiments with two groups, a Student t test was used. The level of statistical significance was set at $p < 0.05$. For the statistical analysis along the sessions divided in bin-periods, the analysis of variance repeated measures was used.

## Results

### DOR knockdown in the BLA does not affect anxiety-related and despair-like behaviors

Oprd$^{fl/fl}$ mice were treated with AAV-Cre or AAV-eGFP bilaterally, and submitted to a battery of tests namely the light-dark box, elevated plus maze and tail suspension tests in that order. Behavioral data analysis shown in Figure 3.3 and 3.4 were performed after histological analysis.

We first investigated the effect of BLA-DOR knockdown in the light-dark box test. AAV-Cre injected mice showed comparable anxiety levels in comparison with control AAV-eGFP injected mice. The time spent in the lit compartment was comparable between the two groups ($p>0.05$, Student *t*-test, Figure 3.3A). Both group displayed an equivalent number of entries in the light side ($p>0.05$, Student *t*-test, Figure 3.3B) as well as a comparable latency for first entry ($p>0.05$, Student *t*-test, Figure 3.3C). Altogether, AAV-Cre mice tended to explore less, as reflected by the number of dark/light transitions, but this effect was not statistically significant ($p>0.05$, Student *t*-test, Figure 3.3D).

Secondly, we addressed the effect of viral knockdown in the elevated plus maze test. AAV-Cre mice displayed similar anxiety-related behaviors in comparison with their control mice, as shown by comparable number of entries ($p>0.05$, Student *t*-test, Figure 3.3E) and time spent in the open arms ($p>0.05$, Student *t*-test, Figure 3.3F).

Both groups were then submitted to the tail suspension test in order to assess despair-like behaviors. AAV-Cre and AAV-eGFP mice showed comparable despair-like behavior levels, as reflected by the time of immobility ($p>0.05$, one-way ANOVA Figure 3.4A and Student *t*-test, Figures 3.4B). In addition, the latency before the first immobilization remained similar in both groups ($p>0.05$, Student *t*-test, Figure 3.4C).

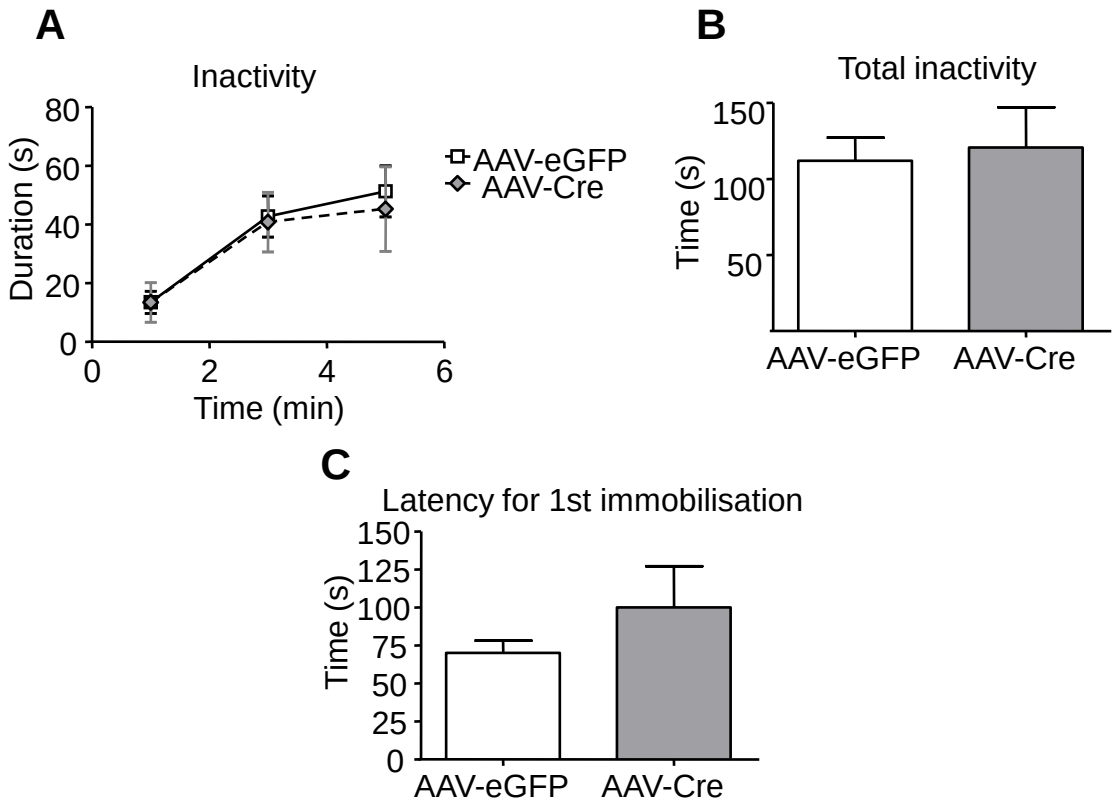



Fig. 3.4: **Despair-like behavior.** Tail suspension test was used to explore emotional responses in BLA-injected AAV-Cre animals. **(A-C)** Tail suspension. No significant differences was found across groups for **(A)** immobility over time. Comparable levels were also observed on **(B)** the total immobility and **(C)** latency to for the first immobilization episode. n= 6-7 per condition.

Our results suggest that DOR expressed at the level of BLA are not required for the phenotype previously observed in the DOR KO mice. $AAV_2$ vectors are described with a neuronal tropism with no evidence for retrograde transport (Burger, Gorbatyuk et al. 2004). Our viral treatment, therefore likely inactivated the few locally synthesized receptors, but does not modify receptors located on presynaptic terminals, which are likely responsible for the anxiogenic effect of DOR antagonists injected locally in the BLA. To further strengthen the histological analysis performed to verify the accurate injection sites, the precise quantification of virally-mediated DOR knock-down in the BLA is currently underway and preliminary data are consistent with less than 10% decrease.

**BLA receive massive projections from the insular cortex**

We examined whether DOR-eGFP proteins detected in the BLA may be synthesized in the cell bodies of BLA-afferent projecting neurons. For this purpose, we started a retrograde tracing experiment in the BLA of DOR-eGFP knockin mice using unilateral injection of Cholera Toxin subunit B. Retrogradely labeled neurons were observed in the olfactory bulb, prelimbic cortex, cingulate cortex, paraventricular nucleus of the thalamus, dorsal endopiriform cortex, piriform cotex, insular cortex (see Figure 3.5). In the contralateral injected hemisphere only a few retrogradely labeled neurons were observed in the piriform cortex, insular cortex and BLA (see figure 3.5). We then obtained high-resolution images by confocal microscopy, and detected DOR-eGFP positive cells that were also retrogradely labeled with CTB (see Figure 3.6). We semi-quantified DOR-eGFP and CTB positive cells in potential BLA-afferent regions and data are summarized in Table 1.

In our experiment, strongest CTB labeling was in the insular cortex, suggesting that BLA receives massive projection from this area of the cortex. Moreover, the insular cortex showed highest number of colocalized cells. Full quantification in the insular cortex (Figure 3.8) indicated that 260.4 ± 20.8 cells per $mm^2$ were retrogradely labeled, 84.43 ± 8.27 cells per $mm^2$ expressed DOR-eGFP and 17.36 ± 3.16 cells per $mm^2$ showed colocalization of CTB and DOR-eGFP signals under our experimental conditions. Neurons with colocalization, therefore, may represent a main DOR-regulated insular cortex-BLA pathway.

| Structures | DOR-eGFP positive neurons | CTB positive neurons | Colocalization | |
|---|---|---|---|---|
| | | | % neurons DOR-eGFP retrogradely labeled | % neurons CTB positive expressing DOR-eGFP |
| Olfactory bulb | +++ | ++ | 0% | 0% |
| Prelimbic cortex | + | + | 0% | 0% |
| Cingulate cortex | + | + | 0% | 0% |
| Paraventricular nucleus of the thalamus | 0 | + | 0% | 0% |
| Insular cortex | ++++ | +++++ | > 25% | > 25% |
| Dorsal endopiriform cortex | ++ | ++ | > 25% (to be confirmed) | > 25% (to be confirmed) |
| Piriform cortex | + | ++ | > 25% (to be confirmed) | > 25% (to be confirmed) |
| Controlateral BLA | +++ | ++ | 0% | 0% |

| ***Estimated cell bodies*** | |
|---|---|
| +++++ | : ~ 200 à 400 neurons / mm² |
| ++++ | : ~ 100 à 200 neurons / mm² |
| +++ | : ~ 50 à 100 neurons / mm² |
| ++ | : ~ 20 à 50 neurons / mm² |
| + | : ~ 1 à 20 neurons / mm² |
| 0 | : 0 neuron / mm² |
| ***Colocalization*** | |
| 100% | : total colocalization |
| > 75% | |
| 25-75% | |
| < 25% | |
| 0% | : no colocalisation detected |

Table 1: **Table of semi-quantitative values in DOR-eGFP knockin mice injected unilaterally with CTB retrograde tracer.** DOR-eGFP knockin mice were pretreated with SNC80 (10mg/kg, subcutaneously) to facilitate the visualization of positive cell bodies. DOR-eGFP positive neurons estimation is consistent with data previously obtrained in the lab (Massotte D., data not shown). Insular cortex showed the highest number of CTB positive cells. Endopiriform and piriform cortex areas remain to be quantified but observations suggest a colocalization in lower extend than in insular cortex. (n = 4 animals / 5-20 sections per animals / 1 count per section)

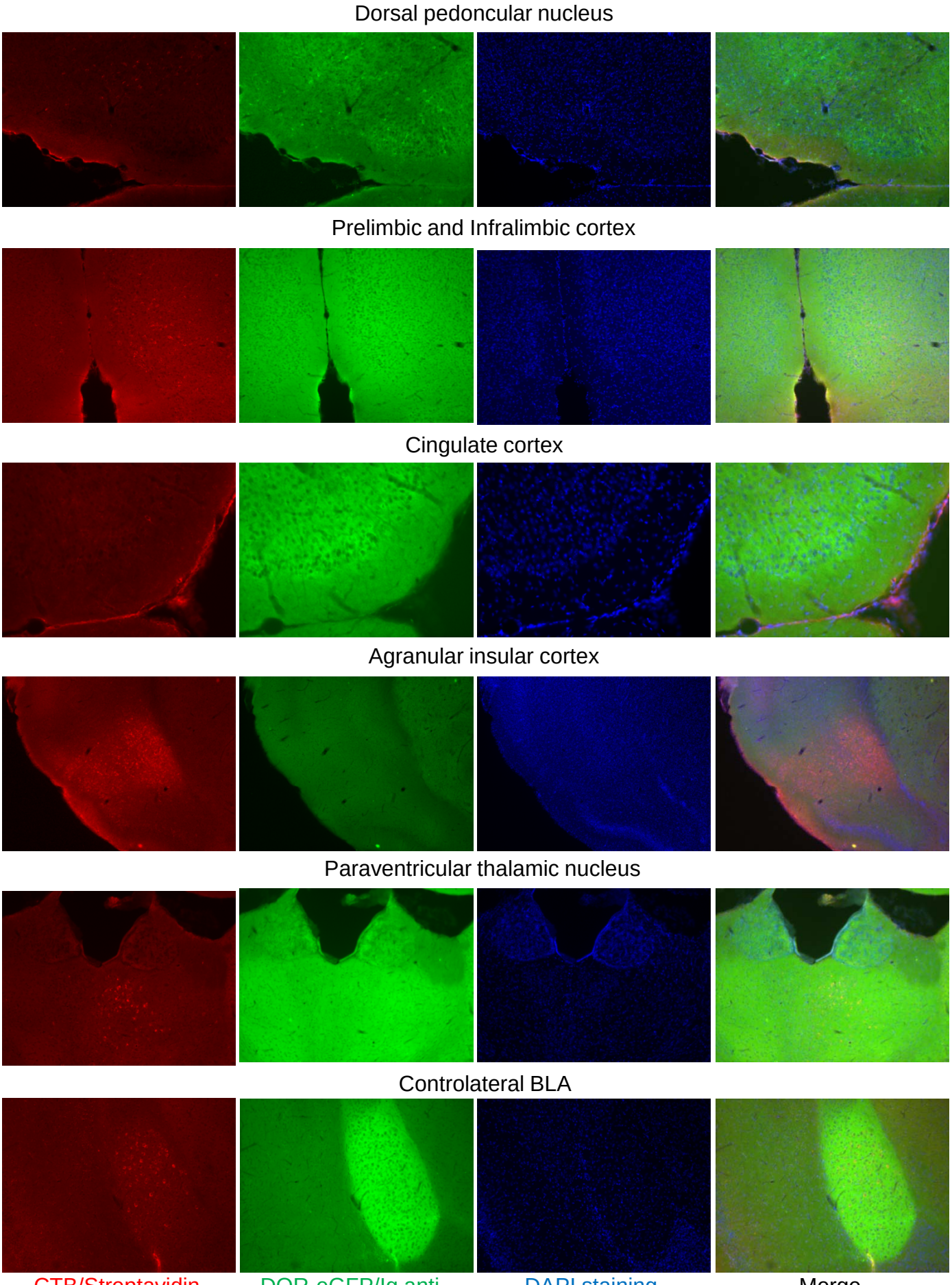



Fig. 3.5: DOR-eGFP unilaterally in the BLA with the retrograde tracer CTB and analyzed by epifluorescence microscopy X days later. Representative images of both DOR-eGFP and CTB signals are shown.

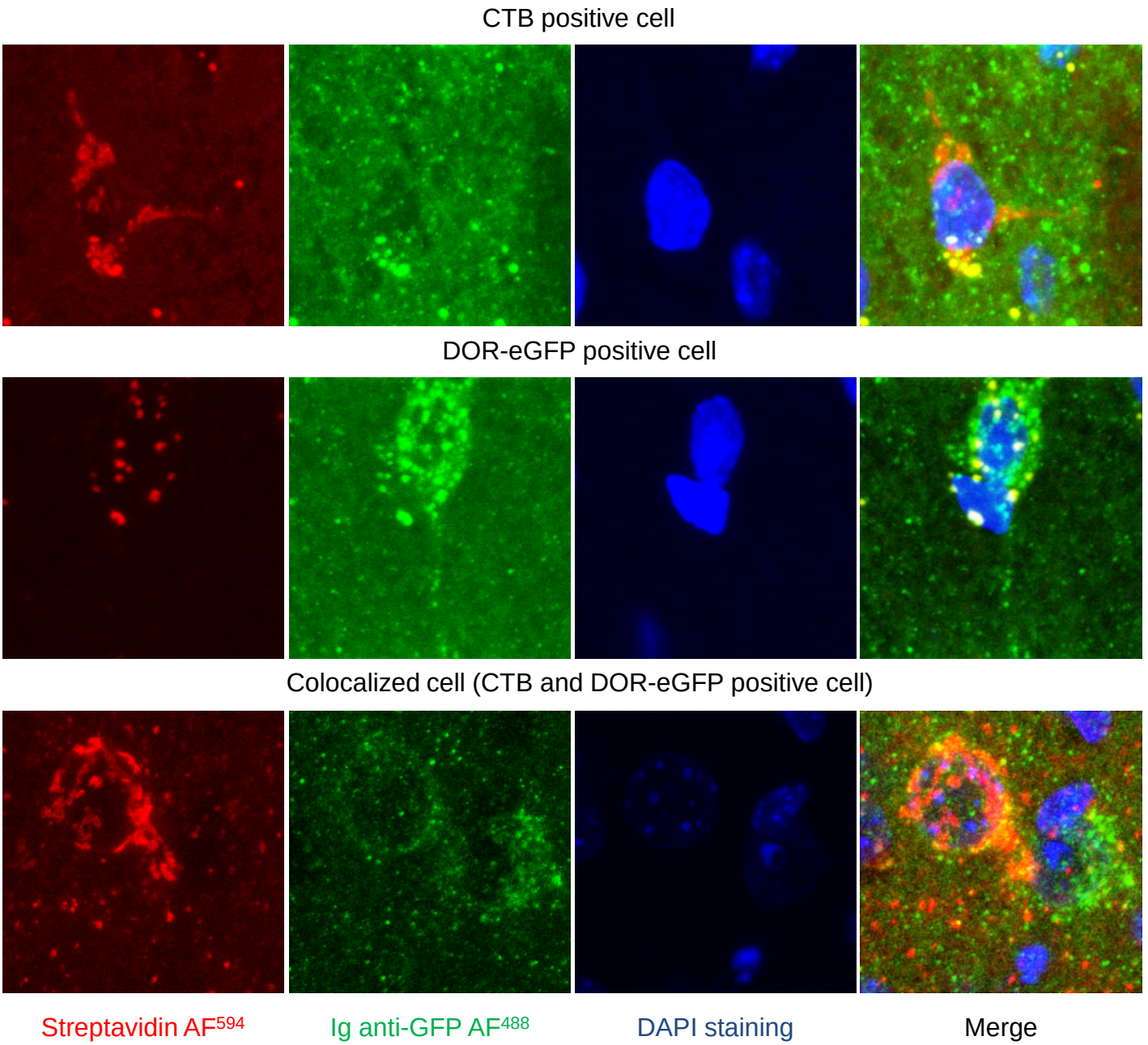



Fig. 3.6: **High-resolution images acquired on confocal microscope in the insular cortex**. Top panel correspond to a cell retrogradly labeled which does not express DOR-eGFP. Middle panel correspond to DOR-eGFP positive cell that do not project to the BLA (CTB negative). Bottom panel shows colocalized cell retrogradely labeled and expressing DOR-eGFP.

