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Laura-Joy BOULOS
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Mu opioid receptors in the habenula: Dissecting reward and aversion in addiction

Supervisor: Dr. Brigitte KIEFFER (McGill)
Co-Supervisor: Dr. Claire GAVÉRIAUX-RUFF (University of Strasbourg)

Members of the Jury:
Dr. Jean-Christophe CASSEL (University of Strasbourg)
Dr. Catherine LE MOINE (University of Bordeaux)
Dr. Marco LEYTON (McGill)

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“No matter how thoroughly you understood the physics of feathers,
you wouldn’t have been able to predict a murmuration of starlings without first seeing it happen.
So it is with the brain: you have to study the behavior first.”

John Krakauer rephrasing David Marr

Content

General introduction

General introduction

I- The opioid system

1. Overview

The opioid system is composed of three opioid receptors –mu (Oprm1), delta (Oprd1) and kappa (Oprk1) (Pert, Pasternak et al. 1973, Simon 1973)- and the three opioid peptide families –enkephalins, dynorphins and β-endorphin- that act on these receptors with more or less affinity. The opioid peptides share a common amino-terminal sequence Tyr-Gly-Gly-Phe called the opioid motif (Akil, Owens et al. 1998) and the opioid receptors are G-Coupled Protein Receptors (GPCR) with 7 transmembrane domains critical for ligand binding and receptor signaling (Befort, Tabbara et al. 1996). The crystal structure of the three receptors was only discovered and characterized recently, revealing a message/address model that describes conserved elements of ligand recognition as well as structural features associated with ligand-subtype selectivity (Granier, Manglik et al. 2012, Manglik, Kruse et al. 2012, Wu, Wacker et al. 2012).

2. Anatomical distribution

While ligand autoradiography studies have demonstrated that all three opioid receptors are broadly expressed throughout the brain (Kitchen, Slowe et al. 1997), in situ hybridization techniques has shown that mRNA expression generally matches the receptors protein distribution, suggesting that a majority of opioid containing neurons are local. Some brain regions however express mRNA but not the protein, suggesting that presynaptic receptors can also be transported to projection structures (Mansour, Fox et al. 1994). Detailed distribution is available in **Figure 1A**. Furthermore, the distribution of opioid peptide immunoreactivity is also broadly visualized throughout the brain (Charbogne, Kieffer et al. 2014) but there is an important mismatch between peptide immunoreactivity and cell body localization, suggesting that a substantial portion of peptides are released by projection neurons (Le Merrer, Becker et al. 2009). This has been reviewed by Kieffer and Evans, 2009 (Kieffer and Evans 2009).

Interestingly and beyond their overlap, there are some disparities across the expression of the three opioid receptors and mu opioid receptors (MORs) stands out as the most broadly and abundantly expressed in mesocorticolimbic structures as well as in habenular

pathways (Kitchen, Slowe et al. 1997, Le Merrer, Becker et al. 2009, Gardon, Faget et al. 2014, Erbs, Faget et al. 2015) (**Figure 1B**), two critical circuitries in reward, aversion and addiction. We will thus focus on mu opioid receptors. For functional implication of other opioid receptors as well as opioid peptides, see recent reviews of the literature (Lutz and Kieffer 2013, Lutz and Kieffer 2013, Bodnar 2017).

II- Mu opioid receptors

1. Pharmacology

Mu opioid receptors (MORs) have numerous ligands, some of which are endogenous peptides whereas others are synthetized for therapeutic, euphoric or research purposes. Morphine is the prototypic mu agonist used in the clinic of pain for its major analgesic properties (Spetea, Asim et al. 2013). It is metabolized into three active metabolites: morphine-3-glucuronide, morphine-6-glucuronide and nor-morphine(Pergolizzi, Boger et al. 2008). Morphine however possesses strong addictive effects that chemists and more recently neuroscientists have been trying to circumvent with substitutive opiate molecules. Results are generally not successful as heroin has even stronger addictive effects, codeine can only be used for mild pain and oxycodone, a semi-synthetic opioid drug in which the clinic had initially put a lot of hope, has triggered a new wave of opioid crisis in the USA and everywhere around the world during the less decade (Raffa and Pergolizzi 2010, Raffa, Pergolizzi et al. 2010). MORs have nonetheless become a target of choice in the treatment of addiction. Partial agonists such as buprenorphine are used alone (Subutex®) or in combination with a MORs antagonist such as naloxone (Suboxone®) in the maintenance therapy of addiction (Modesto-Lowe, Brooks et al. 2010, Li, Shorter et al. 2014) and antagonist such as naloxone are even starting to be used alone to treat opioid overdoses (Wermeling 2015) as naloxone nasal kits become legal and their public access expand in many states and countries.

2. Cellular mechanisms

MORs are preferentially inhibitory G-Coupled Protein Receptors. Their cellular mechanism is illustrated in **Figure 2**. Their activation by acute application of agonists activates and dissociates the α-G protein subunit through GDP-GTP exchange, which in turn inhibits adenylyl cyclase that decreases cAMP levels and modulate voltage-dependent current to decrease in fine neuronal excitability. MORs activation also leads to the activation/dissociation of βγ-G protein subunits which in turn activate potassium channels and particularly G protein-activated inwardly rectifying potassium channels (GIRKs), thus contributing to the hyperpolarization of the neuron they are located on (Williams, Christie et al. 2001). Other signaling pathways have also been described, including mitogen-activate protein kinase (MAPK), the activation of which enhances phosphorylation of transcription factors such as CREB, ERK and c-fos (Haghparast, Taslimi et al. 2011). Therefore activation of MORs first decreases neurotransmitter release and cell excitability then modifies gene expression for long term adaptations. This also means that the general effect of MORs depends on the neurons on which they are located (inhibition of an inhibitory/excitatory transmission) and subsequently, on the brain region in which they are expressed (Fields and Margolis 2015).

3. Anatomical distribution

Mu opioid receptors are expressed throughout the brain with more or less density depending on brain structures (Kitchen, Slowe et al. 1997, Erbs, Faget et al. 2015). They are particularly strongly expressed in the dopaminergic mesocorticolimbic circuitry and their location in this pathway has been well characterized (Nieh, Kim et al. 2013). Importantly, a study has recently revealed that MORs are most highly expressed in the habenula (Gardon, Faget et al. 2014), a central epithalamic small brain structure that is gaining increased interest in neuroscientific fields to which MORs strongly contribute (Boulos, Darcq et al. 2017). The role of habenular MORs has not been studied yet.

3.1. In the mesocorticolimbic circuitry

The mesocorticolimbic circuitry is mainly composed of neurons projecting from the midbrain ventral tegmental area (VTA) to forebrain regions such as the nucleus

accumbens (NAc), the amygdala (Amy) and the prefrontal cortex (PFC) (Meye and Adan 2014). MORs are mostly expressed on GABAergic neurons in these structures (Jaferi and Pickel 2009, Watabe-Uchida, Zhu et al. 2012, Kudo, Konno et al. 2014). They are specifically expressed on striosome medium spiny neurons in the NAc (Cui, Ostlund et al. 2014), in somatodendritic sites of the central nucleus of the amygdala and the bed nucleus of the stria terminalis in the Amy and in dendrites, axons and terminals of mainly GABAergic but also glutamatergic neurons of the VTA. Detailed distribution is available in **Figure 3A**. Their functions have been explored pharmacologically and, more recently, genetically (Charbogne, Gardon et al. 2017).

3.2. In the habenula

Despite its small size, the habenula can be divided into two sub-structures, the lateral and the medial habenula. MORs are mainly expressed in the medial habenula (MHb) and the characterization of a MOR-mcherry knock-in mouse line from our lab reveals that MORs are more specifically present in cell bodies from the basolateral and apical parts of the MHb where the receptor seems to colocalize with both cholinergic and substance P neurons (Gardon, Faget et al. 2014). Additionally, visualization of fluorescent signals in the knock-in mouse line suggests that MORs are also expressed in the lateral and rostral parts of the interpeduncular nucleus, the main brain structure to which the MHb massively projects, as well as in the fasciculus retroflexus, the white matter bundle through which the MHb projects to the IPN. Detailed distribution is available in **Figure 3B**. The remarkably high MOR density mainly in the MHb and associated pathway strongly suggests a physiological importance to this population of receptors.

4. Physiological functions

Consistent with their wide expression throughout the central and peripheral systems, MOR physiological functions are multiple and diverse. These functions have been revealed with numerous approaches ranging from pharmacological targeting to genetic engineering. They include but are not limited to autonomic, endocrinal and immune functions (Bodnar 2016), pain responses and analgesia (Matthes, Maldonado et al. 1996, Weibel, Reiss et al. 2013), physical withdrawal (Corder, Doolen et al. 2013), negative

affect and mood (Lutz and Kieffer 2013), natural (Pecina and Smith 2010) and drug reward (Matthes, Maldonado et al. 1996, Charbogne, Kieffer et al. 2014). Studies to date have emphasized on the analgesic (Pasternak and Pan 2013) and rewarding (Fields and Margolis 2015) properties of MORs and we put our initial focus on the latter.

MORs are essential for attributing a positive value to natural rewards. Activation of MORs enhances both hedonic properties of food and food motivation in mice (Pecina and Smith 2010), whereas antagonism of MORs decreases frequency and severity of binge eating in humans (Nathan and Bullmore 2009). There is also evidence that MORs contribute to social reward (Moles, Kieffer et al. 2004) and sexual behavior (Coolen, Fitzgerald et al. 2004). Beyond this role in natural rewards, MORs are responsible for the rewarding effects of morphine (Matthes, Maldonado et al. 1996), other opiates (Contarino, Picetti et al. 2002) and other non-opioid drugs of abuse including nicotine (Berrendero, Kieffer et al. 2002), cocaine (Becker, Grecksch et al. 2002) and alcohol (Roberts, McDonald et al. 2000, Ben Hamida, Boulos et al. 2017).

In the context of our work herein presented, we focus on the crucial role of these receptors in reward processing as well as their potential contribution to aversive states and how both components can lead to addiction. Physiological roles of MORs that we address in each part of our work will be described in the introduction of the associated chapter.

III- Reward and aversion in addiction

Reward is a universal human experience (infatuation, craving chocolate, feeling euphoric) that greatly impacts our decision-making processes (choosing a partner, buying a car, cooking diner). Repeated overstimulation of the reward system dysregulates neurochemical circuits that underpin the system (dopamine, opioids) and recruits brain stress systems responsible of an aversive state. Reward dysfunction can thus lead to reward-related psychiatric disorders such mood disorders and addiction, as drug taking becomes compulsive-like and the factors that motivate behavior shift from positive reinforcement (/reward) to negative reinforcement (/aversion). How reward and aversion

processes emerge from neuronal brain activity is an incredibly captivating question the answer to which still needs to be refined.