## Discussion

In this study, local knockdown of DOR in the BLA did not alter anxiety-related and despair-like behaviors. Although negative results should be taken with caution, the lack of behavioral effects of Cre-mediated DOR knockdown in the BLA, combined with the observation of very few DOR-eGFP cells bodies in DOR-eGFP mice, supports the notion that the strong DOR expression in BLA is mainly presynaptic. A previous study showed that local infusion of the DOR antagonist naltrindole produces an increase of anxiety-related behaviors, reflecting a tonic anxiolytic role of DOR in the BLA (Narita, Kaneko et al. 2006). Altogether therefore, data suggest that the anxiolytic DOR activity, which operates in BLA, arises from DOR-mediated modulation of presynaptic terminals activity. In the future, it will be interesting to use a Cre-expressing virus with retrograde potential in order to target these presynaptic DORs. Recently, recombinant Pseudorabies virus (PRV) expressing Cre recombinase showed a retrograde infection potential, and were used for the characterization of neuroanatomical pathways (Card, Kobiler et al. 2011; Koyuncu, Perlman et al. 2013). This technology offers the possibility to delete DORs in all neurons projecting to the BLA through stereotaxic injections into the BLA of $Oprd^{fl/fl}$ mice.

Using retrograde tracing, we were able to detect retrogradely labeled neurons in most regions described to project into the BLA (Knapska, Radwanska et al. 2007). Our preliminary results suggest that a large part of BLA DORs may be synthesized at the level of insular cortex, as this cortical area presents the highest number of BLA afferent neurons and a significant number of DOR-eGFP positive cells projecting to the BLA. We are currently performing precise counting in other regions especially the endopiriform cortex.

The insular cortex is one of the high DOR expression sites in the brain, as shown by radiolabelled agonist binding (see Manuscript 1, Table 1). Interestingly, evidence indicates that insular cortex may play an important role in the regulation of emotions (Paulus and Stein 2006; Stein, Simmons et al. 2007; Lamm and Singer 2010). In addition, evidence emphasizes the contribution of insular cortex in

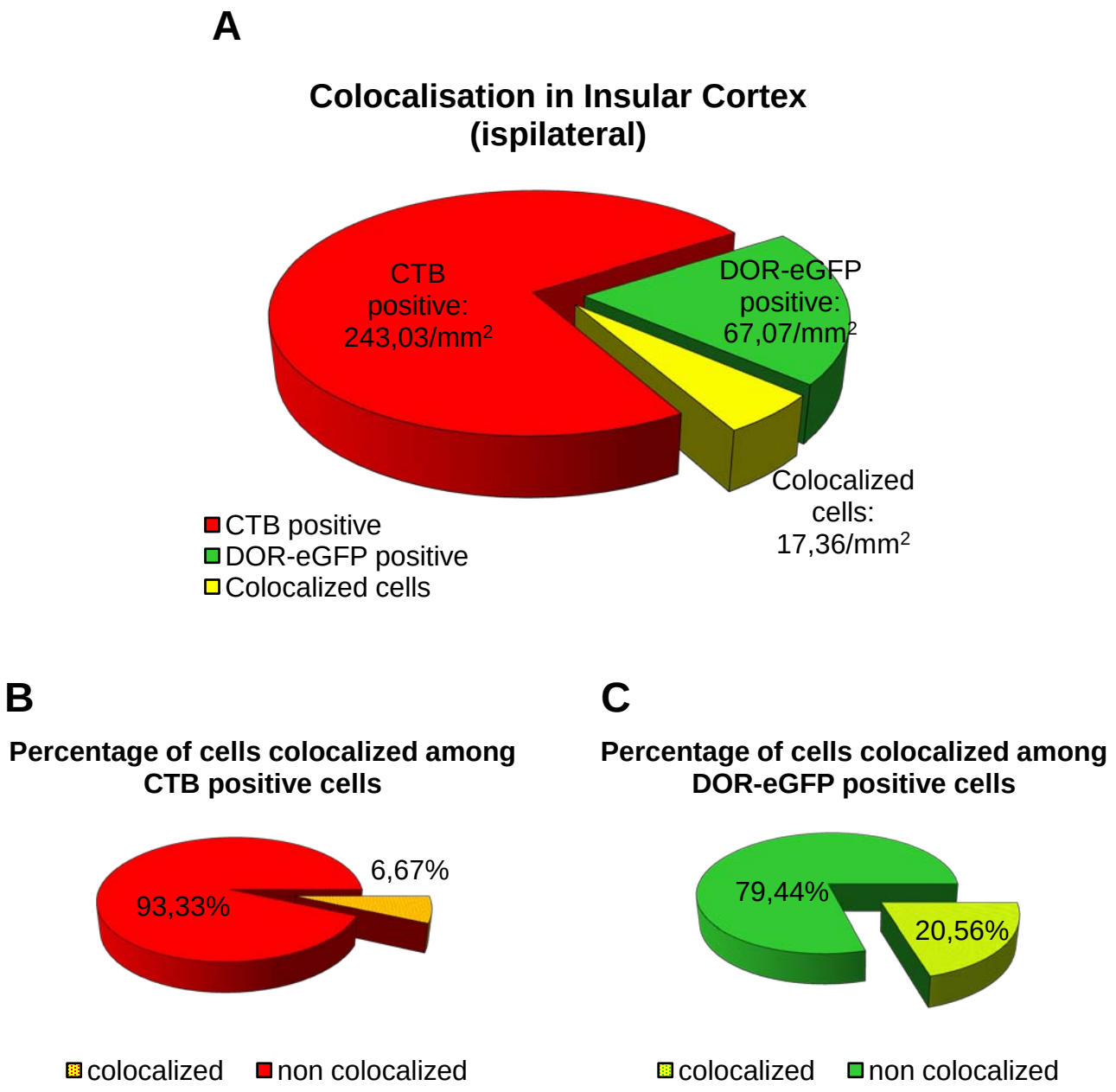



Fig. 3.8: **Quantification values in insular cortex. (A)** Number of cells per $mm^2$ expressing each marker. In the insular cortex , 243.03 neurons/ $mm^2$ positive for CTB tracer alone, 67.07 neurons/ $mm^2$ positive for DOR-eGFP expression alone and 17.36 neurons/ $mm^2$ colocalized for both markers. **(B)** Percentage of CTB positive cells colocalized with DOR-eGFP expression. About 6.7% of CTB positive neurons are expressing DOR-eGFP. **(C)** Percentage of DOR-eGFP positive cells colocalized with retrograde tracer CTB. About 20.5% of cells expressing DOR-eGFP are retrogradely labeled. (n = 4 animals / 5-20 sections per animals / 1 count per section)

substance use disorder (Naqvi and Bechara 2009). The ability to quit smoking has been reported to be easier for smokers with a damage of insular cortex (Naqvi, Rudrauf et al. 2007). A recent study showed that local inactivation of insular cortex by anisomycin injections disrupt amphetamine conditioned place preference in rats which indicate a contribution in context-drug associations, suggesting that the insular cortex might be a critical brain substrate in drug craving (Contreras, Ceric et al. 2007; Contreras, Billeke et al. 2012). The role of DOR expressed in the insular cortex has not been addressed, as yet. We have previously shown reduced morphine and lithium place conditioning in constitutive DOR knockout mice, suggesting that DOR activity normally facilitates drug-context associations independently from the affective value of the drug (Le Merrer, Faget et al. 2012). DORs expressed at the level of insular cortex and transported at presynaptic terminals in the BLA may well be critical for this activity and further experiments will be designed to test this hypothesis. The role of insular cortex is raising increasing interest in the context of substance use disorder, and DORs may be essential for regulation at this brain site.

In conclusion, our study indicates that DORs are expressed on neurons from insular cortex projecting to the BLA, thus demonstrating an expression at presynaptic terminals. The insular cortex-BLA pathway is an important circuit in emotional control and drug-context associations where DOR activity could play a substantial role. Further genetic approaches targeting this particular pathway will determine whether DORs represent an important molecular player regulating this neural microcircuitry.

Angelucci, A., F. Clasca, et al. (1996). "Anterograde axonal tracing with the subunit B of cholera toxin: a highly sensitive immunohistochemical protocol for revealing fine axonal morphology in adult and neonatal brains." J Neurosci Methods **65**(1): 101-112.

Balleine, B. W. and S. Killcross (2006). "Parallel incentive processing: an integrated view of amygdala function." Trends Neurosci **29**(5): 272-279.

Brown, C. E. and R. H. Dyck (2005). "Retrograde tracing of the subset of afferent connections in mouse barrel cortex provided by zincergic neurons." J Comp Neurol **486**(1): 48-60.

Burger, C., O. S. Gorbatyuk, et al. (2004). "Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system." Mol Ther **10**(2): 302-317.

Chu Sin Chung, P. and B. L. Kieffer (2013). "Delta opioid receptors in brain function and diseases." Pharmacol Ther.
Conte, W. L., H. Kamishina, et al. (2009). "The efficacy of the fluorescent conjugates of cholera toxin subunit B for multiple retrograde tract tracing in the central nervous system." Brain Struct Funct **213**(4-5): 367-373.
Del Boca, C., P. E. Lutz, et al. (2012). "Cholecystokinin knock-down in the basolateral amygdala has anxiolytic and antidepressant-like effects in mice." Neuroscience.
Ehrlich, I., Y. Humeau, et al. (2009). "Amygdala inhibitory circuits and the control of fear memory." Neuron **62**(6): 757-771.
Erbs, E., L. Faget, et al. (2012). "Distribution of delta opioid receptor-expressing neurons in the mouse hippocampus." Neuroscience **221**: 203-213.
Faget, L., E. Erbs, et al. (2012). "In vivo visualization of delta opioid receptors upon physiological activation uncovers a distinct internalization profile." J Neurosci **32**(21): 7301-7310.
Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.
Goncalves, M. A. (2005). "Adeno-associated virus: from defective virus to effective vector." Virol J **2**: 43.
Goody, R. J., S. M. Oakley, et al. (2002). "Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice." Brain Res **945**(1): 9-19.
Kaufling, J., P. Veinante, et al. (2009). "Afferents to the GABAergic tail of the ventral tegmental area in the rat." J Comp Neurol **513**(6): 597-621.
Killcross, S., T. W. Robbins, et al. (1997). "Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala." Nature **388**(6640): 377-380.
Knapska, E., K. Radwanska, et al. (2007). "Functional internal complexity of amygdala: focus on gene activity mapping after behavioral training and drugs of abuse." Physiol Rev **87**(4): 1113-1173.
Le Merrer, J., J. A. Becker, et al. (2009). "Reward processing by the opioid system in the brain." Physiol Rev **89**(4): 1379-1412.
Le Merrer, J., L. Faget, et al. (2012). "Cues predicting drug or food reward restore morphine-induced place conditioning in mice lacking delta opioid receptors." Psychopharmacology (Berl) **223**(1): 99-106.
LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci **23**: 155-184.
Lencer, W. I. and B. Tsai (2003). "The intracellular voyage of cholera toxin: going retro." Trends Biochem Sci **28**(12): 639-645.
Mamiya, N., H. Fukushima, et al. (2009). "Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory." J Neurosci **29**(2): 402-413.
Mansour, A., C. A. Fox, et al. (1995). "Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications." Trends in Neurosciences **18**: 22-29.
McDonald, A. J. (1982). "Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat." J Comp Neurol **212**(3): 293-312.
McDonald, A. J. (1998). "Cortical pathways to the mammalian amygdala." Prog Neurobiol **55**(3): 257-332.

McDonald, A. J. (2003). "Is there an amygdala and how far does it extend? An anatomical perspective." Ann N Y Acad Sci **985**: 1-21.
Narita, M., C. Kaneko, et al. (2006). "Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala." Neuropsychopharmacology **31**(4): 739-750.
Narita, M., N. Kuzumaki, et al. (2006). "Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction." J Neurochem **97**(5): 1369-1378.
Parkinson, J. A., T. W. Robbins, et al. (2000). "Dissociable roles of the central and basolateral amygdala in appetitive emotional learning." Eur J Neurosci **12**(1): 405-413.
Paterna, J. C., J. Feldon, et al. (2004). "Transduction profiles of recombinant adeno-associated virus vectors derived from serotypes 2 and 5 in the nigrostriatal system of rats." J Virol **78**(13): 6808-6817.
Pradhan, A. A., J. A. Becker, et al. (2009). "In vivo delta opioid receptor internalization controls behavioral effects of agonists." PLoS One **4**(5): e5425.
Randall-Thompson, J. F., K. A. Pescatore, et al. (2010). "A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors." Psychopharmacology (Berl) **212**(4): 585-595.
Rezai, X., L. Faget, et al. (2012). "Mouse delta opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells." Cell Mol Neurobiol **32**(4): 509-516.
Rogan, M. T. and J. E. LeDoux (1996). "Emotion: systems, cells, synaptic plasticity." Cell **85**(4): 469-475.
Scherrer, G., N. Imamachi, et al. (2009). "Dissociation of the opioid receptor mechanisms that control mechanical and heat pain." Cell **137**(6): 1148-1159.
Scherrer, G., P. Tryoen-Toth, et al. (2006). "Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo." Proc Natl Acad Sci U S A **103**(25): 9691-9696.
Solati, J., M. R. Zarrindast, et al. (2010). "Dorsal hippocampal opioidergic system modulates anxiety-like behaviors in adult male Wistar rats." Psychiatry Clin Neurosci **64**(6): 634-641.
Tenenbaum, L., A. Chtarto, et al. (2004). "Recombinant AAV-mediated gene delivery to the central nervous system." J Gene Med **6 Suppl 1**: S212-222.
Zhang, G., V. Gurtu, et al. (1996). "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells." Biochem Biophys Res Commun **227**(3): 707-711.

# Discussion Generale

The aim of my thesis was to investigate DOR roles and identify neuronal populations, brain areas or circuits supporting physiological functions of the receptor. For this purpose, we genetically targeted *Oprd1* gene in forebrain GABAergic neurons using a Dlx5/6-Cre driver and generated a conditional knockout mouse line. Analysis of DOR neuroanatomical distribution in this mutant mouse demonstrated a dramatic reduction of DOR expression in the olfactory bulb, caudate putamen and nucleus accumbens as well as a partial reduction in hippocampus.

Behavioural analysis of Dlx-DOR mice reveals an unexpected emotional phenotype. Our data suggest that DORs expressed in GABAergic neurons contribute to increase anxiety-related behavior and decrease risky behaviors, a role that potentially opposes the well-established anxiolytic function of DORs. In addition, we report that the same DOR population mediates locomotor stimulant effects of SNC80, and exert a tonic suppressive effect on D1 receptor-mediated locomotor stimulation. Further, we show the pro-seizure activity of high-internalizing, but not low-internalizing agonist via electroencephalogram recordings, and demonstrate that ablation of DOR in forebrain GABAergic neurons is sufficient to suppress these events. Our last part of the work, finally, highlights the insular cortex-basolateral amygdala pathway as a potential site for DOR-mediated mood control.

Behavioral deficits in Dlx-DR mice differ from those observed in total knockout mice (distinct or absent). Our set of experiments, therefore, also emphasizes the interest of targeting DOR in neuronal populations other than forebrain GABAergic neurons to elucidate further DOR functions. The basolateral amygdala is essentially composed of glutamatergic and GABAergic neurons (McDonald and Mascagni 2001; Sah and Lopez De Armentia 2003). Striatum contains mainly GABAergic medium spiny neurons as well as few cholinergic neurons (Matamales, Bertran-Gonzalez et al. 2009). The neuroanatomical characterization of conditional knockout mice revealed intact DOR expression in the BLA as well as few remaining receptors in the striatum. Altogether, this anatomical evidence and our finding that Dlx-DOR phenotypes do not exactly match constitutive phenotypes, suggest that DORs expressed in glutamatergic and cholinergic neurons may significantly contribute to DOR function, at least at the level of amygdala and striatum respectively.

In the striatum, DORs are expressed both in GABAergic medium spiny neurons and cholinergic neurons (Scherrer, Tryoen-Toth et al. 2006). In Dlx-DOR mice, the stimulating effect of D1/D3 dopamine receptor agonist is facilitated, suggesting that DORs expressed in D1-positive GABAergic medium spiny neurons normally inhibit the direct striatonigral output pathway. It is likely that a genetic deletion of DOR in striatal cholinergic neurons would have a very different effect on locomotor activity. A previous study proposed that activation of DOR on cholinergic interneurons suppresses Ach release and, in turn, decreases nAChR activity on DA terminals (Britt and McGehee 2008). Another study described enhanced electrical field stimulation-evoked ACh release after naltrindole infusion on striatal slices suggesting a tonic inhibition on cholinergic striatal neurons (Sandor, Lendvai et al. 1992). In addition, DOR agonists were described to inhibit glutamate-evoked Ach release (Arenas, Alberch et al. 1990). Altogether these studies highlight the important role of DORs in cholinergic neurons of the striatum. Comparing consequences of a conditional DOR deletion in GABAergic versus cholinergic neurons would definitely clarify DOR-mediated mechanisms regulating.

The likely modulatory role of DOR on striatal acetylcholine/dopamine (Ach/DA) balance may be relevant to the low fear/high risk-taking phenotype of Dlx-DOR mice. This phenotype could also be viewed as an altered avoidance-approach phenotype. Enhanced dopamine release has been related to approach behaviours mostly through D1 receptors (Durieux, Schiffmann et al. 2012). Further, acetylcholine release is hypothesized to counteract the excessive DA-mediated approach behavior especially in the context of drugs of abuse and therefore mediates state of anxiety that prevent over-responding (Hoebel, Avena et al. 2007).Therefore, tonic DOR activity may prevent the cholinergic release to counterbalance dopaminergic effects perhaps to maintain approach-avoidance behaviours appropriate regarding external stimulus. In potentially threatening environment for example, DOR-mediated inhibition of cholinergic neurons may favor escape behavior, whereas DOR-mediated inhibition ofD1 neurons would support freezing behavior. This hypothesis, in the future, may be strengthened using a larger set of conflict behavioral paradigms.