1. Reward and positive reinforcement

Reward is classically measured with approach behavior that is thought to illustrate a positive/salient state. Although reward is often confused with positive reinforcement, it can have three different meanings. First, it can be used to describe stimuli with appetitive (desirable) consequences. Second, it can refer to a learning situation in which a given response leads to an appetitive stimulus; this is the closest definition to positive reinforcement and it can be defined in opposition to punishment. Third, reward is also referred to as an internal pleasurable or hedonic state (Everitt and Robbins 2005). Positive reinforcement on the other hand is a broader construct defined as the process by which the presentation of a stimulus increases the probability of a response (Hyman 2005) = acting to obtain something.

2. Aversion and negative reinforcement

Aversion is classically measured with avoidance behavior that is thought to illustrate a negative somatic and/or affective state. Aversive processes are responses to the aversive states in which a subject can be due to aversive properties of a given stimulus or context. There is an interesting literature on aversive properties of drugs of abuse in rodents (Davis, de Brugada et al. 2010, Davis and Riley 2010) and humans (Jones, Hall et al. 2010, Verendeev and Riley 2011, Verendeev and Riley 2012, Gore-Langton, Flax et al. 2015), all of which converge to say that these properties are not fixed or static but rather a function of different variables. This is particularly salient because, at a different dose and in different circumstances, a same stimulus can act as a positive or a negative reinforcement. Negative reinforcement is thus defined as the process by which the removal of an aversive stimulus or state increases the probability of a response (Sanchis-Segura and Spanagel 2006) = acting to avoid something.

3. Reward and aversion in addiction

Addiction is a chronic, relapsing disease of the brain that includes compulsive drug-seeking and consumption and the emergence of negative emotional states when access

to the drug is prevented (Koob and Le Moal 1997, Koob 2017). Major addiction theories thus converge to say that this brain disorder can be conceptualized as a three-stage cycle of intoxication, withdrawal and preoccupation/anticipation (Koob and Le Moal 1997). Positive (reward) and negative (aversion) reinforcements are hypothesized to contribute to the compulsion of drug seeking with a switch from the former to the latter over time. This negative affective state and the subsequent learning to consume a drug in order to avoid the negativity is one of the main distinction between addiction and recreational drug use (Koob and Le Moal 2005). Hence both reward and aversion processing occupy critical positions in the development, maintenance and relapse of addiction. This model is illustrated in **Figure 4A**.

Other major theories in addiction argue that positive and negative reinforcement are not sufficient to account for the compulsive behavioural patterns observed in drug seeking and drug taking (Robinson and Berridge 2000). They further stipulate that critical neuroadaptations render the brain reward systems hypersensitive to drugs and associated stimuli (Robinson and Berridge 2001) and that sensitized brain systems do not mediate the pleasurable effects of drugs but instead mediate a subcomponent of reward termed “incentive salience” (Robinson and Berridge 2008). This model is illustrated in **Figure 4B and C**.

While both psychobiological theories are relevant in their own way, we argue that incentive salience can happen in parallel to aversion-enhanced compulsivity. They both spring from overstimulation of the reward system and they both lead to addiction.

4. Circuitry underlying addiction

While they probably operate simultaneously, different components of addiction could either be mediated by similar or by distinct brain circuitry. Dopamine (DA) and opioids are the most widely explored and accepted underpinning mechanisms to drug addiction due to reward/motivation mechanisms in the mesocorticolimbic circuitry, with models stressing on the importance of dopamine mostly in motivation and incentive sensitization versus an importance of opioids in hedonia (Robinson and Berridge 2008, Lutz and Kieffer 2013, Fields and Margolis 2015, Chen, Nong et al. 2017) . To improve the

understanding of the role of DA and opioid receptors in reward pathways, specific brain regions, including the ventral tegmental area (VTA), nucleus accumbens (NAc) and, more recently, the habenula, are being explored as well as their associated networks. Interestingly, some of these brain structures seem to underpin aversive processes as well (Zweifel, Fadok et al. 2011, Lammel, Lim et al. 2012, Pignatelli and Bonci 2015). Given the fact that many stimuli offer a mix of both appetitive and aversive properties depending on the doses (Davis and Riley 2010), it is not surprising then that the neural circuitry responsible for the processing of aversive stimuli overlaps with brain regions that have been shown to govern reward processes (Doremus-Fitzwater and Spear 2016). Moreover, the identification of structures and pathways contributing to both reward and aversion could shed new light on the link between these two systems.

5. The link with cognition and self-control disorders

We defined addiction as a “loss of control despite negative consequences”. This definition signifies that cognitive control, a complex function that allows overriding of impulses in order to make decisions and take actions based on goals rather than habits, is strongly impaired in addiction (Jentsch, Ashenhurst et al. 2014, Jentsch and Pennington 2014). Indeed, if human beings can be motivated to obtain a reward they are often motivated, too, to avoid potential aversive consequences of drug consumption. These attempts to avoid drug seeking depend on effortful, voluntary inhibition of a certain behavior towards drugs and drug-related cues (Dalley and Robbins 2017), a function that is severely altered in addiction (Izquierdo and Jentsch 2012). Evidence points at both drug-induced alterations in molecular, cellular, circuit mechanisms that mediate cognitive control (Goldstein and Volkow 2002, Ersche, Roiser et al. 2008) and native inter-individual differences in inhibitory control of drug users (Sher, Bartholow et al. 2000, Tarter, Kirisci et al. 2003). At the intersection of risk factors and drug-induced alterations we find that poor inhibitory control is linked to low striatal dopamine D2 receptor availability and other associated dopaminergic impairments (Volkow 2004, Volkow, Fowler et al. 2004). Current research is now identifying mechanisms upstream and downstream of dopamine as well as the involvement of other neurotransmitter systems acting alone or in concert with dopamine on cognitive control. Namely, there has been a growing interest in

understanding the role of the habenula in reward processing and cognitive control (Baker, Jhou et al. 2016, Ortega, Solano et al. 2017). Specifically, genetic ablation of MHb neurons impairs inhibitory control and impulsive risky decision in mice (Kobayashi, Sano et al. 2013). Importantly, MORs constitutive knockout mice also showed relatively low inhibitory control (Olmstead, Ouagazzal et al. 2009). This means that MORs –and potentially, more specifically, MORs from the MHb- may potentially contribute to cognitive control.

An exploration of the role of MORs in reward-related cognitive functions in normal and maladaptive behavior will point to a deeper understanding of the initiation and maintenance of addiction and will further benefit emerging avenues of clinical research such as high comorbidity between addiction and attention deficit hyperactivity disorder (ADHD), which is in essence a disorder of self-control (Groman, James et al. 2009, Wilens, Adler et al. 2011).

IV- The habenula

1. Why this structure?

The habenula has gained scientific visibility after the discovery of its direct impact on reward prediction errors (RPE). RPE, a parameter that captures discrepancies between expectations and actual outcomes (Dagher 2017), is in fact just the word cognitive scientists use to refer to surprises. RPE include all at once reward, aversion and the gap between both, a gap that is bridged through cognitive reinforcement learning. RPE are thus central to addiction (Langdon, Sharpe et al. 2017), and so seem to be the habenula (Mathuru 2017). The following section reviews the literature on the habenula, a brain structure that has grown to be more complex than just RPE (Boulos, Darcq et al. 2017).

Review

Biological Psychiatry

Translating the Habenula—From Rodents to Humans

Laura-Joy Boulos, Emmanuel Darcq, and Brigitte Lina Kieffer

ABSTRACT

The habenula (Hb) is a central structure connecting forebrain to midbrain regions. This microstructure regulates monoaminergic systems, notably dopamine and serotonin, and integrates cognitive with emotional and sensory processing. Early preclinical data have described Hb as a brain nucleus activated in anticipation of aversive outcomes. Evidence has now accumulated to show that the Hb encodes both rewarding and aversive aspects of external stimuli, thus driving motivated behaviors and decision making. Human Hb research is still nascent but develops rapidly, alongside with the growth of neuroimaging and deep brain stimulation techniques. Not surprisingly, Hb dysfunction has been associated with psychiatric disorders, and studies in patients have established evidence for Hb involvement in major depression, addiction, and schizophrenia, as well as in pain and analgesia. Here, we summarize current knowledge from animal research and overview the existing human literature on anatomy and function of the Hb. We also discuss challenges and future directions in targeting this small brain structure in both rodents and humans. By combining animal data and human experimental studies, this review addresses the translational potential of preclinical Hb research.

Keywords: Addiction, Depression, Habenula, Human, Reward, Rodent

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The habenula (Hb) is a bilateral epithalamic structure, evolutionary conserved among vertebrates (1–3). This small brain nucleus is composed of two subdivisions—the medial (MHb) and the lateral Hb (LHb)—and has a central anatomic position in the brain, connecting the forebrain to the ventral midbrain and hindbrain (4,5). The Hb regulates midbrain monoaminergic systems, notably dopamine and serotonin, and integrates cognitive with emotional and sensory processing.

A key study in rhesus monkeys originally described the structure as a brain nucleus that is activated in anticipation of aversive outcomes, or failure to obtain reward, and in turn suppresses motor behavior (6). Hb function has since attracted increasing attention in both neuroscience and the clinic. Preclinical data have now accumulated to show that Hb encodes both rewarding and aversive aspects of external stimuli. The general view from animal research is that Hb activity prevents behaviors leading to negative reward such as punishment, while reinforcing behaviors with positive reward value (7), thus driving motivated behaviors and decision making (8). Consequent to this highly integrative function, Hb also contributes to learning and memory (9) and to a range of other behaviors (8,10). Not surprisingly, therefore, Hb dysfunction has been associated with psychiatric disorders, and studies in patients have established evidence for Hb involvement in major depression (11,12), addiction (11,13), and schizophrenia (14), as well as in pain and analgesia (10).

Although still limited, human Hb research is expected to develop rapidly in the next decade, and knowledge on Hb anatomy, connectivity, and function in nonhuman primates and rodents is increasing exponentially (15). Here, we briefly summarize current knowledge from animal research and extensively review the existing human literature on Hb structure and function. Focus is on psychiatric disorders, and a section on pain and analgesia is also proposed (Supplement). We also discuss the translational potential of preclinical research to understand Hb function in humans and for psychiatry.

ANATOMY

Rodents

Most knowledge on Hb connectivity, as well as structural characteristics and neurochemistry of Hb neurons, stems from studies in animals. In brief, retrograde and anterograde tracing studies in rodents (4,16) and electrophysiological studies in nonhuman primates (5) have provided a detailed description of afferent and efferent connections of the Hb complex, summarized in Figure 1. Because of their distinct input/output structures, the LHb and MHb seem to form parallel channels, regulating the information flow from forebrain to midbrain.

Electrophysiological and morphologic analyses of rat Hb slices show distinct intrinsic circuitries within the two nuclei, confirming different information processing at the two sites, and also reveals asymmetrical MHb projections to the LHb within the Hb complex (17). The latter observation, which deserves further investigation, suggests potential interactions

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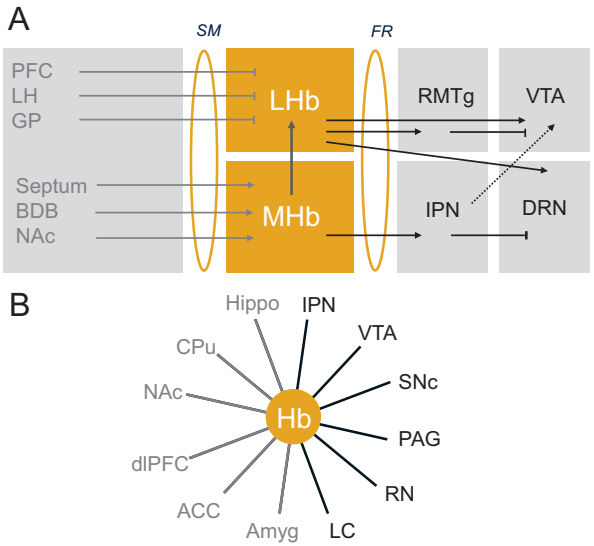

Figure 1. Habenula (Hb) connectivity in rodents and humans. Key pathways connecting medial Hb (MHb) and lateral Hb (LHb), the two subdivisions of the Hb, to other brain structures. Hb connectivity is embedded in brain circuits classically described as reward and emotion circuits, whose dysfunction is associated to psychiatric diseases reviewed here. **(A)** Structural connectivity in animal studies. The LHb receives inhibitory inputs from the prefrontal cortex (PFC), ventral pallidum, globus pallidus (GP), and lateral hypothalamus (LH) through the stria medullaris (SM) and, in turn, sends information to monoaminergic nuclei (5). Projections of LHb to dopaminergic neurons have been best described and include direct [ventral tegmental area (VTA) (99)] and indirect [tail VTA (100,101)] projections. A recent tracing study further revealed an equal number of LHb projections to either dopaminergic (VTA) or serotonergic [dorsal raphe nucleus (DRN) and median raphe nucleus (MnR)] nuclei, which are mostly but not exclusively segregated, indicating that LHb regulates the two monoamine nuclei either independently (most LHb projecting neurons) or jointly (few heterogeneously distributed LHb projecting neurons) (102); both projections are excitatory (11,103). The MHb circuitry is less well known. The medial nucleus receives mainly excitatory inputs from the septum, nucleus accumbens (NAc), and broca diagonal band (BDB) (4,5) and has excitatory projections to the rostromedial tegmental nucleus (RMTg) but mainly and massively to the interpeduncular nucleus (IPN), which in turn projects to the VTA and possibly the raphe nuclei (103). Thus, both MHb and LHb regulate in turn the VTA, DRN, and possibly other midbrain and hindbrain structures such as the locus coereulus (LC) (102). Asymmetrical projections from MHb to LHb have been described (17). **(B)** Functional connectivity in human studies. Hb connectivity is established for both forebrain (in gray) and midbrain/hindbrain (in black) structures by functional magnetic resonance imaging (10,20,104). ACC, anterior cingulate cortex; Amy, amygdala; CPu, caudate putamen; dlPFC, dorsolateral prefrontal cortex; FR, fasciculus retroflexus; Hippo, hippocampus; PAG, periaqueductal gray; RN, raphe nucleus; SNC, substantia nigra compacta.

across the two circuitries whose functional implications remain unknown. Whether similar parallel and potentially interacting LHb/MHb networks operate in humans is unknown.

The analysis of LHb cytoarchitecture in rat brain slices shows high morphologic heterogeneity, which is unrelated to electrophysiological characteristics of the neurons (18). The latter appear surprisingly homogenous throughout the LHb nucleus and include neuron populations with silent, tonic, or bursting spontaneous activities, as well as neuroglialform cells that could be interneurons (18). MHb cells are classified into only two types based on their dendritic structural characteristics, and, regardless of their anatomy, all show similar electrophysiological activity (17). Notably, the latter study also shows the existence of asymmetrical projections from MHb to LHb only (17).

Immunostaining, in situ hybridization, and anterograde tracing experiments show that LHb neurons are mostly glutamatergic, with some gamma-aminobutyric acidergic (GABAergic) neurons (16). LHb neurons are also characterized by heterogeneous expression of monoaminergic receptors across subnuclei, mainly dopaminergic D_2 receptors and serotonin 5-HT_{2C} receptors (16). Similarly, MHb contains mainly glutamatergic neurons distributed into three phenotypically distinct populations, that is, neurons expressing glutamate alone or coexpressing either substance P or acetylcholine (16,19).

Humans

Anatomic description of Hb in the human brain remains limited. As for the rodent Hb, the human Hb is also located next to the third ventricle above the thalamus and is approximately 5–9 mm in diameter with a total volume in the range of 30–36 mm^3 (20) [mouse Hb is 0.8 mm in height and width for comparison (21)]. Histologic examination of postmortem human brain shows partition of Hb into medial and lateral parts, connected by the Hb commissure, similarly to the partition observed in rodents (22). Another morphologic and immunohistochemical analysis showed that overall, the MHb subnuclear organization in humans is similar to that observed in rodents, whereas the shape, relative size, and intranuclear organization of the LHb show significant difference (23). One important difference resides in the substantially enlarged dorsal part of the human LHb that shows that $GABA_B$ receptors are immunoreactive cells. This growth in size possibly indicates increased influence of limbic and striatal afferents into the LHb of humans compared with rodents (23).

Apart from these postmortem studies and owing to its particularly small size, the human Hb was difficult to investigate structurally until recently. Ultra-high-resolution magnetic resonance imaging (hr-MRI) at 7T now allows researchers to visualize and explore the structure noninvasively.

With the use of 7T hr-MRI, Strotmann *et al.* (24) were able to discriminate MHb, LHb, and the habenular commissure in vivo and also explored the structural connectivity of the Hb. Tractographic analysis of diffusion-weighted MRI data revealed fiber tracts connecting Hb to other brain regions for both MHb (anterior posterior direction, in the form of the retroflexus fasciculus identified in rodents) and LHb (anterior posterior direction and superior inferior direction) (24). The general topography of Hb connecting forebrain and mid/hindbrain, therefore, appears similar in rodents and humans. In another study these researchers used 7T ultra hr-MRI ex vivo to further differentiate subnuclei within the Hb. High-resolution T1- and T2-weighted images with 300- and 60-μm isotropic resolution, respectively, revealed LHb heterogeneity with two distinct lateral and medial substructures (25). Ideally, these ex vivo results should help in interpreting in vivo structural MRI data (24,25).

Because an increasing number of functional MRI (fMRI) studies, performed at 3T, are reporting neural activation of human Hb (see next sections), it is critical to isolate this structure from adjacent thalamic areas. A study by Lawson *et al.* (26) offers a set of guidelines to anatomically define the Hb for in vivo hr-MRI at 3T in conjunction with a stereotactic atlas of the human brain. This analysis in native space, as opposed to voxelwise approaches, aims at minimizing reductions in spatial specificity and avoiding localization errors during preprocessing (26). Overall, the ability to identify human Hb and its connections by using MRI and tractography has largely confirmed neuroanatomic findings in experimental animals (see the Supplement) and, altogether, supports the notion that structural and functional Hb characteristics are essentially translatable from rodents to humans. Interestingly, transcriptome analysis also identifies genes expressed in both rodent and human MHb and/or LHb, which also have translational potential for Hb research (Figure 2).

DEPRESSION

Rodents

In animal research, the notion that LHb hyperactivity is associated with depressive-like symptoms, whereas LHb inhibition improves depressive-like behaviors, is well established [reviewed in (12)]. In the late 1980s, a first rat study showed elevated deoxyglucose metabolism in LHb across three behavioral models of depression (27,28). Among main further findings, an LHb lesion study showed reduced depressive-like behaviors and increased 5-HT turnover in the dorsal raphe nucleus of rats subjected to chronic stress procedures (29). These findings were replicated using other procedures. Similar consequences of LHb lesion were reported in a 6-hydroxydopamine rat model of Parkinson's disease (29), while on the contrary, LHb activation using a 5-HT_{2C} agonist decreased monoamine levels and increased depressive-like behaviors in hemiparkinsonian rats (31). Pharmacologic inhibition of LHb by the GABA agonist muscimol had antidepressant effects in congenital helpless rats (32), and opposite metabolic alterations in Hb (high) and ventral tegmental area (VTA) (low) were observed in these rats (33). A very recent study also showed that enhancement of GABA-GIRK (G-protein-coupled inwardly rectifying potassium) function in the LHb ameliorates depressive-like behaviors in mice (34).

Further evidence stems from deep brain stimulation (DBS) experiments in rats. Repeated stimulation of LHb afferences in animals displaying learned helplessness suppressed synaptic drive onto VTA-projecting LHb neurons and increased escape behavior in an active avoidance task (35). Hb DBS also improved depressive-like behaviors and increased monoamine concentrations (dopamine and serotonin) in rats exposed to chronic mild stressors (36). Rats also showed reduced anxiety levels and increased motivation for food when LHb was stimulated (37), substantiating the notion that DBS treatment of the LHb effectively improves depressive symptoms in rats. LHb DBS in a rat model of depression was further shown to alter signaling pathways involving Ca^{2+}/calmodulin-dependent protein kinase, glycogen synthase kinase 3, and adenosine monophosphate–activated protein kinase, and the phosphorylation status of these molecules was associated with the antidepressant actions of DBS (38).

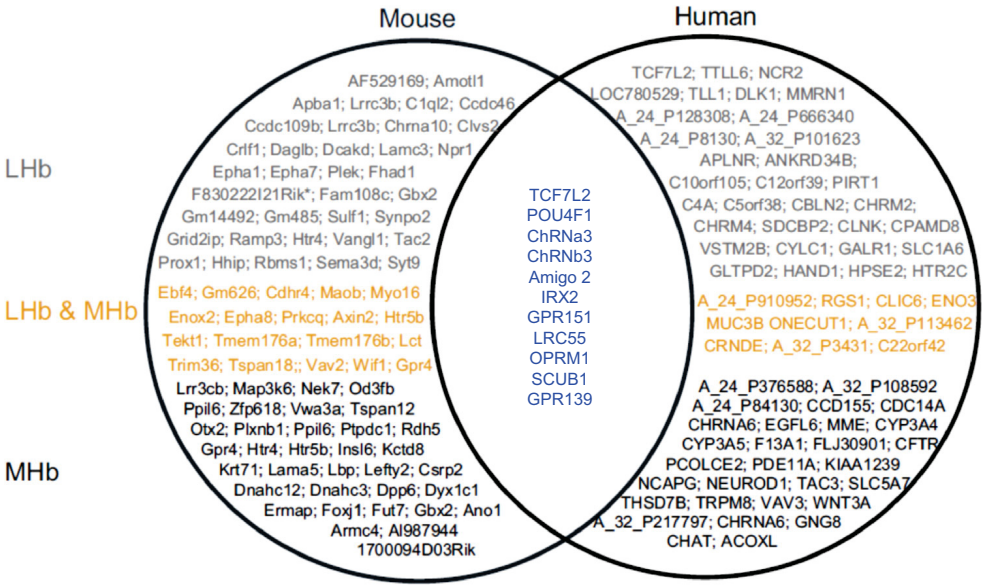

Figure 2. Gene transcriptome in the habenula (Hb). Genome-wide gene expression studies in rodents show differing expression patterns between the lateral Hb (LHb) and medial Hb (MHb), as exemplified by the study of Wagner *et al.* (105) or large-scale gene mapping studies (see Allen Brain Atlas or GENSAT). In our own analysis, data extracted from both Allen Brain Atlas and Brain Star show the top 100 genes with strongest expression in mouse (left) and human (right) transcriptomes. Genes from these groups detected in the LHb (in gray), MHb (in black), or both (in yellow) are indicated. Our analysis of mouse databases confirms differential gene expression in the MHb and LHb, with a pool of common genes detected across the two Hb subdivisions. Our analysis of highly expressed human genes using the same AllenBrain and BrainStar databases unveils differential gene expression in the LHb and MHb in the human brain, supporting the notion of separate functions for the two main Hb nuclei. Interestingly, comparison of mouse and human transcriptome data reveals a cluster of highly expressed Hb genes common to humans and rodents (in blue). This cluster includes *Gpr139* encoding an orphan G protein coupled receptor and *Scub1* encoding a ribosomal protein highly expressed in MHb as well as several other genes encoding, notably, the mu opioid receptor, the orphan receptor GPR151, or subunits of the nicotinic acetylcholine receptors. Further studies are obviously required, but overall, all the genes expressed in both species have translational value for rodent Hb research and potential clinical development.