DORs are involved in several physiological processes that contribute to substance abuse (see review in(Chu Sin Chung and Kieffer 2013). Constitutive DOR knockout mice showed enhanced ethanol intake, interpreted as self-medication to alleviate anxiety (Roberts, Gold et al. 2001), suggesting that DOR anxiolytic effects may limit excessive alcohol intake. Further, recent data from our laboratory indicate that DOR knockout mice show enhanced emotional deficits upon protracted abstinence to heroin (Lutz et al, in preparation), demonstrating a protective role of DORs during the development of a negative affect associated to addiction. Also, reduced morphine conditioned place preference and lithium place aversion in DOR knockout mice suggest that DORs facilitate drug-context associations (Le Merrer, Plaza-Zabala et al. 2011). Finally, DOR knockout mice showed increased motor impulsivity, indicating that DORs enhance self-control (Olmstead 2009). Altogether, knockout studies performed in the past decade indicate that DOR activity influences emotional states, context learning and inhibitory controls, all having strong relevance to substance abuse. The notion that DOR activity may promote risk-taking behaviors (present study) also has important implications for drug abuse (Schultz 2011) and adds to the many facets of DOR functions that may impact addictive behaviors.

# Bibliography


Abrahamsen, B., J. Zhao, et al. (2008). "The cell and molecular basis of mechanical, cold, and inflammatory pain." Science **321**(5889): 702-705.

Akil, H., C. Owens, et al. (1998). "Endogenous opioids: overview and current issues." Drug Alcohol Depend **51**(1-2): 127-140.

Ambroggi, F., A. Ishikawa, et al. (2008). "Basolateral amygdala neurons facilitate reward-seeking behavior by exciting nucleus accumbens neurons." Neuron **59**(4): 648-661.

Angelucci, A., F. Clasca, et al. (1996). "Anterograde axonal tracing with the subunit B of cholera toxin: a highly sensitive immunohistochemical protocol for revealing fine axonal morphology in adult and neonatal brains." J Neurosci Methods **65**(1): 101-112.

Arenas, E., J. Alberch, et al. (1990). "Effect of opioids on acetylcholine release evoked by K+ or glutamic acid from rat neostriatal slices." Brain Res **523**(1): 51-56.

Asok, A., L. W. Ayers, et al. (2013). "Immediate early gene and neuropeptide expression following exposure to the predator odor 2,5-dihydro-2,4,5-trimethylthiazoline (TMT)." Behav Brain Res **248**: 85-93.

Atici, S., I. Cinel, et al. (2005). "Liver and kidney toxicity in chronic use of opioids: an experimental long term treatment model." J Biosci **30**(2): 245-252.

Audet, N., I. Charfi, et al. (2012). "Differential association of receptor-Gbetagamma complexes with beta-arrestin2 determines recycling bias and potential for tolerance of delta opioid receptor agonists." J Neurosci **32**(14): 4827-4840.

Balleine, B. W. and S. Killcross (2006). "Parallel incentive processing: an integrated view of amygdala function." Trends Neurosci **29**(5): 272-279.

Baxter, M. G. and E. A. Murray (2002). "The amygdala and reward." Nat Rev Neurosci **3**(7): 563-573.

Bechara, A., H. Damasio, et al. (2000). "Emotion, decision making and the orbitofrontal cortex." Cereb Cortex **10**(3): 295-307.

Befort, K., D. Filliol, et al. (2008). "Mu-opioid receptor activation induces transcriptional plasticity in the central extended amygdala." Eur J Neurosci **27**(11): 2973-2984.

Befort, K., M. K. Mahoney, et al. (2011). "Effects of delta opioid receptors activation on a response inhibition task in rats." Psychopharmacology (Berl) **214**(4): 967-976.

Befort, K., M. G. Mattei, et al. (1994). "Chromosomal localization of the delta opioid receptor gene to human 1p34.3-p36.1 and mouse 4D bands by in situ hybridization." Genomics **20**(1): 143-145.

Befort, K., L. Tabbara, et al. (1996). "The conserved aspartate residue in the third putative transmembrane domain of the delta-opioid receptor is not the anionic counterpart for cationic opiate binding but is a constituent of the receptor binding site." Mol Pharmacol **49**(2): 216-223.

Befort, K., C. Zilliox, et al. (1999). "Constitutive activation of the δ opioid receptor by mutations in transmembrane domains III and VII." Journal of Biological Chemistry **274**(26): 18574-18581.

Benbouzid, M., C. Gaveriaux-Ruff, et al. (2008). "Delta-opioid receptors are critical for tricyclic antidepressant treatment of neuropathic allodynia." Biol Psychiatry **63**(6): 633-636.
Bilecki, W., A. Wawrzczak-Bargiela, et al. (2004). "Activation of AP-1 and CRE-dependent gene expression via mu-opioid receptor." J Neurochem **90**(4): 874-882.
Bilsky, E. J., S. N. Calderon, et al. (1995). "SNC 80, a selective, nonpeptidic and systemically active opioid delta agonist." J Pharmacol Exp Ther **273**(1): 359-366.
Bodnar, R. J. (2004). "Endogenous opioids and feeding behavior: a 30-year historical perspective." Peptides **25**(4): 697-725.
Bohn, L. M., R. R. Gainetdinov, et al. (2004). "G protein-coupled receptor kinase/beta-arrestin systems and drugs of abuse: psychostimulant and opiate studies in knockout mice." Neuromolecular Med **5**(1): 41-50.
Bohn, L. M., R. R. Gainetdinov, et al. (2000). "Mu-opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence." Nature **408**(6813): 720-723.
Bonica, J. J. (1979). "The need of a taxonomy." Pain **6**(3): 247-248.
Bourin, M. and M. Hascoet (2003). "The mouse light/dark box test." Eur J Pharmacol **463**(1-3): 55-65.
Britt, J. P. and D. S. McGehee (2008). "Presynaptic opioid and nicotinic receptor modulation of dopamine overflow in the nucleus accumbens." J Neurosci **28**(7): 1672-1681.
Broom, D. C., J. F. Nitsche, et al. (2002). "Comparison of receptor mechanisms and efficacy requirements for delta-agonist-induced convulsive activity and antinociception in mice." J Pharmacol Exp Ther **303**(2): 723-729.
Brown, C. E. and R. H. Dyck (2005). "Retrograde tracing of the subset of afferent connections in mouse barrel cortex provided by zincergic neurons." J Comp Neurol **486**(1): 48-60.
Bruchas, M. R., B. B. Land, et al. (2009). "CRF1-R activation of the dynorphin/kappa opioid system in the mouse basolateral amygdala mediates anxiety-like behavior." PLoS One **4**(12): e8528.
Bryant, S. D., S. Salvadori, et al. (1998). "New delta-opioid antagonists as pharmacological probes." Trends Pharmacol Sci **19**(2): 42-46.
Bueno, L. and J. Fioramonti (1988). "Action of opiates on gastrointestinal function." Baillieres Clin Gastroenterol **2**(1): 123-139.
Bunzow, J. R., C. Saez, et al. (1994). "Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type." FEBS Lett **347**(2-3): 284-288.
Buot, A. and J. Yelnik (2012). "Functional anatomy of the basal ganglia: limbic aspects." Rev Neurol (Paris) **168**(8-9): 569-575.
Burger, C., O. S. Gorbatyuk, et al. (2004). "Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system." Mol Ther **10**(2): 302-317.
Burns, L. H., B. J. Everitt, et al. (1999). "Effects of excitotoxic lesions of the basolateral amygdala on conditional discrimination learning with primary and conditioned reinforcement." Behav Brain Res **100**(1-2): 123-133.

Callaway, C. W., R. L. Hakan, et al. (1991). "Distribution of amygdala input to the nucleus accumbens septi: an electrophysiological investigation." J Neural Transm Gen Sect **83**(3): 215-225.
Cardinal, R. N., J. A. Parkinson, et al. (2002). "Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex." Neurosci Biobehav Rev **26**(3): 321-352.
Carey, A. N., A. M. Lyons, et al. (2009). "Endogenous kappa opioid activation mediates stress-induced deficits in learning and memory." The Journal of neuroscience : the official journal of the Society for Neuroscience **29**(13): 4293-4300.
Chang, K. J., G. C. Rigdon, et al. (1993). "A novel, potent and selective nonpeptidic delta opioid receptor agonist BW373U86." J Pharmacol Exp Ther **267**(2): 852-857.
Chang, S. D., D. Y. Chen, et al. (2008). "Infusion of lidocaine into the dorsal hippocampus before or after the shock training phase impaired conditioned freezing in a two-phase training task of contextual fear conditioning." Neurobiol Learn Mem **89**(2): 95-105.
Chavkin, C. (2011). "The therapeutic potential of kappa-opioids for treatment of pain and addiction." Neuropsychopharmacology **36**(1): 369-370.
Chieng, B. and J. T. Williams (1998). "Increased opioid inhibition of GABA release in nucleus accumbens during morphine withdrawal." J Neurosci **18**(17): 7033-7039.
Christie, M. J., R. J. Summers, et al. (1987). "Excitatory amino acid projections to the nucleus accumbens septi in the rat: a retrograde transport study utilizing D[3H]aspartate and [3H]GABA." Neuroscience **22**(2): 425-439.
Chu Sin Chung, P. and B. L. Kieffer (2013). "Delta opioid receptors in brain function and diseases." Pharmacol Ther.
Chu Sin Chung, P. and B. L. Kieffer (2013). "Delta opioid receptors in brain function and diseases." Pharmacol Ther **140**(1): 112-120.
Coco, M. L., C. M. Kuhn, et al. (1992). "Selective activation of mesoamygdaloid dopamine neurons by conditioned stress: attenuation by diazepam." Brain Res **590**(1-2): 39-47.
Comb, M., P. H. Seeburg, et al. (1982). "Primary structure of the human Met- and Leu-enkephalin precursor and its mRNA." Nature **295**(5851): 663-666.
Comer, S. D., E. M. Hoenicke, et al. (1993). "Convulsive effects of systemic administration of the delta opioid agonist BW373U86 in mice." J Pharmacol Exp Ther **267**(2): 888-895.
Conte, W. L., H. Kamishina, et al. (2009). "The efficacy of the fluorescent conjugates of cholera toxin subunit B for multiple retrograde tract tracing in the central nervous system." Brain Struct Funct **213**(4-5): 367-373.
Cryan, J. F. and F. F. Sweeney (2011). "The age of anxiety: role of animal models of anxiolytic action in drug discovery." Br J Pharmacol **164**(4): 1129-1161.
Danielsson, I., M. Gasior, et al. (2006). "Electroencephalographic and convulsant effects of the delta opioid agonist SNC80 in rhesus monkeys." Pharmacol Biochem Behav **85**(2): 428-434.
Davidson, R. J. (2002). "Anxiety and affective style: role of prefrontal cortex and amygdala." Biol Psychiatry **51**(1): 68-80.
Davis, M. (2006). "Neural systems involved in fear and anxiety measured with fear-potentiated startle." Am Psychol **61**(8): 741-756.

Day, R., K. A. trujillo, et al. (1993). Prodynorphin biosynthesis and posttranslational processing. Opioids I. H. A. Berlin heidelberg New York, Springer Verlag. **104/I:** 449-470.
Deacon, R. M., D. M. Bannerman, et al. (2002). "Anxiolytic effects of cytotoxic hippocampal lesions in rats." Behav Neurosci **116**(3): 494-497.
Decaillot, F. M., K. Befort, et al. (2003). "Opioid receptor random mutagenesis reveals a mechanism for G protein-coupled receptor activation." Nat Struct Biol **10**(8): 629-636.
Del Boca, C., P. E. Lutz, et al. (2012). "Cholecystokinin knock-down in the basolateral amygdala has anxiolytic and antidepressant-like effects in mice." Neuroscience.
Del Rio, J. A., E. Soriano, et al. (1992). "Development of GABA-immunoreactivity in the neocortex of the mouse." J Comp Neurol **326**(4): 501-526.
Dragunow, M. and R. Faull (1989). "The use of c-fos as a metabolic marker in neuronal pathway tracing." J Neurosci Methods **29**(3): 261-265.
Duncan, G. E., D. J. Knapp, et al. (1996). "Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety." Brain Res **713**(1-2): 79-91.
Durieux, P. F., S. N. Schiffmann, et al. (2012). "Differential regulation of motor control and response to dopaminergic drugs by D1R and D2R neurons in distinct dorsal striatum subregions." EMBO J **31**(3): 640-653.
Ehrlich, I., Y. Humeau, et al. (2009). "Amygdala inhibitory circuits and the control of fear memory." Neuron **62**(6): 757-771.
Endler, N. S. and N. L. Kocovski (2001). "State and trait anxiety revisited." J Anxiety Disord **15**(3): 231-245.
Erbs, E., L. Faget, et al. (2012). "Distribution of delta opioid receptor-expressing neurons in the mouse hippocampus." Neuroscience **221**: 203-213.
Evans, C. J., D. E. Keith, Jr., et al. (1992). "Cloning of a delta opioid receptor by functional expression." Science **258**(5090): 1952-1955.
Everitt, B. J., R. N. Cardinal, et al. (2003). "Appetitive behavior: impact of amygdala-dependent mechanisms of emotional learning." Ann N Y Acad Sci **985**: 233-250.
Faget, L., E. Erbs, et al. (2012). "In vivo visualization of delta opioid receptors upon physiological activation uncovers a distinct internalization profile." J Neurosci **32**(21): 7301-7310.
Feil, R., J. Brocard, et al. (1996). "Ligand-activated site-specific recombination in mice." Proc Natl Acad Sci U S A **93**(20): 10887-10890.
File, S. E., A. S. Lippa, et al. (2004). "Animal tests of anxiety." Curr Protoc Neurosci **Chapter 8**: Unit 8 3.
File, S. E. and P. Seth (2003). "A review of 25 years of the social interaction test." Eur J Pharmacol **463**(1-3): 35-53.
Filliol, D., S. Ghozland, et al. (2000). "Mice deficient for delta- and mu-opioid receptors exhibit opposing alterations of emotional responses." Nat Genet **25**(2): 195-200.
Fournier, N. M. and R. S. Duman (2013). "Illuminating hippocampal control of fear memory and anxiety." Neuron **77**(5): 803-806.
Franklin, K. B. J. and G. Paxinos (1997). The Mouse Brain in Stereotaxic Coordinated. San Diego, CA, Academic Press.
Fredriksson, R., M. C. Lagerström, et al. (2003). "The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints." Molecular Pharmacology **63**(6): 1256-1272.

Fricker, L. D. and L. Devi (1993). "Posttranslational processing of carboxypeptidase E, a neuropeptide-processing enzyme, in AtT-20 cells and bovine pituitary secretory granules." J Neurochem **61**(4): 1404-1415.
Gao, C. J., L. Niu, et al. (2012). "Hypoxic preconditioning attenuates global cerebral ischemic injury following asphyxial cardiac arrest through regulation of delta opioid receptor system." Neuroscience **202**: 352-362.
Gaveriaux-Ruff, C. (2013). "Opiate-Induced Analgesia: Contributions from Mu, Delta and Kappa Opioid Receptors Mouse Mutants." Curr Pharm Des.
Gaveriaux-Ruff, C., L. A. Karchewski, et al. (2008). "Inflammatory pain is enhanced in delta opioid receptor-knockout mice." Eur J Neurosci **27**(10): 2558-2567.
Gaveriaux-Ruff, C. and B. L. Kieffer (2007). "Conditional gene targeting in the mouse nervous system: Insights into brain function and diseases." Pharmacol Ther **113**(3): 619-634.
Gaveriaux-Ruff, C. and B. L. Kieffer (2011). "Delta opioid receptor analgesia: recent contributions from pharmacology and molecular approaches." Behav Pharmacol **22**(5-6): 405-414.
Gaveriaux-Ruff, C., C. Nozaki, et al. (2011). "Genetic ablation of delta opioid receptors in nociceptive sensory neurons increases chronic pain and abolishes opioid analgesia." Pain **152**(6): 1238-1248.
Gaveriaux, C., J. Peluso, et al. (1995). "Identification of kappa- and delta-opioid receptor transcripts in immune cells." FEBS Lett **369**(2-3): 272-276.
Gether, U. (2000). "Uncovering molecular mechanisms involved in activation of G protein-coupled reeceptors." Endocrine Reviews **21**(1): 90-113.
Ghozland, S., H. W. Matthes, et al. (2002). "Motivational effects of cannabinoids are mediated by mu-opioid and kappa-opioid receptors." J Neurosci **22**(3): 1146-1154.
Gillett, K., E. Harshberger, et al. (2013). "Protracted withdrawal from ethanol and enhanced responsiveness stress: Regulation via the dynorphin/kappa opioid receptor system." Alcohol **47**(5): 359-365.
Goeldner, C., P. E. Lutz, et al. "Impaired emotional-like behavior and serotonergic function during protracted abstinence from chronic morphine." Biol Psychiatry **69**(3): 236-244.
Goeldner, C., D. Reiss, et al. (2009). "Activation of nociceptin opioid peptide (NOP) receptor impairs contextual fear learning in mice through glutamatergic mechanisms." Neurobiol Learn Mem **91**(4): 393-401.
Goldstein, A., S. Tachibana, et al. (1979). "Dynorphin (1-13), an extraordinarily potent opioid peptide." Proceedings of the National Academy of Science USA **76**: 6666-6670.
Goncalves, M. A. (2005). "Adeno-associated virus: from defective virus to effective vector." Virol J **2**: 43.
Goody, R. J., S. M. Oakley, et al. (2002). "Quantitative autoradiographic mapping of opioid receptors in the brain of delta-opioid receptor gene knockout mice." Brain Res **945**(1): 9-19.
Granier, S., A. Manglik, et al. (2012). "Structure of the delta-opioid receptor bound to naltrindole." Nature **485**(7398): 400-404.
Grobe, N., M. Lamshoft, et al. (2010). "Urinary excretion of morphine and biosynthetic precursors in mice." Proc Natl Acad Sci U S A **107**(18): 8147-8152.
Gross, C. and R. Hen (2004). "The developmental origins of anxiety." Nat Rev Neurosci **5**(7): 545-552.