Optogenetic stimulation of GABAergic and glutamatergic neurons projecting to the LHb indicated that LHb activity is controlled by co-release of the two neurotransmitters (39). The GABA/glutamate ratio was reduced in a mouse model of depression, and in contrast, mice chronically treated with an antidepressant showed a high GABA/glutamate co-release ratio, further supporting the notion that inhibition/activation balance of LHb activity is key to mood control and depression. Finally, a recent fluorodeoxyglucose positron emission tomography live imaging study in the rat showed coordinated increased metabolic activity in septum (projecting on MHb) and Hb during uncontrollable stress that correlated with subsequent learned helplessness behavior (40).

Humans

Human research has identified the Hb as a brain structure contributing to mood disorders. An early positron emission tomography study showed enhanced coupling between Hb and raphe activities in volunteer patients experiencing transient depressive relapse on tryptophan depletion (41). This report provided the first evidence for Hb implication in mood regulation in humans. Further data reporting structural changes in depressed patients are emerging. A postmortem histologic study showed decreased volume of both LHb and MHb in depressed patients diagnosed with major depressive disorder (MDD) or bipolar disorder (BD), and a reduction of neuron number in the Hb (22). The researchers also processed postmortem tissues from schizophrenic patients and found no change (22), suggesting that robust structural Hb alterations are specific to depressive states. Another study using hr-MRI at 3T to analyze Hb volumes also showed a decrease of Hb volumes for unmedicated patients with BD, as well as unmedicated female patients with MDD (20). Another volumetric MRI study reported increased volume of Hb white matter for women with a first episode of MDD (42). Recently, a structural MRI study used gray matter MR images to predict the diagnostic status of subjects with treatment-resistant depression compared with healthy control subjects (43). In this study, major brain regions supporting the diagnosis classification were caudate, insula, and Hb. Finally, a 7T MRI study linked the increase of Hb volumes with disease severity in unmedicated patients with MDD but not in medicated individuals, further supporting that changes in Hb volumes are linked to disease development (44). Of note is that volumetric changes of Hb have not been reported in animals to date; therefore, mechanisms underlying this phenomenon have not been studied yet. Whether structural changes in Hb relate to functional modifications in depressed patients remains open.

Despite the paucity of Hb-focused human fMRI data in the area of depression, there is evidence that the Hb is activated in aversive learning (45,46). With the use of high-resolution fMRI in conjunction with a reinforcement learning paradigm, Lawson *et al.* (45) demonstrated positive Hb responses to the changing values of cues signaling punishments (painful electric shocks). Another study investigated the role of the dopaminergic midbrain (mainly the VTA) and Hb in the processing aversive events in humans. With the use of high-resolution cardiac-gated fMRI (3T), the researchers measured functional activity in the VTA and Hb, as well as other midbrain structures, while participants were experiencing rewarding, aversive, and neutral stimuli. Results showed strong Hb activation and increased functional coupling between Hb and VTA in response to aversive stimuli (46). Although none of these studies directly addresses depressive states, it is possible that Hb overactivity on chronic aversive learning contributes to the development of structural modifications observed in patients with MDD and BD. A recent high-resolution fMRI study examining Hb responses to potential and experienced negative outcome in MDD confirmed Hb activation during prediction of future losses in a probabilistic guessing task with healthy patients, but this was not observed in depressed patients (47). The latter finding demonstrates abnormal Hb activation in response to negative outcome and definitely links aversive learning to MDD.

Finally, a remarkable success, and perhaps the best example to date for translational Hb research, comes from DBS studies (48). Kiening and Sartorius (49) and Sartorius *et al.* (50) tested the potential benefit of inhibiting Hb by DBS in two patients with MDD with treatment-resistant depression. DBS of the stria medullaris thalami, the major LHb afferent bundle, in a patient with treatment resistance achieved full and stable remission, and a second patient showed a 50% improvement of depression symptoms (51). Because this finding is consistent with evidence from animal studies (35–37,49), efforts are under way to evaluate the reliability, as well as pros and cons, of this potential therapy.

In the area of depression, therefore, rodent and human data converge to support the notion that Hb hyperactivity contributes to depressive-like symptoms and that these symptoms can be relieved by inhibiting the structure, providing a strong opportunity to treat depression. Further steps toward this aim include a better understanding of molecular and cellular bases for this activity in animal studies, determining genetic and environmental factors that lead to Hb hyperactivity in mood deficits in both rodent models and human patients, and selecting molecular targets that could allow selective reduction of Hb hyperactivity by pharmacologic means.

ADDICTION

Rodents

In addiction research, animal studies have been extraordinarily productive to demonstrate the importance of Hb in neuroadaptations to drugs of abuse and negative consequences of drug dependence. Here, we summarize current knowledge with emphasis on recent studies.

Several rodent studies have proposed a role for LHb in cocaine reward and dependence. In a mouse model of cocaine conditioned place preference, *c-fos* immunohistochemistry showed increased neuronal activation in the LHb of mice undergoing cocaine-primed reinstatement (52). Another study investigated intrinsic properties of LHb neurons after cocaine self-administration (SA) in rats and after short- and long-term withdrawal from cocaine. Membrane neuron excitability was increased after short-term withdrawal and persisted at least 7 days, suggesting that sustained amplification of neuronal signaling in the LHb could be implicated in the long-term negative effects of cocaine use (53). This hypothesis was

strengthened through a recent study of glutamatergic transmission in LHb projections to the rostromedial tegmental nucleus. Cocaine-treated mice showed synaptic potentiation of these neurons for at least 14 days, and virally mediated blockade of GluA1 trafficking in the LHb prevented cocaine-induced depressive-like phenotypes in tail suspension and forced swim tests (54). GluA1 trafficking-dependent plasticity in the LHb is therefore critical for cocaine-driven aversive states.

Although the LHb is mainly associated with cocaine studies, the MHb subdivision has become a main focus of interest in the area of nicotine research (55,56). Several nicotinic receptor subunits are highly expressed in the MHb-interpeduncular pathway, including mainly $\alpha_3\beta_4$ receptors but also α_2 to α_6 and β_2 to β_4 subunits (57), and knockout mouse studies addressing the role of distinct subunits in rodent models of nicotine addiction have been reviewed recently (13). Notably, nicotine acting at $\alpha_3\beta_4$ receptors in the MHb was shown to directly modify mesolimbic dopamine responses (58). Circuit mapping also identified α_5 nicotinic subunits at the level of the interpeduncular nucleus (IPN), the main MHb output structure, forming a possible link to serotonergic centers of the brain (59). A recent study showed that optogenetic silencing of MHb input to the IPN and also pharmacologic blockade of corticotropin-releasing factor receptor 1 receptors in the IPN both reduce nicotine withdrawal-induced anxiety, possibly implicating a VTA-MHb-IPN circuit (60). Together therefore, a large set of rodent studies definitely establish the importance of the MHb-IPN pathway in negative aspects of nicotine dependence.

Mu opioid receptors are strongly expressed in the Hb, mainly within the MHb (19), and likely interact with cholinergic transmission. In rats, blockade of $\alpha_3\beta_4$ nicotinic receptors in the MHb and IPN attenuates sensitization of the dopamine response to repeated morphine administration, and chronic exposure to morphine enhances cholinergic signaling in the MHb (61). Whether the MHb-IPN pathway contributes to opioid addiction, however, remains open, and more generally, a potential role for LHb and MHb in opioid and alcohol reward and dependence has not been studied in rodents as yet.

Finally, to potentially translate rodent research to clinical applications, DBS was used in rats to examine whether LHb stimulation would lead to decreased cocaine consumption in a set of two studies (62,63). In this work, retrograde tracing experiments showed dose-dependent degeneration of the fasciculus retroflexus after extinction and reinstatement of cocaine SA, suggesting decreased LHb-midbrain connectivity on cocaine SA. Focusing on the LHb, the researchers conducted DBS during maintenance, extinction, and reinstatement of cocaine SA and found that DBS reduced cocaine intake and seeking, at least in rats that self-administered low doses of cocaine. The two studies together provide support for LHb-targeted DBS in the treatment of cocaine dependence (64), but there is no reported study in humans as yet. Current studies are evaluating the efficacy of DBS in human addiction and have mainly focused on the nucleus accumbens and subthalamic nucleus; LHb may also be a target of interest in this context (65).

Humans

At present, studies in humans are scarce and will undoubtedly develop in upcoming years. Related to substance use disorders and reward processing are studies addressing reward prediction error (RPE), a fundamental dimension of associative learning. In monkeys, a grounding electrophysiological study by Matsumoto and Hikosaka (6) demonstrated that LHb neurons are excited by negative prediction error (unpleasant event or absence of reward) and inhibited by unexpected reward, therefore encoding RPE rather than reward per se. Recent studies have explored RPE in the context of drug abuse showing correlation between RPE and addiction not only in rodents but also in humans with cocaine (66), cigarette smoking (67,68), and alcohol (69).

To date, two fMRI studies have provided evidence that RPE activates the human Hb (70,71). A pilot study scanned subjects in a 3T MRI scanner during a juice-delivery task, and data revealed Hb activation during negative prediction error; that is, when the juice is not delivered at the expected time (71). Another study further investigated Hb activation using fMRI together with connectivity analysis and demonstrated correlated LHb and VTA activation during a stop-error task measuring the negative prediction error (70). Whether the Hb networks are altered in addicted individuals remains to be studied.

In a very different context, human genetics indirectly implicates the Hb in nicotine addiction (72). Three meta-analyses have simultaneously found significant association between single nucleotide polymorphisms and cigarettes smoked per day, and single nucleotide polymorphisms were included in the α_5-α_3-β_4 nicotinic receptor subunit cluster. Nicotinic receptor subunits encoded by these genes are expressed in several brain areas, but only the MHb and its primary output, the IPN, show coexpression of all three subunits. These findings therefore integrate Hb pathways in human nicotine research.

Overall, rodent data identify Hb as a key brain site for addiction research, whereas human Hb addiction research is still at its infancy. In the latter, an important step will be the mapping of Hb connectivity and activation in dependent and abstinent individuals, in relation to other components of reward and aversion networks. Another potential approach yet to be used is DBS of the Hb for the treatment of craving and relapse representing the greatest challenge in the area of substance use disorders. As was done for MDD, such studies should be done in both rodents and humans using translational designs.

SCHIZOPHRENIA

Because of the complex connectivity of the Hb to multiple forebrain and hindbrain circuits, similar in rodents, non-human primates, and humans, it is anticipated that Hb activity affects multiple dimensions of normal behavior, with implications for disease beyond depression and addiction. Here, we focus on the possible role of the Hb in schizophrenia.

Tightly linked to predicting errors are decision-making processes, and rat studies have demonstrated causal implication of the LHb in subjective decision making. Stopper and Floresco (73) used in vivo electrophysiology to manipulate phasic dopamine signaling during a risk/reward decision-making task and showed that LHb stimulation before choice

redirects the selection of action away from the preferred or rewarded outcome. Conversely, LHb inactivation abolishes the previously described choice biases, favoring random patterns of choice behavior (14,74). This particular function of the Hb may be relevant to schizophrenia research (14), because reinforcement learning deficits and misusing feedback to appropriately guide decision making are integral aspects of schizophrenia (75,76).

In humans, anatomic modifications in the Hb have been linked to schizophrenia. An early computed tomographic study on postmortem human brain slices showed increased calcification in the Hb of schizophrenic patients (77). Postmortem immunochemistry also showed reduced capillary densities, specifically in the Hb of schizophrenic patients, and reduced density of neurons expressing the adenosine triphosphate–binding cassette transporter protein ABCB1, whose malfunction has been associated in schizophrenia (78).

fMRI coupled to a visual-spatial match-to-sample task further showed that patients with schizophrenia lack appropriate modulation of Hb activity in adaptive response to feedback and errors (79). This finding suggests that pronounced deficits observed in schizophrenic patients in situations of problem solving and learning could result from an alteration of Hb-mediated feedback processing. Further studies are necessary to confirm this hypothesis, with perhaps selected schizophrenic patient subgroups.

Of note also is that LHb lesions in the rat induce behavioral deficits in the Morris water maze (80), analogous to deficits of declarative memory in humans known to be impaired in schizophrenia (81), and also lead to disturbed attention in a 5-choice serial reaction time task (82), modeling the continuous performance test of attention in the clinic where patients with schizophrenia score low (83). Although Hb function in learning and memory has been less studied and engages processes distinct from those underlying subjective decision making, evidence from animal studies all support the notion that Hb research is relevant to cognition in the area of schizophrenia.

CHALLENGES AND FUTURE DIRECTIONS

In this review, we have organized rodent and human data in three major psychiatric disease areas: depression, drug dependence, and other potential disease areas of psychiatry, notably schizophrenia. We have also added a section on pain in the Supplement. Table 1 summarizes functional consequences of Hb manipulations in both rodents and humans within the four categories.

Basic research in laboratory animals has revealed the Hb as a core integration center, which influences many aspects of behavior. One current goal of rodent research is the genetic targeting of specific Hb neuron populations to dissect circuit mechanisms underpinning the many Hb-controlled behaviors. Main recent studies demonstrate Hb implication in emotional or cognitive responses that have not been discussed here. For example, in the area of stress, fear, and anxiety, optogenetic activation of LHb efferent neurons to the rostromedial tegmental nucleus induced acute and conditioned avoidance (84), ablation of projection neurons from the triangular septum to the MHb promoted deficits of anxiety-related behavior, and ablation of neurons projecting from the bed nucleus of the anterior commissure to the MHb led to severe decreases in fear responses and fear learning (85,86). In addition, specific deletion of the *CB1* gene in MHb neurons reduced aversive-acquired responses such as freezing in cued and contextual fear-conditioning experiments (87), whereas optogenetic activation of glutamatergic LHb neurons projecting to the laterodorsal tegmentum generated fear-like responses and regulates olfactory cue-induced innate fear (88). In the context of executive functions, another recent study showed that ablation of MHb neurons increases impulsivity and impairs cognition-dependent functions, including aversion to delay and effort, deficits in long-term memory, and reduced flexibility (89). The LHb also contributes to behavioral flexibility using proactive and retroactive information when performing decision-making appetitive tasks (90). Finally, Hb integrity was found essential in processing positive (social play) and negative (social isolation) social information in juvenile rats, with a specific implication of the medial LHb (91). Whether and how these activities relate to psychiatric disorders in humans represents an entire field of investigation for the next decade.

On the human side, a major effort lies in overcoming technical challenges due to the small size of the Hb. hr-MRI and fMRI now allow accurate targeting of the structure (24,26), and manganese-based neuroimaging with minimal toxicity may develop for patients in the future (92). In addition, surgical treatment for psychiatric disorders is being rekindled, and strong efforts are dedicated to DBS in sites involving emotional and behavioral circuitry, among which is the Hb (64,93). Traps and pitfalls of the technique applied to small deep structures are being addressed, and achieving successful surgery is becoming feasible (94). Human Hb research should now focus on sharpening neuroimaging and DBS techniques to increase both functional studies and clinical trials. Together, future studies will promote the use of translational techniques; that is, approaches that can be used across species or at least have a predictive value (predict outcome in humans). A corpus of techniques applicable to both rodents and humans is developing in the field of habenular research, including DBS, MRI, fMRI, and some behavioral tasks or experiments. Efforts are now required on the animal side, particularly in the area of neuroimaging techniques representing the best translatable analysis tool for brain activity (95–97).

In conclusion, Hb research in humans is still in its infancy [see also (98)], but it is developing at a rapid pace. Animal research, however, has become a mature field and has revealed a vast spectrum of Hb functions throughout emotional and cognitive brain processes, opening the way to multiple opportunities in terms of potential implications in the clinic. Upcoming findings in both rodents and humans will contribute to refine our understanding of the role of the Hb, the foundation of which was set in 2007 (6), and perhaps assign a unique integrative role in reward and aversion processing to this intriguing brain structure. Future studies will also determine whether Hb-targeted strategies indeed prove efficient in the treatment of depression and could perhaps surpass mood disorders for broader applications in the area of psychiatric disorders.

Table 1. Functions Mediated by Habenula in Rodents and Humans

Rodents	Functions
Pain	
MHb	Gene knockdown: Medial habenular RSK2 contributes to morphine analgesia (106)
MHb+LHb	Hb: Integrative hub for pain control and regulating nociceptive processes (110)
	Electrical stimulation of the Hb induces analgesia (108,109)
Depression	
LHb	Activation of LHb 5-HT2C receptors increases depressive-like behaviors (31)
	LHb lesion studies (29,30) or pharmacological inhibition (32,33) reduces depressive-like behaviors
	DBS of LHb reduces depressive-like behavior (35,36) by suppressing synaptic drive onto VTA-projecting LHb neurons (35) and increases monoamine concentrations (36)
	Optogenetic stimulations of GABA and Glu neurons projecting to the LHb demonstrate that LHb activity is regulated by corelease of both neurotransmitters (39)
MHb+LHb	^{18}FDG-PET live imaging study shows increased activity of Hb that correlates with subsequent learned helplessness behavior (40)
Addiction	
LHb	A cocaine conditioned place preference study shows increased neuronal activation in the LHb of mice undergoing cocaine-primed reinstatement (52)
	Electrophysiological studies show that cocaine induces synaptic potentiation of LHb neurons (53,54)
	Retrograde labeling shows dose-dependent degeneration of the fasciculus retroflexus after extinction and reinstatement of cocaine self-administration (63)
	DBS of LHb reduces cocaine intake and seeking (62,63)
MHb	Optogenetic silencing of MHb input to the IPN or pharmacological blockade of CRF1 receptors in the IPN reduce nicotine withdrawal-induced anxiety (60)
	Pharmacological blockade of $\alpha3\beta4$ nicotinic receptors in MHb and IPN attenuates sensitization of the dopamine response to repeated morphine administration (111)
	Chronic exposure to morphine enhances cholinergic signaling in the MHb (61)
Schizophrenia	
LHb	Relevant to schizophrenia: LHb stimulation prior to choice redirects the selection of action away from the preferred or rewarded outcome (13)
	LHb lesions in the rat induce memory (31,112) and attentional (80) deficits analogous to cognitive impairments in schizophrenic patients.
Humans	**Functions**
Pain	
MHb+LHb	fMRI: Noxious heat activates Hb (107)
	Resting-state fMRI: Pediatric patients with chronic pain exhibit a reduced Hb rsFC to the rest of the brain and specifically with forebrain area (104)
	Postmortem histological study (22) and structural MRI studies (20,43) show decreased volumes of Hb in MDD and BD patients
	PET study shows enhanced coupling between Hb and raphe activities in volunteer patients experiencing transient depressive relapse upon tryptophan depletion (40)
Depression	
LHb	DBS of the stria medullaris thalami, the major LHb afferent bundle, reduces symptoms of treatment-resistant depression (49,51)
Addiction	
MHb	Genetic meta-analyses found association between $\alpha5$-$\alpha3$-$\beta4$ cluster and cigarettes smoked per day. Only the MHb shows co-expression of all three subunits (72)
Schizophrenia	
MHb+LHb	Computed tomographic study on post-mortem brain slices shows increased calcification in the Hb of schizophrenic patients (77)
	fMRI study shows that patients with schizophrenia lack appropriate modulation of Hb activity in adaptive response to feedback and errors (79)

This table summarizes main preclinical and human studies discussed in this review. Reports for rodents and humans are categorized based on studies in area of pain and analgesia, depression, addiction and in schizophrenia (top to bottom). These studies address the medial habenula (MHb), lateral habenula (LHb), or both. Some of the studies are also reviewed for pain and analgesia (10), for mood and depression (12), for drug dependence (13), and for schizophrenia (14). The parallel between rodent and human findings show promises for effective translation of preclinical research to human psychiatry.

BD, bipolar disorder; CRF1, corticotropin-releasing factor receptor 1; DBS, deep brain stimulation; fMRI, functional magnetic resonance imaging; GABA, gamma-aminobutyric acid; Glu, glutamate; IPN, interpeduncular nucleus; MDD, major depressive disorder; PET, positron emission tomography; rsFC, resting-state functional connectivity; VTA, ventral tegmental area; ^{18}FDG, fluorodeoxyglucose.

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ARTICLE INFORMATION

From the Douglas Hospital Research Center (LJB, ED, BLK), Department of Psychiatry, Faculty of Medicine, McGill University, Montreal, Quebec, Canada; Institut de Génétique et de Biologie Moléculaire et Cellulaire (LJB), Illkirch, France; and Université de Strasbourg (LJB), Illkirch, France.

Address correspondence to Brigitte Kieffer, Ph.D., Douglas Hospital Research Center, Perry Pavilion Room E-3317.1, 6875 Boulevard LaSalle, Montreal, QC H4H 1R3, Canada; E-mail: brigitte.kieffer@douglas.mcgill.ca.