Guo, J., Y. Wu, et al. (2000). "Identification of G protein-coupled receptor kinase 2 phosphorylation sites responsible for agonist-stimulated delta-opioid receptor phosphorylation." Mol Pharmacol **58**(5): 1050-1056.
Hacquemand, R., N. Choffat, et al. (2013). "Comparison between low doses of TMT and cat odor exposure in anxiety- and fear-related behaviors in mice." Behav Brain Res **238**: 227-231.
Haller, J. and M. Alicki (2012). "Current animal models of anxiety, anxiety disorders, and anxiolytic drugs." Curr Opin Psychiatry **25**(1): 59-64.
Handa, B. K., A. C. Land, et al. (1981). "Analogues of beta-LPH61-64 possessing selective agonist activity at mu-opiate receptors." Eur J Pharmacol **70**(4): 531-540.
He, X., H. K. Sandhu, et al. (2013). "Neuroprotection against hypoxia/ischemia: delta-opioid receptor-mediated cellular/molecular events." Cell Mol Life Sci **70**(13): 2291-2303.
Hoebel, B. G., N. M. Avena, et al. (2007). "Accumbens dopamine-acetylcholine balance in approach and avoidance." Curr Opin Pharmacol **7**(6): 617-627.
Holahan, M. R., J. Nichol, et al. (2008). "Spatial information processing consequences of DAMGO injections into the dorsal striatum." Neurobiol Learn Mem **90**(2): 434-442.
Holmes, A., C. C. Wrenn, et al. (2002). "Behavioral profiles of inbred strains on novel olfactory, spatial and emotional tests for reference memory in mice." Genes Brain Behav **1**(1): 55-69.
Hoshino, K., D. A. Uga, et al. (2004). "The compulsive-like aspect of the head dipping emission in rats with chronic electrolytic lesion in the area of the median raphe nucleus." Braz J Med Biol Res **37**(2): 245-250.
Huang, A. C., B. C. Shyu, et al. (2013). "Neural substrates of fear conditioning, extinction, and spontaneous recovery in passive avoidance learning: a c-fos study in rats." Behav Brain Res **237**: 23-31.
Huang, R. Q., C. L. Bell-Horner, et al. (2001). "Pentylenetetrazole-induced inhibition of recombinant gamma-aminobutyric acid type A (GABA(A)) receptors: mechanism and site of action." J Pharmacol Exp Ther **298**(3): 986-995.
Hudzik, T. J., C. Maciag, et al. (2011). "Preclinical pharmacology of AZD2327: a highly selective agonist of the delta-opioid receptor." J Pharmacol Exp Ther **338**(1): 195-204.
Hughes, J., T. W. Smith, et al. (1975). "Identification of two related pentapeptides from the brain with potent opiate agonist activity." Nature **258**(5536): 577-580.
Inglis, F. M. and B. Moghaddam (1999). "Dopaminergic innervation of the amygdala is highly responsive to stress." J Neurochem **72**(3): 1088-1094.
Ingram, S. L., C. W. Vaughan, et al. (1998). "Enhanced opioid efficacy in opioid dependence is caused by an altered signal transduction pathway." J Neurosci **18**(24): 10269-10276.
Ingram, S. L. and J. T. Williams (1994). "Opioid inhibition of Ih via adenylyl cyclase." Neuron **13**(1): 179-186.
Ishii, H., S. Ohara, et al. (2012). "Inactivating anterior insular cortex reduces risk taking." J Neurosci **32**(45): 16031-16039.
Johansen, J. P., S. B. Wolff, et al. (2012). "Controlling the elements: an optogenetic approach to understanding the neural circuits of fear." Biol Psychiatry **71**(12): 1053-1060.
Jutkiewicz, E. M., M. G. Baladi, et al. (2006). "The convulsive and electroencephalographic changes produced by nonpeptidic delta-opioid

agonists in rats: comparison with pentylenetetrazol." J Pharmacol Exp Ther **317**(3): 1337-1348.
Jutkiewicz, E. M., S. T. Kaminsky, et al. (2005). "Differential behavioral tolerance to the delta-opioid agonist SNC80 ([(+)-4-[(alphaR)-alpha-[(2S,5R)-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-(3-me thoxyphenyl)methyl]-N,N-diethylbenzamide) in Sprague-Dawley rats." J Pharmacol Exp Ther **315**(1): 414-422.
Jutkiewicz, E. M., K. C. Rice, et al. (2005). "Separation of the convulsions and antidepressant-like effects produced by the delta-opioid agonist SNC80 in rats." Psychopharmacology (Berl) **182**(4): 588-596.
Kakidani, H., Y. Furutani, et al. (1982). "Cloning and sequence analysis of cDNA for porcine beta-neo-endorphin/dynorphin precursor." Nature **298**(5871): 245-249.
Kaufling, J., P. Veinante, et al. (2009). "Afferents to the GABAergic tail of the ventral tegmental area in the rat." J Comp Neurol **513**(6): 597-621.
Kelly, J. P., A. S. Wrynn, et al. (1997). "The olfactory bulbectomized rat as a model of depression: an update." Pharmacol Ther **74**(3): 299-316.
Kenakin, T. (2007). "Functional selectivity through protean and biased agonism: who steers the ship?" Mol Pharmacol **72**(6): 1393-1401.
Kenakin, T. (2011). "Functional selectivity and biased receptor signaling." J Pharmacol Exp Ther **336**(2): 296-302.
Kennedy, B. L., J. J. Schwab, et al. (2001). "Assessment of state and trait anxiety in subjects with anxiety and depressive disorders." Psychiatr Q **72**(3): 263-276.
Kent, J. M. and S. L. Rauch (2003). "Neurocircuitry of anxiety disorders." Curr Psychiatry Rep **5**(4): 266-273.
Kieffer, B. L. (1995). "Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides." Cellular and molecular Neurobiology **15**(6): 615-633.
Kieffer, B. L., K. Befort, et al. (1992). "The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization." Proc Natl Acad Sci U S A **89**(24): 12048-12052.
Kieffer, B. L. and C. J. Evans (2009). "Opioid receptors: from binding sites to visible molecules in vivo." Neuropharmacology **56 Suppl 1**: 205-212.
Kieffer, B. L. and C. Gaveriaux-Ruff (2002). "Exploring the opioid system by gene knockout." Prog Neurobiol **66**(5): 285-306.
Killcross, S., T. W. Robbins, et al. (1997). "Different types of fear-conditioned behaviour mediated by separate nuclei within amygdala." Nature **388**(6640): 377-380.
Kitchen, I., F. M. Leslie, et al. (1995). "Development of delta-opioid receptor subtypes and the regulatory role of weaning: radioligand binding, autoradiography and in situ hybridization studies." The Journal of Pharmacology and Experimental Therapeutics **275**(3): 1597-1607.
Kitchen, I., S. J. Slowe, et al. (1997). "Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene." Brain Res **778**(1): 73-88.
Kitchen, I., S. J. Slowe, et al. (1997). "Quantitative autoradiographic mapping of mu-, delta- and kappa-opioid receptors in knockout mice lacking the mu-opioid receptor gene." Brain Research **778**(1): 73-88.
Knapska, E., K. Radwanska, et al. (2007). "Functional internal complexity of amygdala: focus on gene activity mapping after behavioral training and drugs of abuse." Physiol Rev **87**(4): 1113-1173.

Kobayakawa, K., R. Kobayakawa, et al. (2007). "Innate versus learned odour processing in the mouse olfactory bulb." Nature **450**(7169): 503-508.
Koch, T. and V. Hollt (2008). "Role of receptor internalization in opioid tolerance and dependence." Pharmacol Ther **117**(2): 199-206.
Koch, T., A. Widera, et al. (2005). "Receptor endocytosis counteracts the development of opioid tolerance." Mol Pharmacol **67**(1): 280-287.
Konig, M., A. M. Zimmer, et al. (1996). "Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin." Nature **383**(6600): 535-538.
Kovoor, A., V. Nappey, et al. (1997). "Mu and delta opioid receptors are differentially desensitized by the coexpression of beta-adrenergic receptor kinase 2 and beta-arrestin 2 in xenopus oocytes." J Biol Chem **272**(44): 27605-27611.
Kreil, G., D. Barra, et al. (1989). "Deltorphin, a novel amphibian skin peptide with high selectivity and affinity for delta opioid receptors." Eur J Pharmacol **162**(1): 123-128.
Kreitzer, A. C. and R. C. Malenka (2008). "Striatal plasticity and basal ganglia circuit function." Neuron **60**(4): 543-554.
Kumar, V., Z. A. Bhat, et al. (2013). "Animal models of anxiety: A comprehensive review." J Pharmacol Toxicol Methods **68**(2): 175-183.
Lalonde, R. and C. Strazielle (2012). "Brain regions and genes affecting myoclonus in animals." Neurosci Res.
Lamm, C. and T. Singer (2010). "The role of anterior insular cortex in social emotions." Brain Struct Funct **214**(5-6): 579-591.
Langston, R. F., C. H. Stevenson, et al. (2010). "The role of hippocampal subregions in memory for stimulus associations." Behav Brain Res **215**(2): 275-291.
Laurent, V., B. Leung, et al. (2012). "mu- and delta-opioid-related processes in the accumbens core and shell differentially mediate the influence of reward-guided and stimulus-guided decisions on choice." J Neurosci **32**(5): 1875-1883.
Laux, A., A. H. Muller, et al. (2011). "Mapping of endogenous morphine-like compounds in the adult mouse brain: Evidence of their localization in astrocytes and GABAergic cells." J Comp Neurol **519**(12): 2390-2416.
Law, P. Y., O. M. Kouhen, et al. (2000). "Deltorphin II-induced rapid desensitization of delta-opioid receptor requires both phosphorylation and internalization of the receptor." J Biol Chem **275**(41): 32057-32065.
Le Bourdonnec, B., R. T. Windh, et al. (2008). "Potent, orally bioavailable delta opioid receptor agonists for the treatment of pain: discovery of N,N-diethyl-4-(5-hydroxyspiro[chromene-2,4'-piperidine]-4-yl)benzamide (ADL5859)." J Med Chem **51**(19): 5893-5896.
Le Bourdonnec, B., R. T. Windh, et al. (2009). "Spirocyclic delta opioid receptor agonists for the treatment of pain: discovery of N,N-diethyl-3-hydroxy-4-(spiro[chromene-2,4'-piperidine]-4-yl) benzamide (ADL5747)." J Med Chem **52**(18): 5685-5702.
Le Merrer, J., J. A. Becker, et al. (2009). "Reward processing by the opioid system in the brain." Physiol Rev **89**(4): 1379-1412.
Le Merrer, J., L. Faget, et al. (2012). "Cues predicting drug or food reward restore morphine-induced place conditioning in mice lacking delta opioid receptors." Psychopharmacology (Berl) **223**(1): 99-106.
Le Merrer, J., S. Gavello-Baudy, et al. (2007). "Morphine self-administration into the lateral septum depends on dopaminergic mechanisms: Evidence from pharmacology and Fos neuroimaging." Behav Brain Res **180**(2): 203-217.

Le Merrer, J., A. Plaza-Zabala, et al. (2011). "Deletion of the delta opioid receptor gene impairs place conditioning but preserves morphine reinforcement." Biol Psychiatry **69**(7): 700-703.
Le Merrer, J., X. Rezai, et al. (2013). "Impaired Hippocampus-Dependent and Facilitated Striatum-Dependent Behaviors in Mice Lacking the Delta Opioid Receptor." Neuropsychopharmacology.
Lecoq, I., N. Marie, et al. (2004). "Different regulation of human delta-opioid receptors by SNC-80 [(+)-4-[(alphaR)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-meth oxybenzyl]-N,N-diethylbenzamide] and endogenous enkephalins." J Pharmacol Exp Ther **310**(2): 666-677.
LeDoux, J. E. (2000). "Emotion circuits in the brain." Annu Rev Neurosci **23**: 155-184.
LeDoux, J. E., P. Cicchetti, et al. (1990). "The lateral amygdaloid nucleus: sensory interface of the amygdala in fear conditioning." J Neurosci **10**(4): 1062-1069.
Lencer, W. I. and B. Tsai (2003). "The intracellular voyage of cholera toxin: going retro." Trends Biochem Sci **28**(12): 639-645.
Livak, K. J. and T. D. Schmittgen (2001). "Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method." Methods **25**(4): 402-408.
Loh, H. H., L. F. Tseng, et al. (1976). "beta-endorphin is a potent analgesic agent." Proc Natl Acad Sci U S A **73**(8): 2895-2898.
Lord, J. A., A. A. Waterfield, et al. (1977). "Endogenous opioid peptides: multiple agonists and receptors." Nature **267**(5611): 495-499.
Loscher, W., D. Honack, et al. (1991). "The role of technical, biological and pharmacological factors in the laboratory evaluation of anticonvulsant drugs. III. Pentylenetetrazole seizure models." Epilepsy Res **8**(3): 171-189.
Lutz, P. E. and B. L. Kieffer (2012). "Opioid receptors: distinct roles in mood disorders." Trends Neurosci.
Lydiard, R. B. (2003). "The role of GABA in anxiety disorders." J Clin Psychiatry **64 Suppl 3**: 21-27.
Madamba, S. G., P. Schweitzer, et al. (1999). "Dynorphin selectively augments the M-current in hippocampal CA1 neurons by an opiate receptor mechanism." J Neurophysiol **82**(4): 1768-1775.
Mamiya, N., H. Fukushima, et al. (2009). "Brain region-specific gene expression activation required for reconsolidation and extinction of contextual fear memory." J Neurosci **29**(2): 402-413.
Manglik, A., A. C. Kruse, et al. (2012). "Crystal structure of the micro-opioid receptor bound to a morphinan antagonist." Nature **485**(7398): 321-326.
Mann, E. O. and I. Mody (2008). "The multifaceted role of inhibition in epilepsy: seizure-genesis through excessive GABAergic inhibition in autosomal dominant nocturnal frontal lobe epilepsy." Curr Opin Neurol **21**(2): 155-160.
Mansour, A., C. A. Fox, et al. (1995). "Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications." Trends in Neurosciences **18**: 22-29.
Mansour, A., C. A. Fox, et al. (1994). "Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an in situ hybridization study." J Comp Neurol **350**(3): 412-438.
Margolis, E. B., H. L. Fields, et al. (2008). "Delta-opioid receptor expression in the ventral tegmental area protects against elevated alcohol consumption." J Neurosci **28**(48): 12672-12681.

Marie, N., B. Aguila, et al. (2006). "Tracking the opioid receptors on the way of desensitization." Cell Signal **18**(11): 1815-1833.
Marie, N., I. Lecoq, et al. (2003). "Differential sorting of human delta-opioid receptors after internalization by peptide and alkaloid agonists." J Biol Chem **278**(25): 22795-22804.
Marinelli, P. W., D. Funk, et al. (2010). "Opioid receptors in the basolateral amygdala but not dorsal hippocampus mediate context-induced alcohol seeking." Behav Brain Res **211**(1): 58-63.
Marques, J. M., I. A. Olsson, et al. (2008). "Evaluation of exploration and risk assessment in pre-weaning mice using the novel cage test." Physiol Behav **93**(1-2): 139-147.
Marsicano, G., S. Goodenough, et al. (2003). "CB1 cannabinoid receptors and on-demand defense against excitotoxicity." Science **302**(5642): 84-88.
Martin, M., A. Matifas, et al. (2003). "Acute antinociceptive responses in single and combinatorial opioid receptor knockout mice: distinct mu, delta and kappa tones." Eur J Neurosci **17**(4): 701-708.
Martin, W. R., C. G. Eades, et al. (1976). "The effects of morphine- and nalorphine-like drugs in the non-dependent and morphine-dependent chronic spinal dog." The Journal of Pharmacology and experimental Therapeutics **197**: 517-532.
Maslov, L. N., Y. B. Lishmanov, et al. (2009). "Activation of peripheral delta2 opioid receptors increases cardiac tolerance to ischemia/reperfusion injury Involvement of protein kinase C, NO-synthase, KATP channels and the autonomic nervous system." Life Sci **84**(19-20): 657-663.
Matamales, M., J. Bertran-Gonzalez, et al. (2009). "Striatal medium-sized spiny neurons: identification by nuclear staining and study of neuronal subpopulations in BAC transgenic mice." PLoS One **4**(3): e4770.
Matthes, H. W., R. Maldonado, et al. (1996). "Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene." Nature **383**(6603): 819-823.
McDonald, A. J. (1982). "Neurons of the lateral and basolateral amygdaloid nuclei: a Golgi study in the rat." J Comp Neurol **212**(3): 293-312.
McDonald, A. J. (1998). "Cortical pathways to the mammalian amygdala." Prog Neurobiol **55**(3): 257-332.
McDonald, A. J. (2003). "Is there an amygdala and how far does it extend? An anatomical perspective." Ann N Y Acad Sci **985**: 1-21.
McDonald, A. J. and F. Mascagni (2001). "Colocalization of calcium-binding proteins and GABA in neurons of the rat basolateral amygdala." Neuroscience **105**(3): 681-693.
Mehendale, S. R. and C. S. Yuan (2006). "Opioid-induced gastrointestinal dysfunction." Dig Dis **24**(1-2): 105-112.
Metzger, D. and P. Chambon (2001). "Site- and time-specific gene targeting in the mouse." Methods **24**(1): 71-80.
Monory, K., F. Massa, et al. (2006). "The endocannabinoid system controls key epileptogenic circuits in the hippocampus." Neuron **51**(4): 455-466.
Morris, R. G., P. Garrud, et al. (1982). "Place navigation impaired in rats with hippocampal lesions." Nature **297**(5868): 681-683.
Mosberg, H. I., R. Hurst, et al. (1983). "Conformationally constrained cyclic enkephalin analogs with pronounced delta opioid receptor agonist selectivity." Life Sci **32**(22): 2565-2569.