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Aim of the Thesis

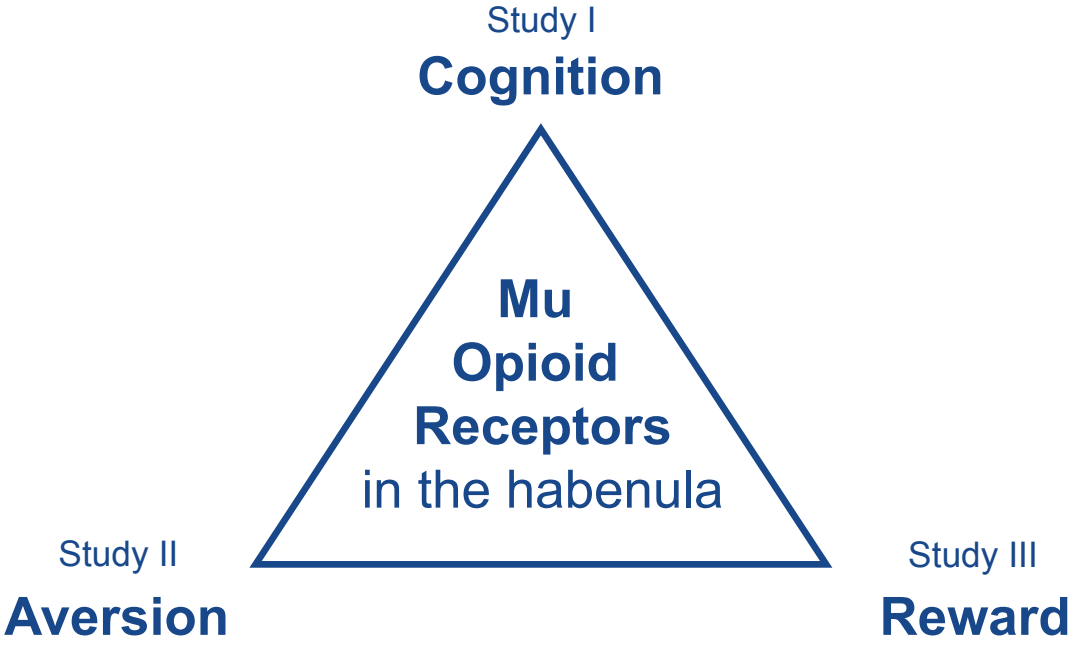
My thesis explores the contribution of MORs in cognition, aversion and reward with a focus on MORs from the habenula.

First we address the poorly studied role of MORs in cognition using our constitutive MOR knockout mouse. We demonstrate novel potential implication of MORs in compulsion, in addition to reward/motivation.

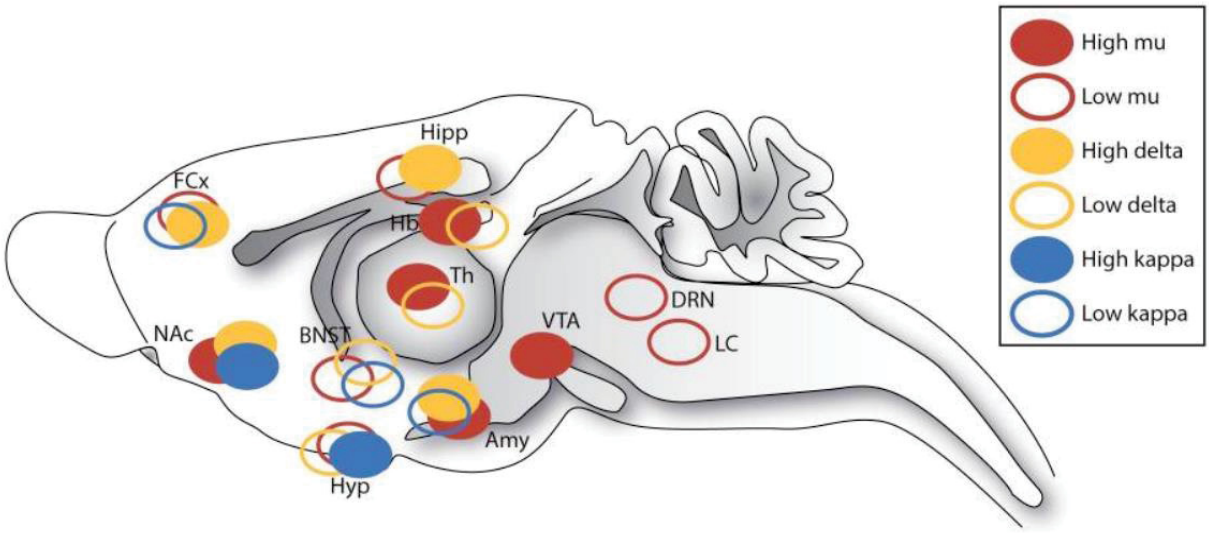
Second, entering the circuits, we investigate the functions of habenular MORs using a conditional knockout MOR mouse that we generated for the purpose of this study and that lacks MORs solely in the medial habenula. Given the high density of their expression in a structure that seems crucial to reward, aversion and cognition, we hypothesize that habenular MORs are in a central position to control these brain functions and their output on normal and maladaptive behavior. Our data reveal changes in aversive processing but not in reward or cognitive functions. Hence we demonstrate for the first time that a population of MORs in the habenula controls both somatic and affective aversion.

Third, we examine the role of MORs in alcohol reward-related using another conditional knockout model generated in our lab and that lacks MORs mainly in the striatum. Our study on alcohol reveals the importance of striatal MORs in alcohol consumption and reward.

Our work on MORs is particularly salient in the clinical context of substance use and other self-control and mood disorders.

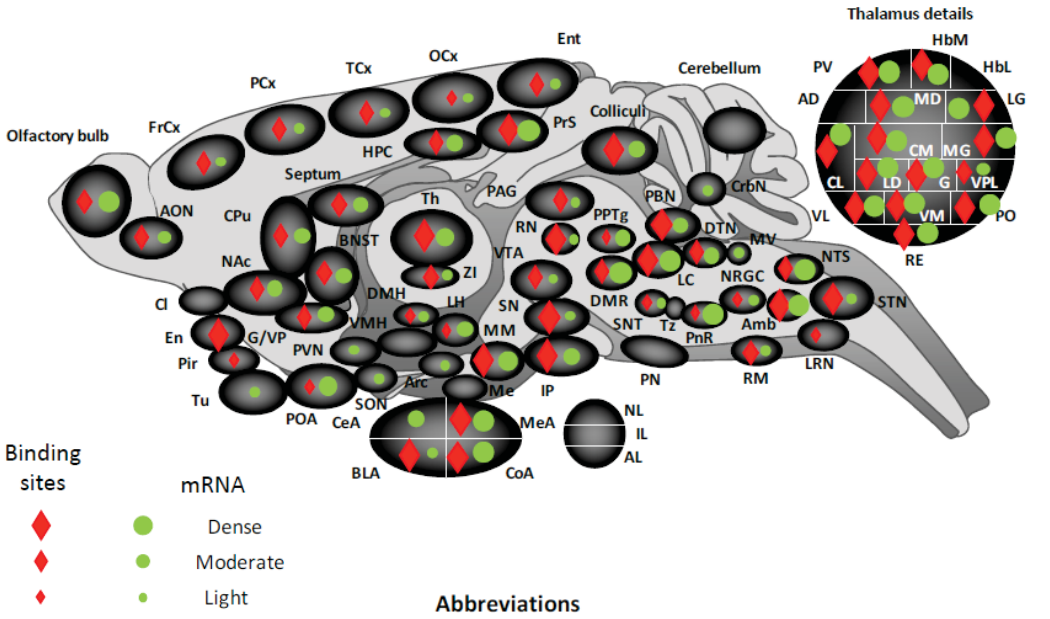


A



Amy, amygdala; BNST, bed nucleus of the stria terminalis; DRN, dorsal raphe nuclei; FC, frontal cortex; Hb, habenula; Hipp, hippocampus; Hyp, hypothalamus; LC, locus coeruleus; Nac, nucleus accumbens; Th, thalamus

B



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 1. Opioids distribution

A- Mu, delta and kappa receptor proteins show overlapping but distinct distribution (adapted from Lutz and Kieffer, 2013)

B- Mu receptor protein and mu receptor mRNA show overlapping anatomical distribution (adapted from Le Merrer et al, 2009)

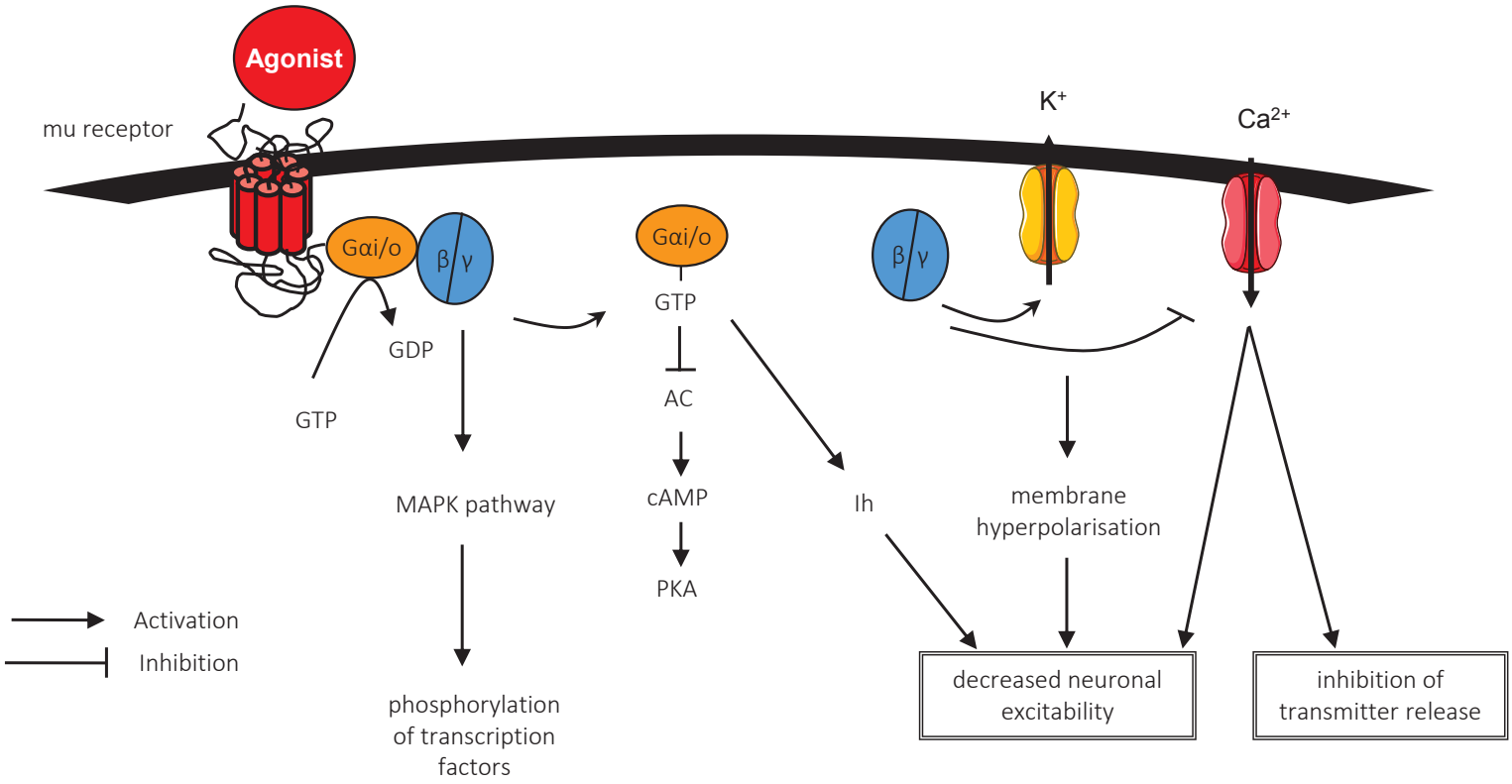


Figure 2. Signal transduction induced by mu opioid receptor activation
Ligand induced mu opioid receptor activation leads to activation of G-protein subunits. Consequences are inhibition of adenylate cyclase, activation of potassium conductance, inhibition of calcium conductance and inhibition of transmitters release (adapted from Williams et al, 2001).
Ac, adenlayte cyclase; cAMP, cyclic adenosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Ih, voltage-dependent current; MAPK, mitogen-activated protein kinase; PKA, protein kinase A

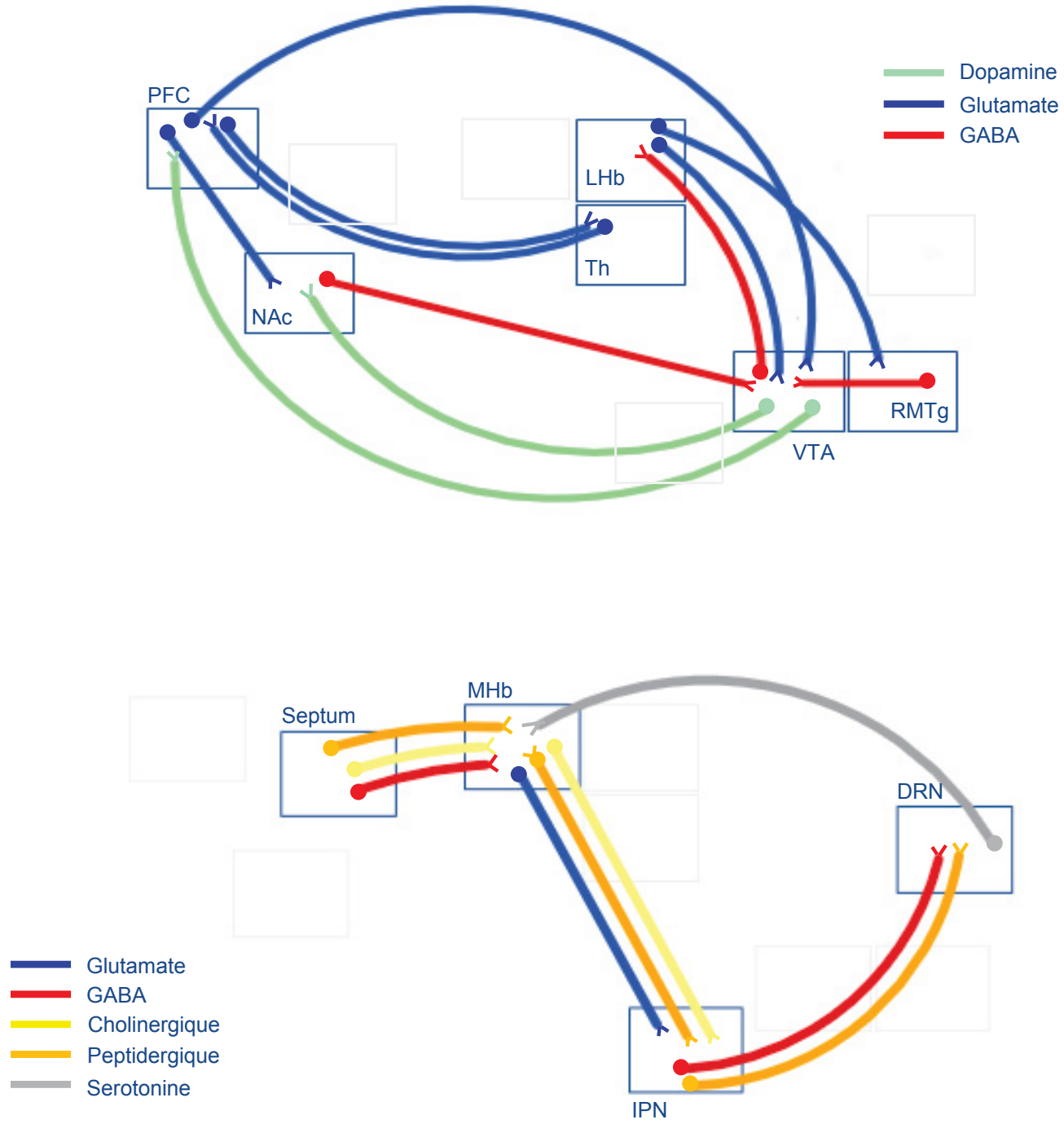


Figure 3. Reward/aversion circuits

A- A simplified diagram of the main connections to and from the mesocorticolimbic system, in a sagittal section of rodent brain (adapted from Pistillo et al, 2015 and other references).

B- A simplified diagram of the main connections to and from the medial habenula (adapted from Antonlin-Fontes et al, 2015 and other references).

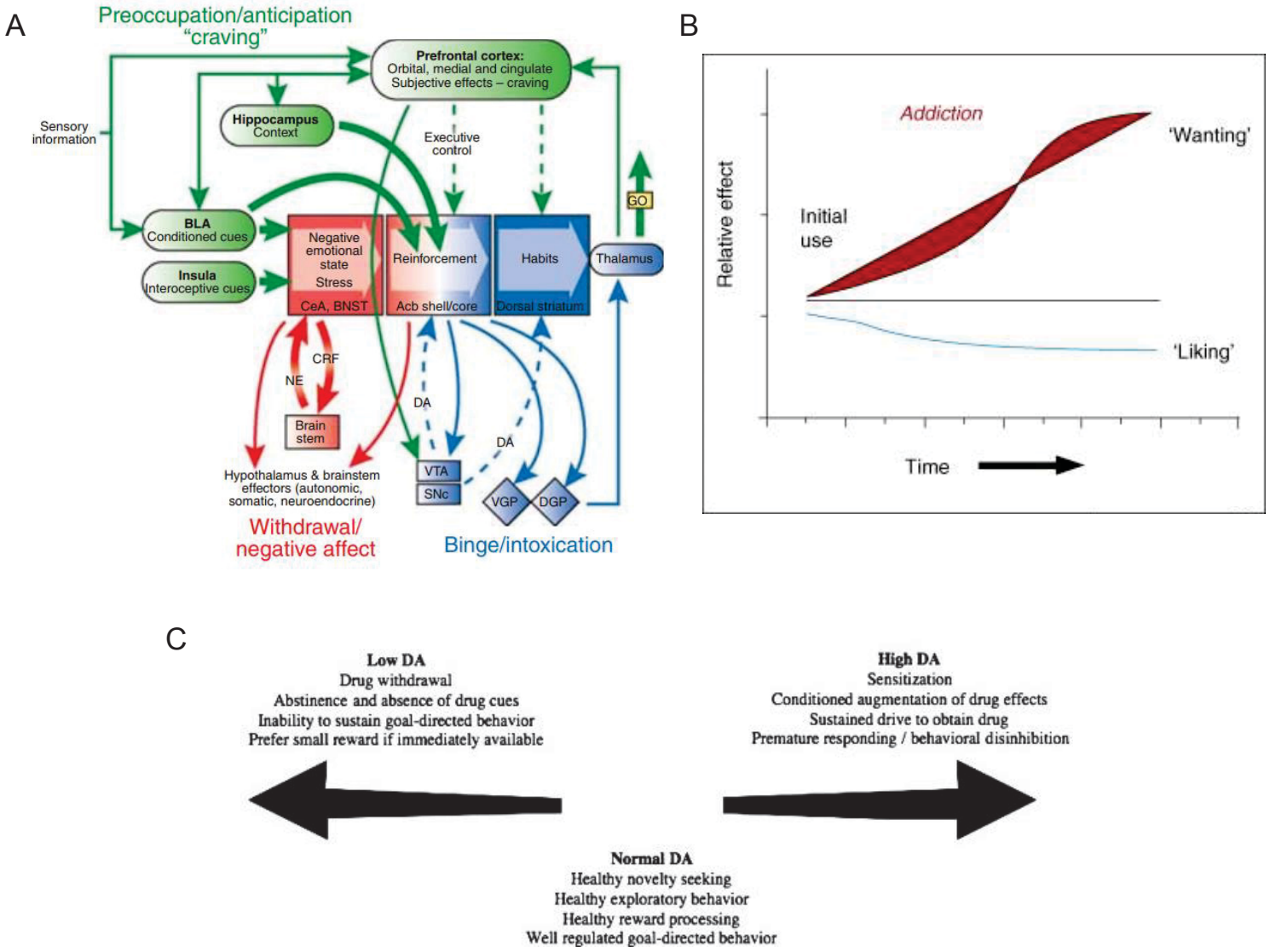
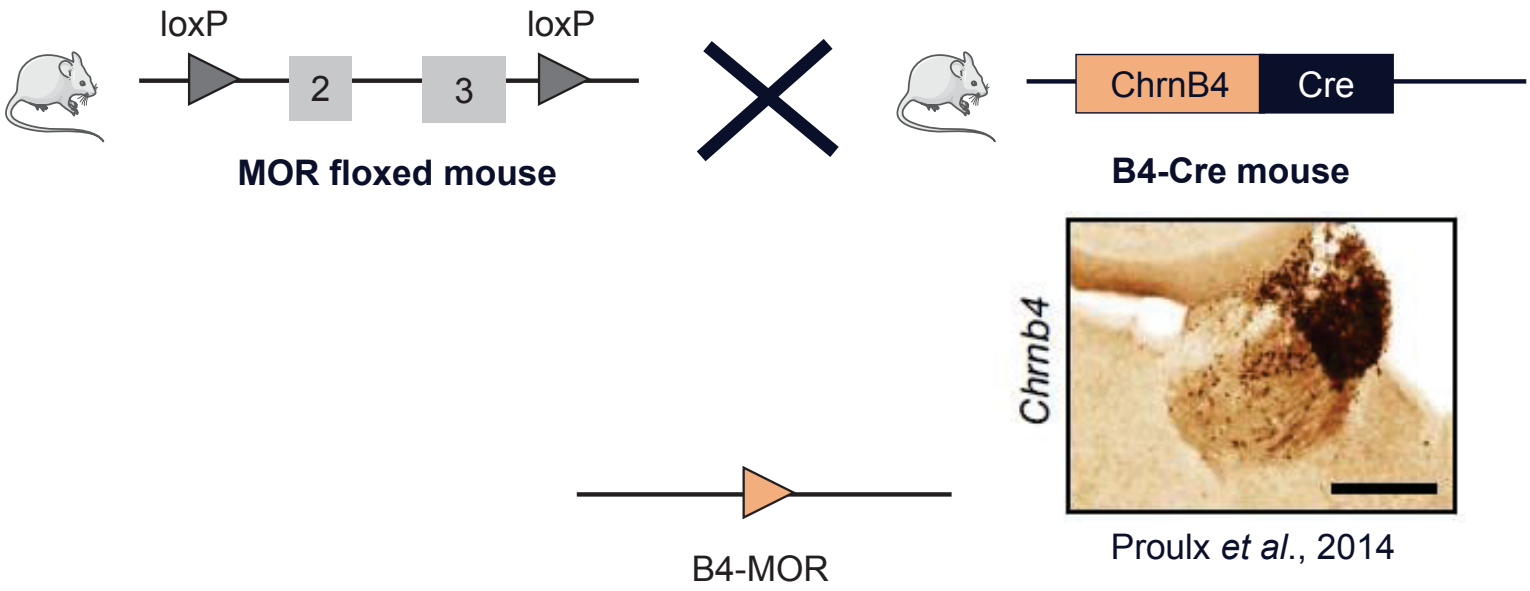