Nadal, X., J. E. Banos, et al. (2006). "Neuropathic pain is enhanced in delta-opioid receptor knockout mice." Eur J Neurosci **23**(3): 830-834.
Nagy, A., L. Mar, et al. (2009). "Creation and use of a cre recombinase transgenic database." Methods Mol Biol **530**: 365-378.
Nakanishi, S., A. Inoue, et al. (1979). "Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor." Nature **278**(5703): 423-427.
Narita, M., C. Kaneko, et al. (2006). "Chronic pain induces anxiety with concomitant changes in opioidergic function in the amygdala." Neuropsychopharmacology **31**(4): 739-750.
Narita, M., N. Kuzumaki, et al. (2006). "Chronic pain-induced emotional dysfunction is associated with astrogliosis due to cortical delta-opioid receptor dysfunction." J Neurochem **97**(5): 1369-1378.
Nozaki, C., B. Le Bourdonnec, et al. (2012). "delta-Opioid mechanisms for ADL5747 and ADL5859 effects in mice: analgesia, locomotion, and receptor internalization." J Pharmacol Exp Ther **342**(3): 799-807.
Oldham, W. M. and H. E. Hamm (2008). "Heterotrimeric G protein activation by G-protein-coupled receptors." Nat Rev Mol Cell Biol **9**(1): 60-71.
Oliveira, A. M., J. D. Hawk, et al. (2010). "Post-training reversible inactivation of the hippocampus enhances novel object recognition memory." Learn Mem **17**(3): 155-160.
Olmstead, M. C., A. M. Ouagazzal, et al. (2009). "Mu and delta opioid receptors oppositely regulate motor impulsivity in the signaled nose poke task." PLoS ONE **4**(2): e4410.
Pare, D. and S. Duvarci (2012). "Amygdala microcircuits mediating fear expression and extinction." Curr Opin Neurobiol **22**(4): 717-723.
Parker, J. G., M. J. Wanat, et al. (2011). "Attenuating GABA(A) receptor signaling in dopamine neurons selectively enhances reward learning and alters risk preference in mice." J Neurosci **31**(47): 17103-17112.
Parkinson, J. A., T. W. Robbins, et al. (2000). "Dissociable roles of the central and basolateral amygdala in appetitive emotional learning." Eur J Neurosci **12**(1): 405-413.
Paterna, J. C., J. Feldon, et al. (2004). "Transduction profiles of recombinant adeno-associated virus vectors derived from serotypes 2 and 5 in the nigrostriatal system of rats." J Virol **78**(13): 6808-6817.
Patil, S. S., B. Sunyer, et al. (2009). "Evaluation of spatial memory of C57BL/6J and CD1 mice in the Barnes maze, the Multiple T-maze and in the Morris water maze." Behav Brain Res **198**(1): 58-68.
Paulus, M. P. and M. B. Stein (2006). "An insular view of anxiety." Biol Psychiatry **60**(4): 383-387.
Peckys, D. and G. B. Landwehrmeyer (1999). "Expression of mu, kappa, and delta opioid receptor messenger RNA in the human CNS: a 33P in situ hybridization study." Neuroscience **88**(4): 1093-1135.
Pellow, S., P. Chopin, et al. (1985). "Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat." J Neurosci Methods **14**(3): 149-167.
Peng, J., S. Sarkar, et al. (2012). "Opioid receptor expression in human brain and peripheral tissues using absolute quantitative real-time RT-PCR." Drug Alcohol Depend **124**(3): 223-228.
Perrine, S. A., B. A. Hoshaw, et al. (2006). "Delta opioid receptor ligands modulate anxiety-like behaviors in the rat." Br J Pharmacol **147**(8): 864-872.

Pert, C. B. and S. H. Snyder (1973). "Opiate receptor: demonstration in nervous tissue." Science **179**: 1011-1014.
Pitman, R. K., A. M. Rasmusson, et al. (2012). "Biological studies of post-traumatic stress disorder." Nat Rev Neurosci **13**(11): 769-787.
Poole, D. P., J. C. Pelayo, et al. (2011). "Localization and regulation of fluorescently labeled delta opioid receptor, expressed in enteric neurons of mice." Gastroenterology **141**(3): 982-991 e981-988.
Porsolt, R. D., G. Anton, et al. (1978). "Behavioural despair in rats: a new model sensitive to antidepressant treatments." Eur J Pharmacol **47**(4): 379-391.
Portoghese, P. S., M. Sultana, et al. (1988). "Naltrindole, a highly selective and potent non-peptide δ opioid receptor antagonist." Eur. J. Pharmacol. **146**: 185-186.
Pradhan, A. A., J. A. Becker, et al. (2009). "In vivo delta opioid receptor internalization controls behavioral effects of agonists." PLoS One **4**(5): e5425.
Pradhan, A. A., K. Befort, et al. (2011). "The delta opioid receptor: an evolving target for the treatment of brain disorders." Trends Pharmacol Sci **32**(10): 581-590.
Pradhan, A. A., M. L. Smith, et al. (2012). "Ligand-directed signalling within the opioid receptor family." Br J Pharmacol **167**(5): 960-969.
Pradhan, A. A., W. Walwyn, et al. (2010). "Ligand-directed trafficking of the delta-opioid receptor in vivo: two paths toward analgesic tolerance." J Neurosci **30**(49): 16459-16468.
Ramos, A. (2008). "Animal models of anxiety: do I need multiple tests?" Trends Pharmacol Sci **29**(10): 493-498.
Randall-Thompson, J. F., K. A. Pescatore, et al. (2010). "A role for delta opioid receptors in the central nucleus of the amygdala in anxiety-like behaviors." Psychopharmacology (Berl) **212**(4): 585-595.
Rawls, S. M., J. M. Hewson, et al. (2005). "Brain delta2 opioid receptors mediate SNC-80-evoked hypothermia in rats." Brain Res **1049**(1): 61-69.
Refojo, D., M. Schweizer, et al. (2011). "Glutamatergic and dopaminergic neurons mediate anxiogenic and anxiolytic effects of CRHR1." Science **333**(6051): 1903-1907.
Reiter, E., S. Ahn, et al. (2012). "Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors." Annu Rev Pharmacol Toxicol **52**: 179-197.
Rezai, X., L. Faget, et al. (2012). "Mouse delta opioid receptors are located on presynaptic afferents to hippocampal pyramidal cells." Cell Mol Neurobiol **32**(4): 509-516.
Roberts, A. J., L. H. Gold, et al. (2001). "Increased ethanol self-administration in delta-opioid receptor knockout mice." Alcohol Clin Exp Res **25**(9): 1249-1256.
Robles, Y., P. E. Vivas-Mejia, et al. (2003). "Hippocampal gene expression profiling in spatial discrimination learning." Neurobiol Learn Mem **80**(1): 80-95.
Rodefer, J. S. and T. N. Nguyen (2008). "Naltrexone reverses age-induced cognitive deficits in rats." Neurobiol Aging **29**(2): 309-313.
Rogan, M. T. and J. E. LeDoux (1996). "Emotion: systems, cells, synaptic plasticity." Cell **85**(4): 469-475.
Roozendaal, B., B. S. McEwen, et al. (2009). "Stress, memory and the amygdala." Nat Rev Neurosci **10**(6): 423-433.
Rosenkranz, J. A. and A. A. Grace (2001). "Dopamine attenuates prefrontal cortical suppression of sensory inputs to the basolateral amygdala of rats." J Neurosci **21**(11): 4090-4103.

Rosenkranz, J. A. and A. A. Grace (2002). "Cellular mechanisms of infralimbic and prelimbic prefrontal cortical inhibition and dopaminergic modulation of basolateral amygdala neurons in vivo." J Neurosci **22**(1): 324-337.
Rosenkranz, J. A., E. R. Venheim, et al. (2010). "Chronic stress causes amygdala hyperexcitability in rodents." Biol Psychiatry **67**(12): 1128-1136.
Rossier, J. (1993). Biosynthesis of enkephalins and proenkephalin-derived peptides. Opioids I. H. A. Berlin heidelberg New York, Springer Verlag. **104/I:** 423-447.
Sah, P. and M. Lopez De Armentia (2003). "Excitatory synaptic transmission in the lateral and central amygdala." Ann N Y Acad Sci **985**: 67-77.
Saitoh, A., N. Hirose, et al. (2006). "Changes in emotional behavior of mice in the hole-board test after olfactory bulbectomy." J Pharmacol Sci **102**(4): 377-386.
Saitoh, A., Y. Kimura, et al. (2004). "Potential anxiolytic and antidepressant-like activities of SNC80, a selective delta-opioid agonist, in behavioral models in rodents." J Pharmacol Sci **95**(3): 374-380.
Saitoh, A., A. Sugiyama, et al. (2011). "The novel delta opioid receptor agonist KNT-127 produces antidepressant-like and antinociceptive effects in mice without producing convulsions." Behav Brain Res **223**(2): 271-279.
Saitoh, A. and M. Yamada (2012). "Antidepressant-like Effects of delta Opioid Receptor Agonists in Animal Models." Curr Neuropharmacol **10**(3): 231-238.
Saitoh, A., Y. Yoshikawa, et al. (2005). "Role of delta-opioid receptor subtypes in anxiety-related behaviors in the elevated plus-maze in rats." Psychopharmacology (Berl) **182**(3): 327-334.
Sanders, M. J. and M. S. Fanselow (2003). "Pre-training prevents context fear conditioning deficits produced by hippocampal NMDA receptor blockade." Neurobiol Learn Mem **80**(2): 123-129.
Sandor, N. T., B. Lendvai, et al. (1992). "Effect of selective opiate antagonists on striatal acetylcholine and dopamine release." Brain Res Bull **29**(3-4): 369-373.
Santarelli, L., G. Gobbi, et al. (2001). "Genetic and pharmacological disruption of neurokinin 1 receptor function decreases anxiety-related behaviors and increases serotonergic function." Proc Natl Acad Sci U S A **98**(4): 1912-1917.
Saraiva, J., S. M. Oliveira, et al. (2004). "Opioid receptors and preconditioning of the heart." Rev Port Cardiol **23**(10): 1317-1333.
Sauriyal, D. S., A. S. Jaggi, et al. (2011). "Extending pharmacological spectrum of opioids beyond analgesia: multifunctional aspects in different pathophysiological states." Neuropeptides **45**(3): 175-188.
Scherrer, G., N. Imamachi, et al. (2009). "Dissociation of the opioid receptor mechanisms that control mechanical and heat pain." Cell **137**(6): 1148-1159.
Scherrer, G., P. Tryoen-Toth, et al. (2006). "Knockin mice expressing fluorescent delta-opioid receptors uncover G protein-coupled receptor dynamics in vivo." Proc Natl Acad Sci U S A **103**(25): 9691-9696.
Schiller, P. W., G. Weltrowska, et al. (1993). "TIPP[psi]: a highly potent and stable pseudopeptide delta opioid receptor antagonist with extraordinary delta selectivity." J Med Chem **36**(21): 3182-3187.
Schramm, C. L. and C. N. Honda (2010). "Co-administration of delta- and mu-opioid receptor agonists promotes peripheral opioid receptor function." Pain **151**(3): 763-770.
Schultz, W. (2011). "Potential vulnerabilities of neuronal reward, risk, and decision mechanisms to addictive drugs." Neuron **69**(4): 603-617.

Shen, H., Q. Ben, et al. (2012). "Role of delta2 opioid receptor in cardioprotection against hypoxia-reoxygenation injury." J Cardiovasc Pharmacol **60**(3): 253-261.
Shin, L. M. and I. Liberzon (2010). "The neurocircuitry of fear, stress, and anxiety disorders." Neuropsychopharmacology **35**(1): 169-191.
Shippenberg, T. S., A. Zapata, et al. (2007). "Dynorphin and the pathophysiology of drug addiction." Pharmacol Ther **116**(2): 306-321.
Silveira, M. C., G. Sandner, et al. (1993). "Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze." Behav Brain Res **56**(1): 115-118.
Simon, E. J., J. M. Hiller, et al. (1973). "Stereospecific binding of the potent narcotic analgesic (3H) Etorphine to rat-brain homogenate." Proc Natl Acad Sci U S A **70**(7): 1947-1949.
Simonin, F., K. Befort, et al. (1994). "The human delta opioid receptor: genomic organization, cDNA cloning, functional expression and distribution in human brain." Molecular Pharmacology **46**: 1015-1021.
Simonin, F., C. Gaveriaux-Ruff, et al. (1995). "kappa-Opioid receptor in humans: cDNA and genomic cloning, chromosomal assignment, functional expression, pharmacology, and expression pattern in the central nervous system." Proc Natl Acad Sci U S A **92**(15): 7006-7010.
Simonin, F., O. Valverde, et al. (1998). "Disruption of the kappa-opioid receptor gene in mice enhances sensitivity to chemical visceral pain, impairs pharmacological actions of the selective kappa-agonist U-50,488H and attenuates morphine withdrawal." Embo J **17**(4): 886-897.
Singewald, N., P. Salchner, et al. (2003). "Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs." Biol Psychiatry **53**(4): 275-283.
Slowe, S. J., F. Simonin, et al. (1999). "Quantitative autoradiography of mu-,delta- and kappa1 opioid receptors in kappa-opioid receptor knockout mice." Brain Res **818**(2): 335-345.
Smith, J. S., A. G. Schindler, et al. (2012). "Stress-induced activation of the dynorphin/kappa-opioid receptor system in the amygdala potentiates nicotine conditioned place preference." J Neurosci **32**(4): 1488-1495.
Smith, J. S., J. K. Zubieta, et al. (1999). "Quantification of delta-opioid receptors in human brain with N1'-([11C]methyl) naltrindole and positron emission tomography." J Cereb Blood Flow Metab **19**(9): 956-966.
Smith, K. S. and K. C. Berridge (2005). "The ventral pallidum and hedonic reward: neurochemical maps of sucrose "liking" and food intake." J Neurosci **25**(38): 8637-8649.
Smith, K. S. and U. Rudolph (2012). "Anxiety and depression: mouse genetics and pharmacological approaches to the role of GABA(A) receptor subtypes." Neuropharmacology **62**(1): 54-62.
Sodickson, D. L. and B. P. Bean (1998). "Neurotransmitter activation of inwardly rectifying potassium current in dissociated hippocampal CA3 neurons: interactions among multiple receptors." J Neurosci **18**(20): 8153-8162.
Solati, J., M. R. Zarrindast, et al. (2010). "Dorsal hippocampal opioidergic system modulates anxiety-like behaviors in adult male Wistar rats." Psychiatry Clin Neurosci **64**(6): 634-641.
Song, C. and B. E. Leonard (2005). "The olfactory bulbectomised rat as a model of depression." Neurosci Biobehav Rev **29**(4-5): 627-647.

Staples, L. G. (2010). "Predator odor avoidance as a rodent model of anxiety: learning-mediated consequences beyond the initial exposure." Neurobiol Learn Mem **94**(4): 435-445.
Steru, L., R. Chermat, et al. (1985). "The tail suspension test: a new method for screening antidepressants in mice." Psychopharmacology (Berl) **85**(3): 367-370.
Stuber, G. D., D. R. Sparta, et al. (2011). "Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking." Nature **475**(7356): 377-380.
Stumm, R. K., C. Zhou, et al. (2004). "Neuronal types expressing mu- and delta-opioid receptor mRNA in the rat hippocampal formation." J Comp Neurol **469**(1): 107-118.
Sung, Y. H., I. J. Baek, et al. (2012). "Mouse genetics: catalogue and scissors." BMB Rep **45**(12): 686-692.
Surratt, C. K. and W. R. Adams (2005). "G protein-coupled receptor structural motifs: relevance to the opioid receptors." Curr Top Med Chem **5**(3): 315-324.
Svoboda, K. R., C. E. Adams, et al. (1999). "Opioid receptor subtype expression defines morphologically distinct classes of hippocampal interneurons." J Neurosci **19**(1): 85-95.
Svoboda, K. R. and C. R. Lupica (1998). "Opioid inhibition of hippocampal interneurons via modulation of potassium and hyperpolarization-activated cation (Ih) currents." J Neurosci **18**(18): 7084-7098.
Tenenbaum, L., A. Chtarto, et al. (2004). "Recombinant AAV-mediated gene delivery to the central nervous system." J Gene Med **6 Suppl 1**: S212-222.
Terenius, L. (1973). "Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain." Acta Pharmacol Toxicol (Copenh) **33**(5): 377-384.
Terranova, M. L. and G. Laviola (2001). "delta-Opioid modulation of social interactions in juvenile mice weaned at different ages." Physiol Behav **73**(3): 393-400.
Townsend, D. t., P. S. Portoghese, et al. (2004). "Characterization of specific opioid binding sites in neural membranes from the myenteric plexus of porcine small intestine." J Pharmacol Exp Ther **308**(1): 385-393.
Trapaidze, N., D. E. Keith, et al. (1996). "Sequestration of the delta opioid receptor. Role of the C terminus in agonist-mediated internalization." J Biol Chem **271**(46): 29279-29285.
Twitchell, W. A. and S. G. Rane (1993). "Opioid peptide modulation of Ca(2+)-dependent K+ and voltage-activated Ca2+ currents in bovine adrenal chromaffin cells." Neuron **10**(4): 701-709.
Tye, K. M., R. Prakash, et al. (2011). "Amygdala circuitry mediating reversible and bidirectional control of anxiety." Nature **471**(7338): 358-362.
Vergura, R., G. Balboni, et al. (2008). "Anxiolytic- and antidepressant-like activities of H-Dmt-Tic-NH-CH(CH2-COOH)-Bid (UFP-512), a novel selective delta opioid receptor agonist." Peptides **29**(1): 93-103.
Vincent, P. G. and B. F. Engelke (1979). "High pressure liquid chromatographic determination of the five major alkaloids in Papaver somniferum L. and thebaine in Papaver bracteatum Lindl. capsular tissue." J Assoc Off Anal Chem **62**(2): 310-314.
Visiers, I., J. A. Ballesteros, et al. (2002). "Three-dimensional representations of G protein-coupled receptor structures and mechanisms." Methods Enzymol **343**: 329-371.

von Zastrow, M. (2003). "Mechanisms regulating membrane trafficking of G protein-coupled receptors in the endocytic pathway." Life Sciences. Part 1. Physiology and Pharmacology **74**: 217-224.
Vonvoigtlander, P. F., R. A. Lahti, et al. (1983). "U-50,488: a selective and structurally novel non-Mu (kappa) opioid agonist." J Pharmacol Exp Ther **224**(1): 7-12.
Waldhoer, M., S. E. Bartlett, et al. (2004). "Opioid receptors." Annu Rev Biochem **73**: 953-990.
Walf, A. A. and C. A. Frye (2007). "The use of the elevated plus maze as an assay of anxiety-related behavior in rodents." Nat Protoc **2**(2): 322-328.
Wang, J. B., P. S. Johnson, et al. (1994). "Human mu opiate receptor. cDNA and genomic clones, pharmacologic characterization and chromosomal assignment." FEBS Lett **338**(2): 217-222.
Wang, Y., C. A. Dye, et al. (2010). "Dlx5 and Dlx6 regulate the development of parvalbumin-expressing cortical interneurons." J Neurosci **30**(15): 5334-5345.
Weber, R. J., R. Gomez-Flores, et al. (2004). "Immune, neuroendocrine, and somatic alterations in animal models of human heroin abuse." J Neuroimmunol **147**(1-2): 134-137.
Whistler, J. L., P. Tsao, et al. (2001). "A phosphorylation-regulated brake mechanism controls the initial endocytosis of opioid receptors but is not required for post-endocytic sorting to lysosomes." J Biol Chem **276**(36): 34331-34338.
Wilding, T. J., M. D. Womack, et al. (1995). "Fast, local signal transduction between the mu opioid receptor and Ca2+ channels." J Neurosci **15**(5 Pt 2): 4124-4132.
Williams, J. T., M. J. Christie, et al. (2001). "Cellular and synaptic adaptations mediating opioid dependence." Physiol Rev **81**(1): 299-343.
Wu, H., D. Wacker, et al. (2012). "Structure of the human kappa-opioid receptor in complex with JDTic." Nature **485**(7398): 327-332.
Xiang, B., G. H. Yu, et al. (2001). "Heterologous activation of protein kinase C stimulates phosphorylation of delta-opioid receptor at serine 344, resulting in beta-arrestin- and clathrin-mediated receptor internalization." J Biol Chem **276**(7): 4709-4716.
Yang, M. and J. N. Crawley (2009). "Simple behavioral assessment of mouse olfaction." Curr Protoc Neurosci **Chapter 8**: Unit 8 24.
Yasuda, K., R. Espinosa, 3rd, et al. (1994). "Localization of the kappa opioid receptor gene to human chromosome band 8q11.2." Genomics **19**(3): 596-597.
Young, E., D. Bronstein, et al. (1993). Proopiomelanocortin biosynthesis, processing and secretion: functional implications. Opioids I. H. A. Berlin heidelberg New York, Springer Verlag. **104/I:** 393-421.
Zerucha, T., T. Stuhmer, et al. (2000). "A highly conserved enhancer in the Dlx5/Dlx6 intergenic region is the site of cross-regulatory interactions between Dlx genes in the embryonic forebrain." J Neurosci **20**(2): 709-721.
Zhang, G., V. Gurtu, et al. (1996). "An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells." Biochem Biophys Res Commun **227**(3): 707-711.
Zhang, Y., W. Xiong, et al. (2009). "Receptor trafficking induced by mu-opioid-receptor phosphorylation." Neurosci Biobehav Rev **33**(8): 1192-1197.
Zollner, C. and C. Stein (2007). "Opioids." Handb Exp Pharmacol(177): 31-63.

Résumé de Thèse

Les récepteurs opioïde delta (DORs) sont des récepteurs couplés aux protéines G et appartiennent au système d'opioïde. Ces récepteurs sont fortement exprimés au niveau du bulbe olfactif, du cortex, du striatum, du noyau basolateral de l'amygdala et des noyaux du pons (Mansour et al., 1995, *Trends in Neurosciences* ; Le Merrer et al., 2009, *Physiol. Rev.*). Les souris mutantes de première génération (souris knockout, délétion totale du gène) ont déjà permis de démontrer que DOR joue un rôle critique dans le contrôle de la douleur chronique (Gavériaux-Ruff et al., 2011, *Pain*), la régulation de l'activité motrice et des réponses émotionnelles (Filliol et al ., 2000, *Nature Genetics*), l'impulsivité motrice (Olmstead et al., 2009, *PLoS ONE* ; Befort et al., 2011, *Psychopharmacology*) et l'association drogue-contexte (Le Merrer et al., 2011, *Biol. Psy.*). Aujourd'hui, l'approche génétique et la pharmacologie ont fait émerger ce récepteur comme une nouvelle cible thérapeutique. En particulier, l'activation de DOR est anxiolytique et antidépressante, et les premiers composés sont en essais cliniques (Pradhan et al., 2011, *Trends Pharmacol. Sci.*; Chu Sin Chung et al., 2013, *Pharmacol. and Ther.*). Le but de notre étude est d'identifier les circuits neuronaux dans lesquels les DORs contrôlent les processus émotionnels et cognitifs. Nous avons démontré au sein du laboratoire que les DORs sont fortement exprimés sur les neurones GABAergiques, en particulier au niveau de l'hippocampe ansi que du striatum (Scherrer et al., 2006, *PNAS* ; Faget et al., 2012, *J. Neuroscience*). Afin d'étudier la contribution des DORs spécifiquement exprimés dans cette population neuronale sur le contrôle des processus émotionnels et cognitifs, nous avons développé une lignée de souris de deuxième génération, dans laquelle les récepteurs sont supprimés spécifiquement dans les neurones GABAergiques du cerveau antérieur. Nous avons ensuite étudié le rôle des DORs exprimés par ces neurones dans les réponses émotionnelles, locomotrices et la sensibilité aux crises épileptiques.

La première partie de mon projet a consisté à caractériser les souris DOR knockout conditionnelles (Dlx-DOR) pour les récepteurs exprimés dans les neurones

GABAergiques du cerveau antérieur (Dlx5/6-Cre x Oprd1$^{fl/fl}$) (Figure 1). Cette lignée a été obtenue en croisant une lignée de souris floxées dont l'exon 2 du gène codant pour DOR est entouré de sites loxP (Oprd1$^{fl/fl}$) avec une lignée de souris exprimant la Cre recombinase sous le contrôle du promoteur Dlx 5/6 (Dlx-5/6-Cre) (Monory et al., 2006, *Neuron*). Nous avons déterminé la distribution de DOR dans le cerveau des souris Dlx-DOR au niveau de l'ARN messager par PCR quantitative en temps réel, et au niveau de la protéine par des expériences de liaison de ligand (autoradiographie quantitative) et de signalisation (GTPγS [$^{35}$S]). Nous avons observé une très forte réduction de l'expression du récepteur au niveau du bulbe olfactif ainsi que du striatum, tant au niveau de ARN messager qu'au niveau de la protéine. Dans le cortex et l'hippocampe la délétion du récepteur est partielle, et aucun changement n'a été détecté au niveau de l'amygdale, des noyaux du pons et de la moelle épinière. Les résultats montrent donc une délétion essentiellement dans le cerveau antérieur, comme attendu. De plus, la délétion majeure dans le bulbe olfactif et le striatum démontre génétiquement que le récepteur est majoritairement exprimé par les neurones inhibiteurs dans ces régions.

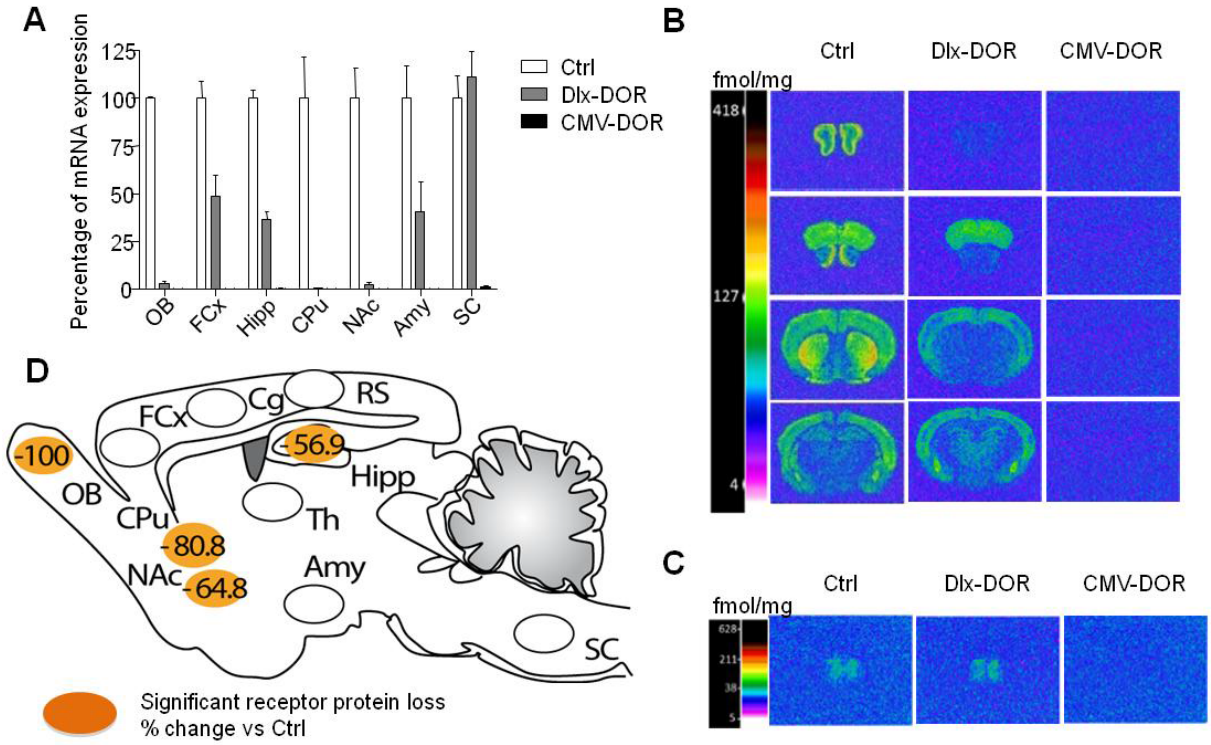



Figure 1 : Caractérisation anatomique des souris mutantes Dlx-DOR. (A) Quantification des niveau d'expression de l'ARN messager codant pour DOR sur des échantillons d'animaux contrôle (Ctrl, barre blanche), Dlx-DOR (barre grise) et de knockout constitutif (CMV-DOR, barre noire) (effecif n=3-

4/génotype). Les données sont normalisées en comparaison avec un gène rapporteur 36B4. Le transcrit n'est plus détectable dans le bulbe olfactif (OB), le noyau caudé-putamen (CPu) et le noyau accumbens (NAc) chez les souris Dlx-DOR. Une diminution partielle de l'expression du transcrit est observée au niveau du cortex préfrontal (PFC), de l'hippocampe (Hipp) et de l'amygdale (AMG). (B-C) Autoradiographie quantitative. Sections cérébrales exposées à un ligand radiomarqué [$^{3}$H] deltorphin-1. La liaison du ligand sur son récepteur est révélée sur un autoradiogramme, puis quantifiée et exprimée en fmol par mg de tissu. Exemples d'autoradiogrammes pour des sections de cerveau (B) ou de moelle épinière (C) des trois génotypes. (D) Représentation schématique du profil d'expression de DOR chez des souris Dlx-DOR. Les régions représentées en orange correspondent aux regions cérébrales présentant une réduction significative de l'expression de DOR et les valeurs représentent le pourcentage de réduction chez des souris Dlx-DOR en comparaison de souris contrôle Ctrl Abbréviations : Amy, amygdale ; Cg, cortex cingulaire ; CPu, noyau caudé-putamen ; FCx, cortex frontal ; Hipp, hippocampe ; NAc, noyau accumbens ; OB, bulbe olfactif ; RS, cortex retrosplenial ; SC, moelle épinière.

Dans une deuxième partie, nous avons réalisé une caractérisation comportementale de la lignée Dlx-DOR orientée vers l'activité locomotrice et les comportements émotionnels. Nous avons comparé les souris mutantes Dlx-DOR (Dlx5/6-Oprd1$^{fl/fl}$) aux souris contrôle (Oprd1$^{fl/fl}$) et aux souris knockout totales ou KO (CMV-Oprd1$^{fl/fl}$) dans plusieurs paradigmes classiquement utilisés pour évaluer les comportements de types anxieux (test de la chambre clair-obscure ; labyrinthe en croix surélevé ; test du champ ouvert), dépressifs (test de la nage forcée ; test de la suspension caudale) ainsi que l'activité locomotrice (cages d'actimétrie).

Nos données ne révèlent aucune différence d'activité locomotrice basale entre les souris Dlx-DOR et contrôle. Nous avons trouvé une tendance pour une activité locomotrice augmentée chez les souris KO, comme précédemment démontré (Filliol et al., 2000, *Nature Genetics*). L'administration d'un agoniste spécifique de DOR, le SNC80, augmente l'activité des souris contrôle comme attendu, mais cet effet n'est pas observé chez les souris Dlx-DOR. Ces résultats démontrent que la population des DORs exprimés par les neurones GABAergiques du cerveau antérieur n'influent pas de manière tonique sur l'activité basale mais sont nécessaires à l'effet hyperlocomoteur de l'agoniste (Figure 2).

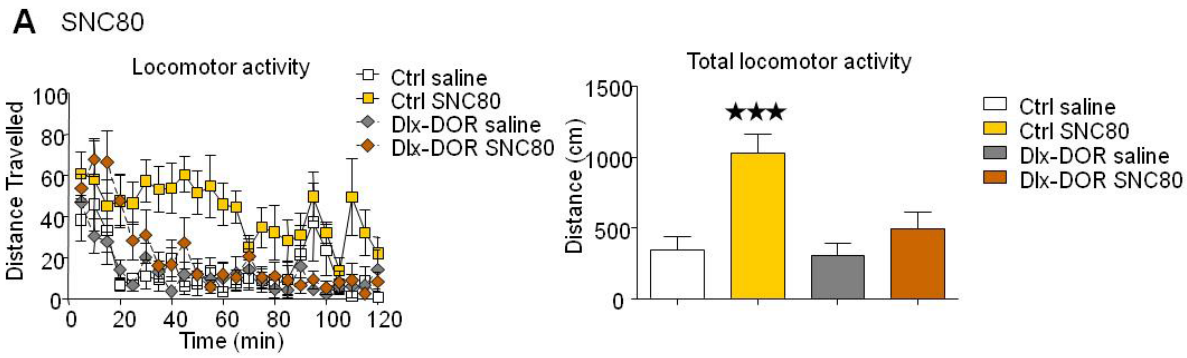



Figure 2 : Activité locomotrice régulée par DOR. Des souris contrôle Ctrl et mutantes Dlx-DOR sont testées dans des cages d'actimetrie pendant 2h suivant l'administration intraperitonéale d'un agoniste du récepteur, SNC80 (10 mg/kg), ou de solution saline (effectif n=9-11 souris par génotype et par traitement). Les souris mutantes montrent une activité similaire aux souris contrôle après administration de solution saline. L'administration de SNC80 provoque une augmentation de l'activité locomotrice chez les souris contrôle uniquement (Analyse de la variance ANOVA à un facteur, trois étoiles, $p<0.001$).

Dans les tests d'anxiété classiques, nos résultats montrent que les souris KO présentent une augmentation du niveau d'anxiété et des comportements de type dépressif, comme précédemment décrit (Filliol et al., 2000, *Nature Genetics*). En revanche, les souris Dlx-DOR montrent un niveau d'anxiété diminué par rapport à des souris contrôle (Figure 3). Ce phénotype inattendu a été confirmé dans un paradigme d'hyponéophagie (test de suppression de la prise alimentaire induite par la nouveauté) également validé pour évaluer le niveau d'anxiété (Santarelli et al., 2003, *Science*). Ainsi, nous observons un phénotype opposé pour les souris KO (plus d'anxiété) et les souris Dlx-DOR (moins d'anxiété). Ce résultat suggère que, contrairement à la notion généralement acceptée que l'activité DOR est anxiolytique, la population de récepteurs exprimés par les neurones GABAergiques du cerveau antérieur ont une activité anxiogène. Ces données démontrent que les mêmes récepteurs exprimés sur des populations neuronales différentes peuvent réguler les états émotionnels de manière opposée.

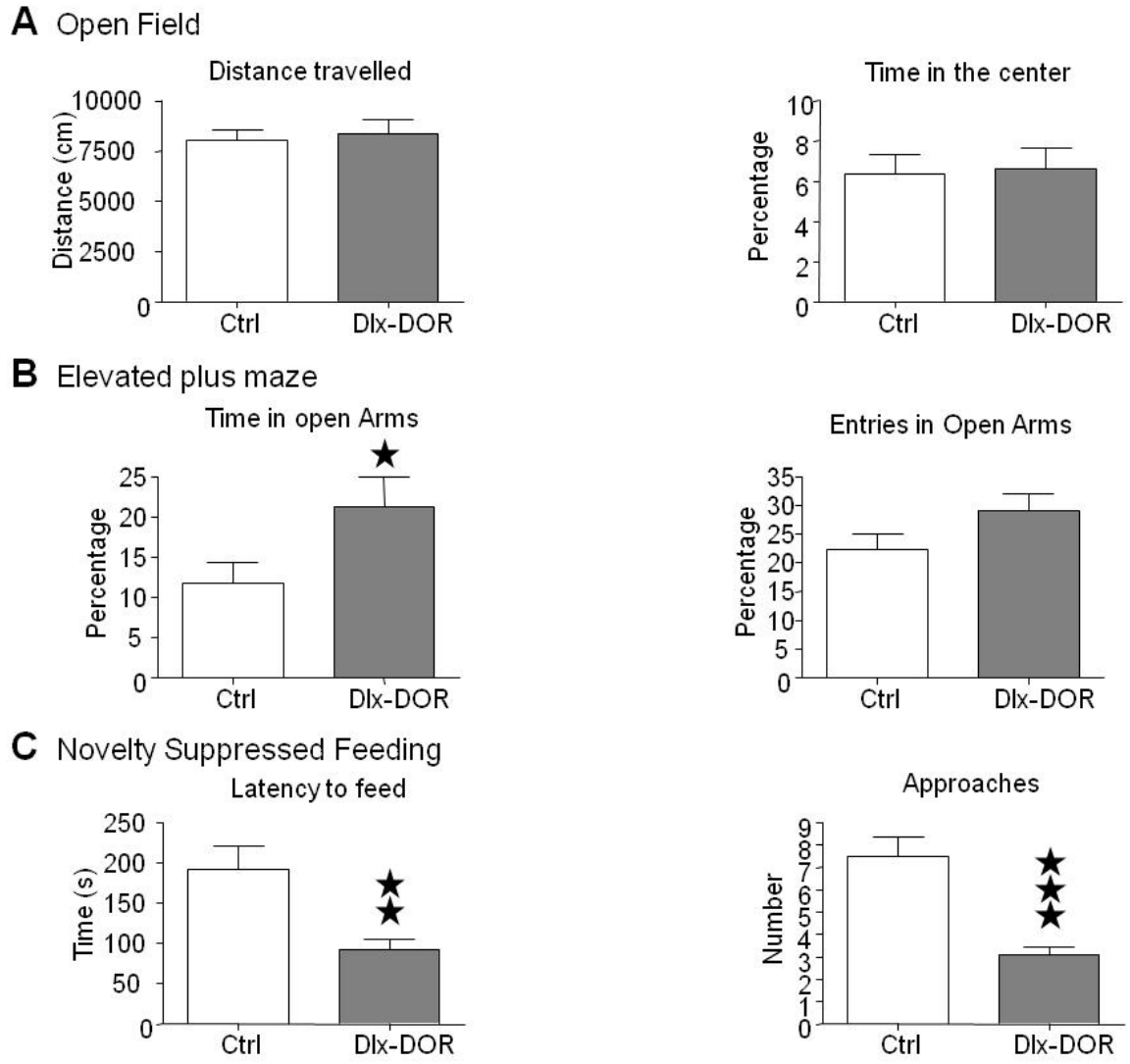



Figure 3 : Analyse des comportements de type anxieux. Les trois paradigmes sont classiquement utilisés pour évaluer les comportements de type anxieux (effectif n=16 par génotype). (A) Open Field (Test du champ ouvert). La distance parcourue (index de l'activité locomotrice) ainsi que le temps passé au centre (index d'anxiété) sont similaires entre les deux génotypes. (B) Elevated plus-maze (Test du labyrinthe en croix sur-élevé). Les souris mutantes Dlx-DOR présentent une augmentation statistiquement significative du temps passé ainsi qu'une tendance à entrer plus souvent dans les bras ouvert, en comparaison des souris contrôles Ctrl. Dans ce paradigme, les deux génotypes présentent des niveaux d'activité similaires (données non présentées). (C) Novelty suppressed feeding (Test de suppression de la prise alimentaire induite par la nouveauté). Les souris mutantes Dlx-DOR montrent une réduction significative du temps requis pour rechercher la nourriture et en conséquence un nombre d'approches également réduit en comparaison des souris contrôles Ctrl. Pour les trois tests, les analyses statistiques sont réalisées par Student *t*-test (une étoile, $p<0.05$ ; deux étoiles, $p<0.01$ ; trois étoiles, $p<0.001$).

Afin de compléter l'analyse comportementale des souris mutantes Dlx-DOR déficiente pour DOR dans les neurones GABAergiques du cerveau antérieur, nous avons réalisé une étude de l'activité neuronale en mesurant l'expression du gène

précoce c-fos par immunohistochimie dans différentes régions cérébrales après le test de suppression de la prise alimentaire induite par la nouveauté (Figure 4). Pour ce faire, les cerveaux de souris contrôle et Dlx-DOR ont été prélevées 90min après la fin du test comportemental, correspondant au pic d'expression de la protéine c-fos. Nous observons une diminution significative du nombre de neurones exprimant la protéine c-fos dans des régions impliquées dans la régulation des émotions, les noyaux basolateraux et centraux de l'amygdale, chez les souris Dlx-DOR en comparaison de sourios contrôles Ctrl. Par ailleurs, nos résultats démontrent une augmentation nombre de neurones c-fos positifs au niveau du noyau accumbens, décrit comme étant impliqué dans le contrôle des processus de récompense.

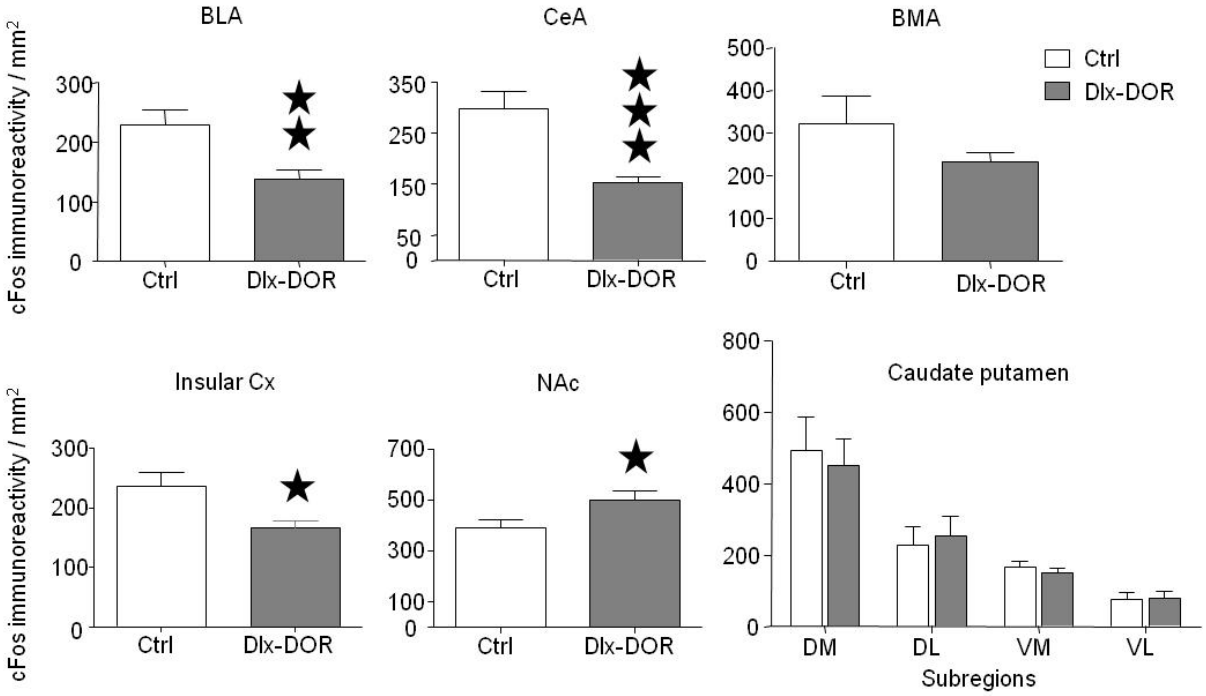



Figure 4 : Activité neuronale mesurée par détection de la protéine c-fos par immunohistochimie après le test de suppression de la prise alimentaire induite par la nouveauté. L'activité neuronale est quantifiée dans 6 régions cérébrales (BLA, amygdale basolatérale ; CeA, amygdale centrale ; BMA, amygdale basomédial ; Insular Cx, cortex insulaire ; NAc, noyau accumbens ; Caudate-putamen, noyau caudé-putamen) et exprimée en nombre de cellules c-fos positive par $mm^2$. Une diminution significative du nombre de cellules c-fos positive est observée au niveau du cortex insulaire, de la BLA et la CeA chez les souris Dlx-DOR en comparaison des souris contrôles Ctrl. Inversement, l'immunoréactivité c-fos est significativement augmentée dans le noyau accumbens des souris mutantes par rapport aux contrôles. Les niveaux d'activité neuronale sont similaires entre les deux génotypes au niveau de la BMA et du noyau caudé-putamen. Effectif n=6-9 souris par génotype / 4-12 sections par régions / 2 valeurs par section. Les analyses statistiques sont réalisées par Student *t*-test (une étoile, $p<0.05$ ; deux étoiles, $p<0.01$ ; trois étoiles, $p<0.001$).

En résumé, l'analyse comportementale de la lignée Dlx-DOR montre que les DORs exprimés sur des neurones inhibiteurs du cerveau antérieur augmentent les niveaux d'anxiété et régulent l'activation motrice au niveau de circuits neuronaux impliquant essentiellement le striatum et les bulbes olfactifs (manuscript en préparation).

La troisième partie de mon projet était d'évaluer la contribution de DOR dans les réponses épileptiques. Les études précédentes ont montré que l'agoniste non-peptidique delta-spécifique SNC80 présentent des propriétés convulsivantes (Jutkiewicz et al., 2006, *J. Pharmacol. Exp. Ther .* ; Jutkiewicz et al., 2005, *Psychopharmacology*). Deux autres agonistes développés plus récemment, ARM390 et ADL5859, n'induisent aucun effet pro-épileptique observable au niveau comportemental (Le Bourdonnec, et al., 2009, *J. Med. Chem.* ; Le Bourdonnec et al., 2008, *J. Med. Chem.*). Nous avons mesuré les effets du SNC80 et de l'ARM390 par des enregistrements électroencéphalographiques (EEG) et une observation comportementale chez les animaux Dlx-DOR, KO et contrôle (Figure 5).

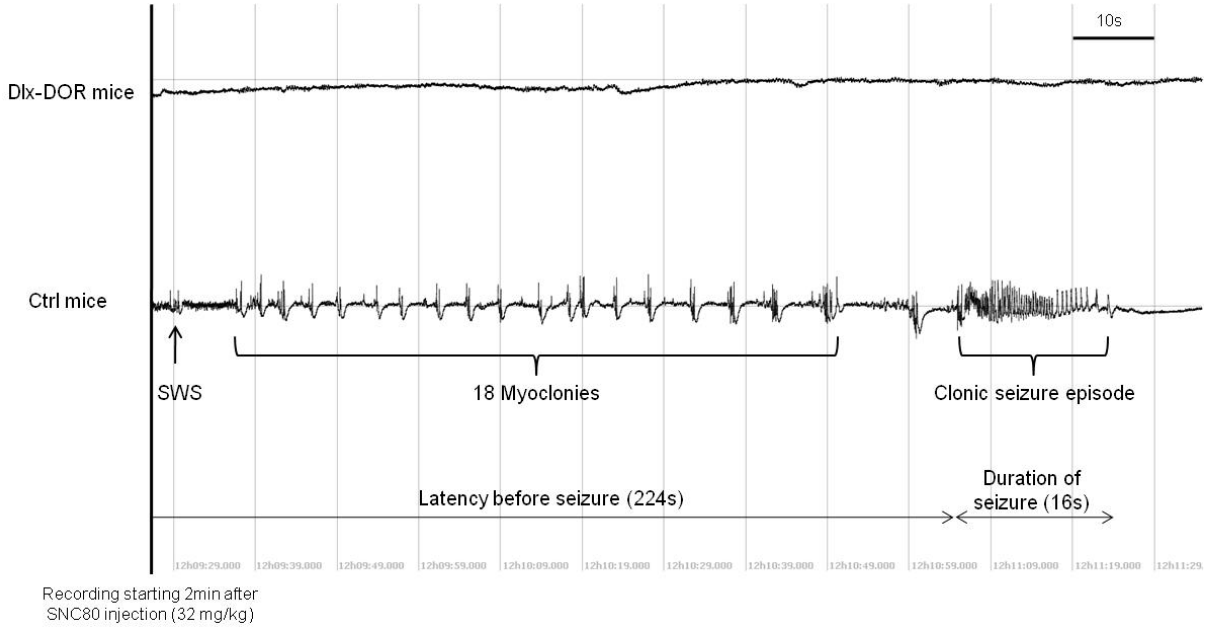



Figure 5 : Exemples d'enregistrements électroencéphalogrammes obtenus après administration de l'agoniste SNC80 chez des souris mutantes Dlx-DOR (tracé supérieur) ou contrôles Ctrl (tracé inférieur). Les décharges de pointes ondes (SWS), crises myocloniques et crises cloniques sont mesurées. Aucun de ces évènements n'est observé chez les souris mutantes.

Nos résultats montrent que les effets pro-épileptiques du SNC80 sont abolis chez les animaux KO et Dlx-DOR. Ces données indiquent que les DORs exprimés sur les neurones GABAergiques du cerveau antérieur sont nécessaires pour les effets épileptogènes de ce ligand. Nous avons également confirmé que l'ARM390 ne provoque aucune perturbation des profils EEG (manuscrit en préparation) (Figure 6).

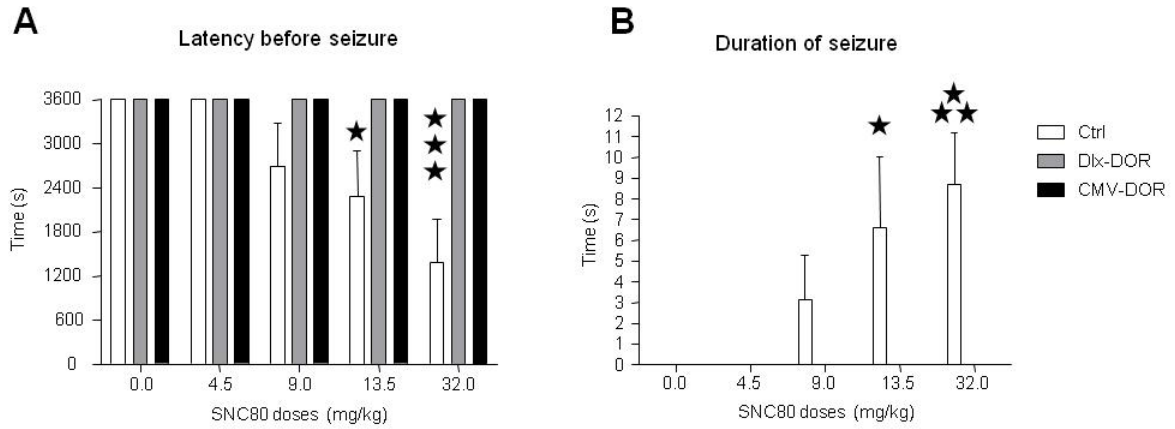



Figure 6 : Crise épileptique induite par le SNC80. (A) Temps de latence avant la première crise clonic et (B) durée de la crise épileptique. Au plus fortes doses (9, 13.5 et 32 mg/kg) l'administration de SNC80 provoque une diminution significative et dose-dépendante de la latence d'apparition de la première crise ainsi qu'une augmentation significative de la durée des crises épileptiques chez les souris contrôles Ctrl (barres blanches), mais ne provoque aucun changement chez les souris Dlx-DOR (barres grises) et les souris knockout constitutifs CMV-DOR (barres noires). Effectif n=8 par génotype. Les analyses statistiques sont réalisées par une analyse de la variance ANOVA à deux facteurs (génotype et traitement) suivi par une analyse *post hoc* Bonferroni/Dunn (une étoile, $p<0.05$ ; deux étoiles, $p<0.01$ ; trois étoiles, $p<0.001$).

La dernière partie de mon projet était de compléter l'analyse des mécanismes neuronaux responsables de l'activité anxiolytique/anxiogénique de DOR en ciblant l'amygdale. Cette région a reçu beaucoup d'attention lors des dernières décennies, en particulier pour son implication dans le conditionnement de peur et le contrôle de l'anxiété (LeDoux, 2000, *Annu Rev Neurosci* ; Tye et al., 2011, *Nature*). Les DORs sont très fortement exprimés au niveau du noyau basolatéral de l'amygdale (BLA) (Kitchen et al., 1997, *Brain Res.* ; Scherrer et al., 2006, *PNAS*) et notons que ces récepteurs sont intacts dans la souris Dlx-DOR analysée dans les projets précédents.

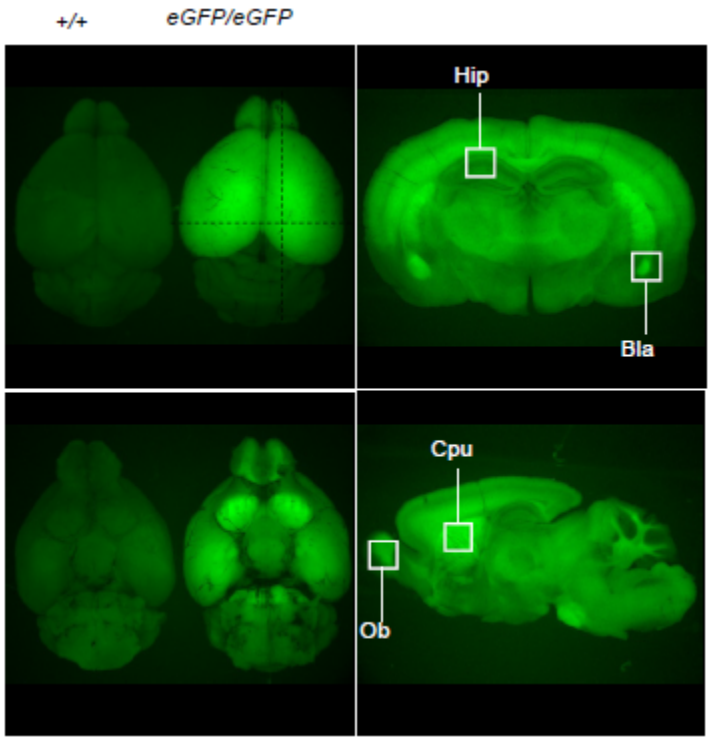



Figure 7 : Souris knock-in exprimant DOR en fusion avec la GFP (green fluorescent protein) (Scherrer et al., 2006, *PNAS*). On observe une expression de DOR-eGFP similaire à l'expression du récepteur DOR natif endogène. DOR-eGFP est fortement exprimé au niveau de l'hippocampe (Hip), de l'amygdale basolatéral (BLA), du bulbe olfactif (OB) et des noyaux caudé-putamen (CPu).

Nous souhaitons tester l'hypothèse que, contrairement aux DORs du striatum et du bulbe olfactif qui sont anxiogènes, les DORs de la BLA présentent une forte activité anxiolytique. Celle-ci serait responsable de l'effet généralement anxiolytique produit par les agonistes deltas lorsqu'ils sont administrés en systémique. L'analyse d'une souris knock-in DOR-eGFP (Scherrer et al., 2006, *PNAS*) nous indique que les DORs de la BLA sont essentiellement localisés au niveau pré-synaptique et exprimés par de neurones afférents. Afin de supprimer génétiquement les récepteurs DORs de la BLA, nous avons entrepris des expériences de traçage rétrograde chez ces souris afin d'identifier l'origine neuronal des DORs de la BLA (Figure 8).

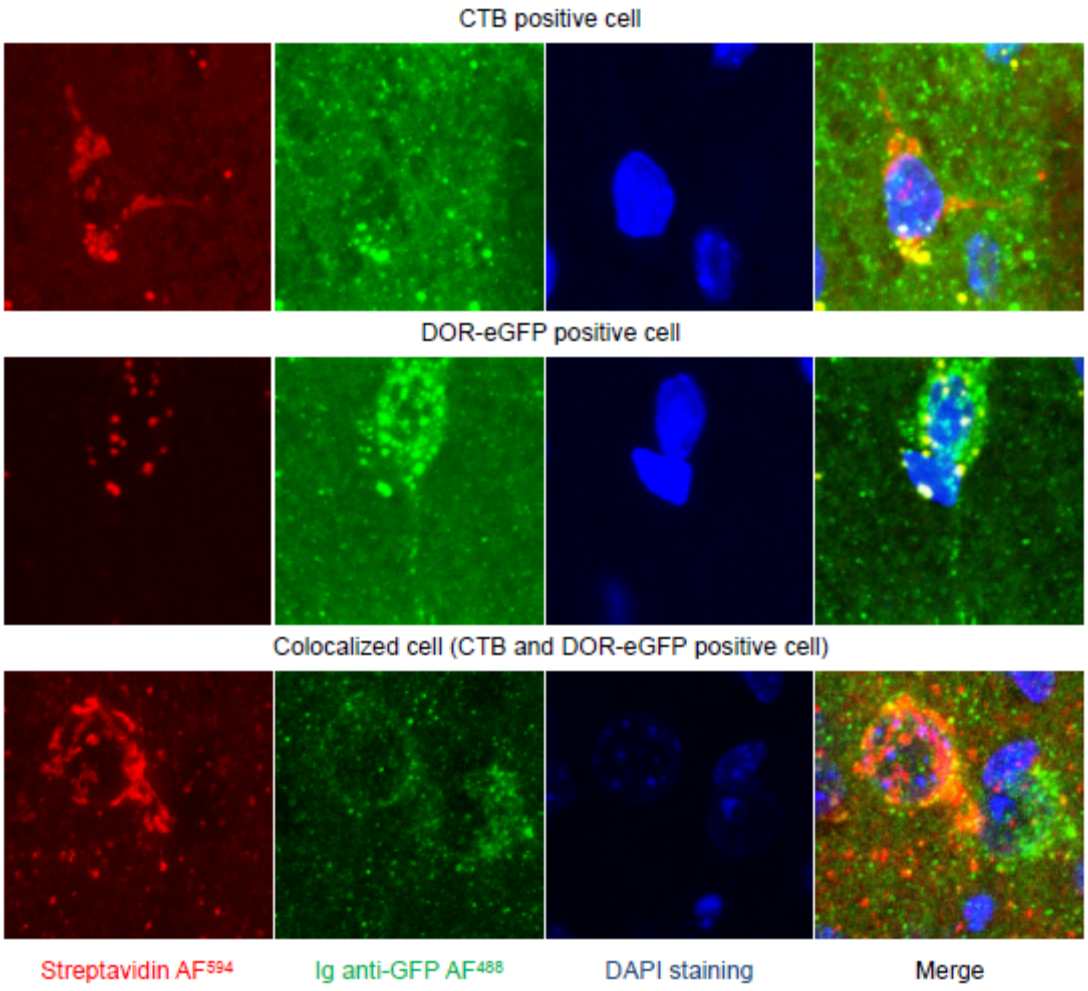



Figure 8 : Images acquises au microscope confocale au niveau du cortex insulaire. Le traceur rétrograde CTB (Cholera Toxin sous-unité B) est révélé par immunohistochimie en utilisant la streptavidin couplé à un Alexa Fluor$^{594}$. Le signal de la DOR-eGFP est amplifié en utilisant un anticorps anti-GFP couplé à un Alexa Fluor$^{488}$. Le marquage au DAPI permet de révéler les noyaux. Le panneau supérieur correspond à un neurone marqué par le traceur rétrograde et qui n'exprime pas la DOR-eGFP. Le panneau central correspond à un neurone exprimant la DOR-eGFP et CTB négatif. Le panneau inférieur représente un neurone dans lequel les signaux DOR-eGFP et CTB colocalisent.

Nos résultats préliminaires indiquent que les projections axonales prédominantes exprimant le récepteur delta proviennent essentiellement des cortex insulaire, piriforme et endopiriforme, qui seront ciblés génétiquement dans des expériences ultérieures (Figure 9).

| Structures | DOR-eGFP positive neurons | CTB positive neurons | Colocalization | |
|---|---|---|---|---|
| | | | % neurons DOR-eGFP retrogradely labeled | % neurons CTB positive expressing DOR-eGFP |
| Olfactory bulb | +++ | ++ | 0% | 0% |
| Prelimbic cortex | + | + | 0% | 0% |
| Cingulate cortex | + | + | 0% | 0% |
| Paraventricular nucleus of the thalamus | 0 | + | 0% | 0% |
| Insular cortex | ++++ | +++++ | > 25% | > 25% |
| Dorsal endopiriform cortex | ++ | ++ | > 25% (to be confirmed) | > 25% (to be confirmed) |
| Piriform cortex | + | ++ | > 25% (to be confirmed) | > 25% (to be confirmed) |
| Controlateral BLA | +++ | ++ | 0% | 0% |

| *Estimated cell bodies* | |
|---|---|
| +++++ | : ~ 200 à 400 neurons / mm² |
| ++++ | : ~ 100 à 200 neurons / mm² |
| +++ | : ~ 50 à 100 neurons / mm² |
| ++ | : ~ 20 à 50 neurons / mm² |
| + | : ~ 1 à 20 neurons / mm² |
| 0 | : 0 neuron / mm² |
| *Colocalization* | |
| 100% | : total colocalization |
| > 75% | |
| 25-75% | |
| < 25% | |
| 0% | : no colocalisation detected |

Figure 9 : Tableau semi-quantitatif pour l'expression de la DOR-eGFP et le marquage rétrograde au CTB dans différentes régions cérébrales. Les souris knockin DOR-eGFP ont été traitées au SNC80 (10 mg/kg, sous-cutanée) 20min avant le sacrifice pour faciliter la visualisation des neurones DOR-eGFP positifs. On observe des neurones qui exprime les deux marquages colocalisés au niveau du cortex insulaire principalement et dans une moindre mesure au niveau des cortex piriforme et endopiriforme. Effectif n=4 souris / 5-20 sections par souris / 1 mesure par section.

La quantification des différents marquages au sein du cortex insulaire montre une moyenne de 243.03 cellules marquées par le traceur rétrograde, 67.07 exprimant le récepteur DOR-eGFP et 17.36 qui colocalisent les deux marquages, par $mm^2$. Nous observons que 6.67% des cellules marquées par le traceur rétrograde exprime également le récepteur DOR-eGFP. Par ailleurs, 20.56% des cellules exprimant la DOR-eGFP sont marquées par le traceur rétrograde et projette donc sur l'amygdale basolatérale (Figure 10).

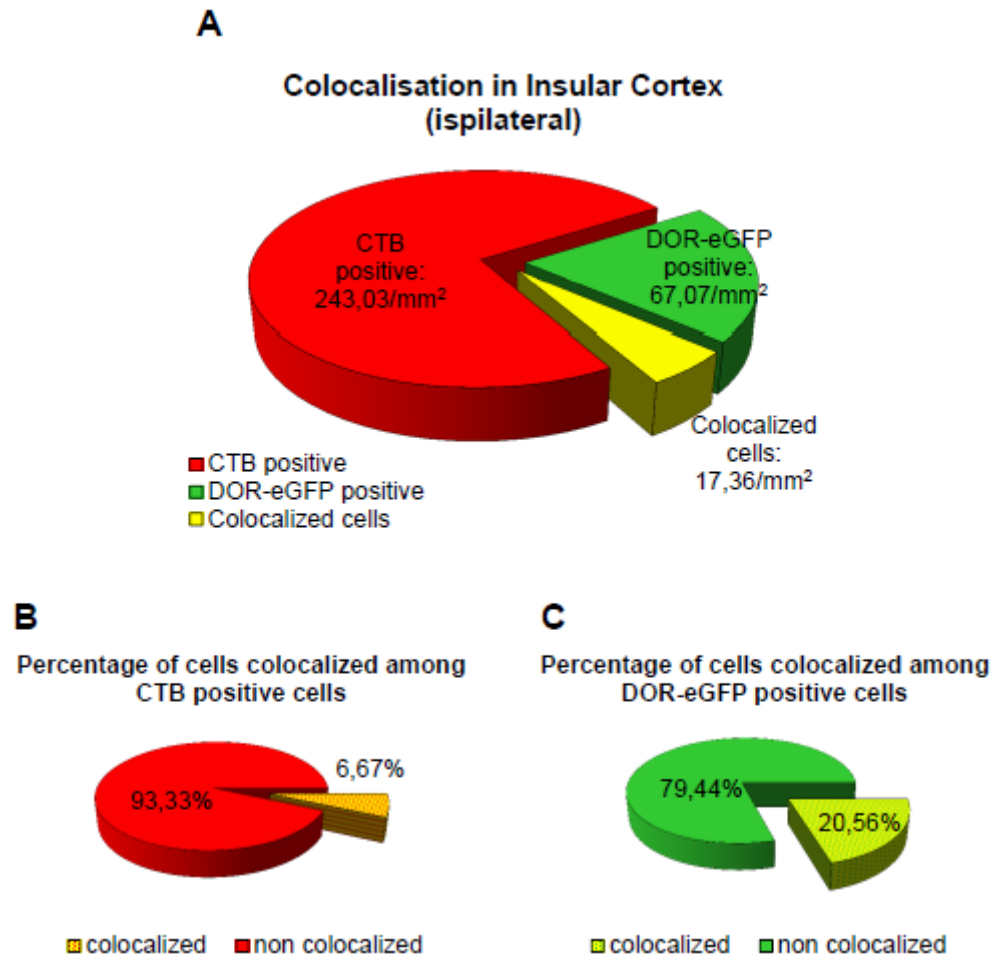



Figure 10 : Quantification de l'expression du récepteur DOR-eGFP et du marquage rétrograde CTB au sein du cortex insulaire. (A) Nombre de cellules positive pour le traceur rétrograde CTB (rouge), la DOR-eGFP (vert) ou qui colocalise les deux marquages (jaune), par $mm^2$. (B) Pourcentage de cellules marqué par le traceur rétrograde et qui exprime ou non la DOR-eGFP. (C) Pourcentage de cellules qui exprime la DOR-eGFP et marqué ou non par le traceur rétrograde. Effectif n=4 souris / 5-20 sections par souris / 1 mesure par section.

En conclusion, ces projets ont contribué à affiner les connaissances actuelles de la fonction de DOR en identifiant le rôle spécifique des récepteurs exprimés dans le cerveau antérieur. Ils mettent à jour de nouveaux mécanismes neuronaux de régulation des réponses émotionnelles qui pourraient avoir des retombées intéressantes dans le traitement des troubles anxieux chez l'homme.

# Abstract

Delta opioid receptors (DORs) are G-protein coupled receptors belonging to the opioid system, which play a central role in chronic pain and emotional responses. DORs are strongly expressed in olfactory bulb, cortex, striatum, basolateral nucleus of the amygdala and pons nuclei. Using constitutive gene knockout, we have previously demonstrated the role of DORs in reducing chronic pain (Gaveriaux-Ruff, Nozaki et al. 2011), anxiety-related behaviors and impulsivity(Olmstead, Ouagazzal et al. 2009), regulating locomotor activity (Filliol, Ghozland et al. 2000) and facilitating context learning (Le Merrer, Faget et al. 2012; Le Merrer, Rezai et al. 2013), Although these functions are well-established, neuronal networks and mechanisms underlying DOR-regulated behaviors remain poorly understood. The aim of this thesis work was to identify neuronal populations and brain circuits that support DOR functions.

Recent evidence showed that DOR is highly expressed in GABAergic neurons, at least in olfactory bulb, striatum (Scherrer, Tryoen-Toth et al. 2006) and hippocampus (Erbs, Faget et al. 2012; Rezai, Faget et al. 2012). We therefore developed a conditional knockout mouse line (Dlx-DOR) to produce a specific deletion of DOR in GABAergic neurons of the forebrain. These mice were obtained upon breeding floxed delta receptor gene (*Oprd1*$^{fl/fl}$) with a driver transgenic Dlx-5/6-Cre line (Monory, Massa et al. 2006).

**In the first part of this work**, we determined brain distribution of delta receptors in Dlx-DOR at mRNA level by RT-qPCR, and protein level by quantitative autoradiography as well as ligand-induced [$^{35}$S]GTPγS binding experiments. We observed a dramatic reduction of receptor expression in olfactory bulb and striatum of Dlx-DOR mice, both at mRNA and protein levels. DOR deletion was partial in cortex and hippocampus, and no change was detected in amygdala, pons nuclei and spinal cord. The behavioural analysis revealed that conditional mutants show no locomotor response upon SNC80 administration and facilitated D1R-mediated locomotor stimulation. In addition, mutant mice displayed lower levels of anxiety and high risk-taking behaviour under stressful conditions, which contrast with higher anxiety levels in constitutive mutants. Our data therefore demonstrate that DORs expressed in forebrain GABAergic neurons exert a significant control on animal motor activity and reveal an unexpected function for this particular receptor population with potential protective effects under threat. This study is presented in a first manuscript: “Delta opioid receptors expressed in forebrain GABAergic neurons increase anxiety and limit risk-taking behaviour.” Chu Sin Chung P., Keyworth H.L., Bailey A., Befort K., Filliol D., Matifas A., Gavériaux-Ruff C., Kitchen I. and Kieffer B.L.

**In the second part,** we tested whether DORs expressed in forebrain GABAergic neurons mediate the well-known epileptogenic effect of SNC80, the prototypal DOR agonist. We showed, indeed, that epileptic events detected on electroencephalogram recordings upon SNC80 administration are abolished in Dlx-DOR mice. This study is presented in a second manuscript: “Delta opioid receptors expressed in forebrain GABAergic neurons are responsible for SNC80-induced seizures.” Chu Sin Chung P., Boehrer A., Stephan A., Befort K., Matifas A., Kieffer B.L.

Motor learning skills and memory performances were further explored in our conditional mutant line and are also presented in this part (Supplementary experiments). Preliminary results suggest that DOR expressed in forebrain GABAergic neurons are not involved in these processes.

**In the third part,** we initiated a project focused on the role of DORs detected at the of basolateral amygdala (BLA), where the receptors are most densely expressed. We first knocked-down DOR using a viral technology and our preliminary results showed no evidence for an involvement of locally synthesized DORs in emotional responses. We then developed retrograde tracing experiments to identify the cellular origin of presynaptic DORs localized in BLA afferent terminals. This study revealed strong DOR expression in an insular cortex-BLA pathway, representing a potential site for DOR-mediate emotional responses and mood control.