Figure 4. Addiction models

A- Neural circuitry associated with the three stages of the addiction cycle. (a) Binge/intoxication stage. Reinforcing effects of drugs may engage reward neurotransmitters and associative mechanisms in the nucleus accumbens shell and core and then engage stimulus–response habits that depend on the dorsal striatum. Two major neurotransmitters mediating the rewarding effects of drugs of abuse are dopamine and opioid peptides. (b) Withdrawal/negative affect stage. The negative emotional state of withdrawal may engage the activation of the extended amygdala. Major neurotransmitters in the extended amygdala hypothesized to have a function in negative reinforcement is corticotropin-releasing factor. (c) Preoccupation/anticipation (craving) stage. This stage involves the processing of conditioned reinforcement in the BLA and the processing of contextual information by the hippocampus. Executive control depends on the prefrontal cortex and includes representation of contingencies, outcomes, and their subjective value. A major neurotransmitter involved in the craving stage is glutamate. Green/blue arrows, glutamatergic projections; orange arrows, dopaminergic projections; pink arrows, GABAergic projections; Acb, nucleus accumbens; BLA, basolateral amygdala; VTA, ventral tegmental area; SNc, substantia nigra pars compacta; VGP, ventral globus pallidus; DGP, dorsal globus pallidus; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; NE, norepinephrine; CRF, corticotropin-releasing factor; PIT, Pavlovian instrumental transfer (taken from Koob and Volkow, 2010).

B- Incentive-sensitization model of addiction. Schematic model of how 'wanting' to take drugs may grow over time independently of 'liking' for drug pleasure as an individual becomes an addict. The transition from casual drug use to compulsive addiction is posited to be owing to drug-induced sensitization of mesocorticolimbic mechanisms of incentive salience (taken from Berridge, 2009).

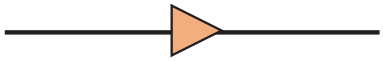
C- Two factor model of dopamine's (DA's) role in drug addiction. The repeated use of psychostimulant drugs of abuse leads to (i) sensitization and increased DA transmission in the presence of drug-paired cues, and (ii) decreased DA transmission in the absence of drug-paired cues. Both neurobiological states might contribute to addiction phenomenology. Increased DA transmission is proposed to over-ride mechanisms of behavioral inhibition, sustain intense craving and focus the drive to obtain drug. Decreased DA transmission is proposed to decrease the ability to sustain goal-directed behavior and degrade decision-making processes thereby increasing the preference for rewards that are easily available (taken from Leyton, 2007).



Transgenic mouse line
lacking mu opioid receptors in the medial habenula

Mixed Genetic Background:
C57Bl/6 and 129Sv/Pas ♂

Figure 6. B4MOR-/- conditional knockout mouse of mu opioid receptors in the habenula
Specific gene inactivation by Cre-loxP system. The promoter *B4*, a nicotinic receptor subunit with specific pattern of expression in the medial habenula, drives the expression of the Cre recombinase that excises the sequence between the two lox P sites of the gene of interest (*Oprm1*). (adapted from Gaveriaux-Ruff and Kieffer, 2007).



Dlx-MOR
Charbogne *et al*, 2017

Transgenic mouse line
lacking mu opioid receptors mainly in the striatum

Mixed Genetic Background:
C57Bl/6 and 129Sv/Pas ♂

Figure 7. DlxMOR-/- conditional knockout mouse of mu opioid receptors in the striatum
Specific gene inactivation by Cre-loxP system. The promoter *dlx5/6* that is expressed in GABAergic neurons of the forebrain, drives the expression of the Cre recombinase that excises the sequence between the two lox P sites of the gene of interest (*Oprm1*). (adapted from Gaveriaux-Ruff and Kieffer, 2007).

Study I- Mu opioid receptors in reward-related cognition

Aim of the study

Both mu opioid receptors and cognitive deficits are extensively associated with addiction but the link between MORs and cognition is poorly studied. Here we explored the contribution of MORs in cognitive functions with emphasis on processes that involve reward sensitivity and contribute to addiction and other self-control conditions.

Why did we look at mu opioid receptors and cognition?

MORs, reward and addiction- In preclinical research, genetic and pharmacological approaches have definitely established the essential role of MORs in mediating drug (Charbogne, Kieffer et al. 2014) and natural (Becker et al., 2014; Moles et al., 2004) reward. More specifically, others and we have demonstrated that rewarding properties of both opiates (Matthes, Maldonado et al. 1996, Roberts, McDonald et al. 2000, Contet, Kieffer et al. 2004) and non-opioid drugs of abuse (Contet, Kieffer et al. 2004) are abolished in constitutive knockout mice lacking the *Oprm1* gene coding for mu opioid receptors (MOR-/- mice). Through their well-established link with reward (Lutz and Kieffer 2013, Charbogne, Kieffer et al. 2014, Fields and Margolis 2015) and potentially through their newly discovered contribution to aversion (Boulos, Darcq et al. 2017), MORs have been tightly implicated in the development and maintenance of addiction. While pharmacogenetic studies have shown that gene variant profiling including MOR gene could help predict treatment responses and assist in developing effective treatments for alcohol, opioid and cocaine addiction, clinical trials are unfolding one by one the beneficial opioid targeting in SUDs, including naltrexone response among alcohol addicted persons (Berrettini 2016, Berrettini 2016) and the long search of the perfect molecule for opiate use disorder (Walwyn, Miotto et al. 2010, Stockton and Devi 2012).

Cognition, reward and addiction- Through the regulation of motivational value, reward can have strong impact on cognition and together they determine executive control and behavior (Diamond 2013). Dysregulation of reward can thus lead to cognitive and executive control deficits, at the heart of a large number of psychiatric disorders, namely self-control conditions including substance use disorders (Jentsch, Ashenhurst et al.

2014). Cognitive control deficits and reward dysregulation, which mainly manifests as anhedonia, are indeed well documented in drug abusers (Cousijn, Wiers et al. 2013, Morie, De Sanctis et al. 2014). Cognitive or executive control is in turn directly linked to a variety of lower-order cognitive functions (Miyake, Emerson et al. 2000, Dickson, Tkacs et al. 2007, Titz and Karbach 2014) that are also altered in addiction (Berridge and Arnsten 2013, Leeman, Robinson et al. 2014, Besson and Forget 2016).

Cognition and MORs- In this context where both MORs and cognition converge towards reward in addiction it is surprising to note the lack of literature when it comes to MORs and cognitive functions.

How did we look at mu opioid receptors and cognition?

To explore the role of MORs in cognition, we decided to use our constitutive MOR knockout mouse model and to focus on behaviors that are known to reflect altered cognitive processes in addiction and that are likely to recruit MORs.

Pavlovian learning- Stimulus-response Pavlovian conditioning is based on the assumption that a given subject (human or rodents) can learn. It is generally accepted that this conditioned learning stems from (i) associative connections between predictive stimuli and the component features of the events that these stimuli predict and (ii) the cognitive functions served by cues an their effect on motivation to perform a specific action (Corbit and Balleine 2016).

Addiction- Pavlovian learning of drug effects is a critical factor in the development and persistence of drug addiction (Everitt and Robbins 2016, Heinz, Schlagenhauf et al. 2016). While clinical studies indicate an important role for thalamocortical loops(Balleine, Morris et al. 2015) and the orbitofrontal cortex (Schoenbaum, Chang et al. 2016) in associative learning dysfunction in addiction, specific mechanisms underlying alterations of basic learning mechanisms in addiction remain elusive.

MORs- A few studies suggest that MORs are involved in the modulation of learning during environmental conditions (Aloyo, Romano et al. 1993) as well as in Pavlovian contextual fear (Sanders, Kieffer et al. 2005, Cole and McNally 2007). A single study

further suggests that, while discriminated behavior is preserved in constitutive MOR knockout mice, a delay could be occurring in the process of learning devaluation in mice lacking MORs (Laurent, Leung et al. 2012). The exact contribution of MORs in pavlovian learning with discrete cues remains however unclear.

Attention- Attentional processes are essential in many everyday occurrences in a constantly changing world (Huttermann and Memmert 2017).

Addiction- While several attentional processes have been explored in the context of addiction, emphasis has been put on attentional bias that can be defined as selective attendance of certain stimuli in the environment versus disregard of other kinds of stimuli (Cox, Fadardi et al. 2014, Cox, Klinger et al. 2015). In the context of addiction, users are distracted by drug-related stimuli (Zilverstand, Parvaz et al. 2016). Beyond playing a vital role in the development and maintenance of addiction, attentional bias serves to enhance craving and subsequent drug use (Weinstein and Cox 2006), participating in the complex cognitive-behavioural model of drug addiction.

MORs- A recent clinical study found that MOR agonism increased while antagonism decreased avert attention to eyes in a face exploration task, suggesting that human MORs promote visual attention to socially significant cues, a process that could involve social reward value (Chelnokova, Laeng et al. 2016). Additionally, older preclinical studies in rodents had demonstrated that MORs in the NAc regulate attentional learning (Iordanova, Killcross et al. 2007) while peripheral opioids regulate attentional functions involved in the earliest stage of Pavlovian learning in rodents (Hernandez, Watson et al. 1997). While this sparse data suggest that MORs potentially modulate fragments of early stage attention, little is known concerning the central opioid regulation of later attentional and associative functions.

Impulsivity/Compulsivity- While impulsivity depends on goal-directed motivational systems that mediate voluntary reward seeking (Wassum, Ostlund et al. 2009), compulsivity can be defined as the maladaptive persistence of responding to a stimulus despite the fact that it is no longer rewarded (Amitai and Markou 2010). Behavioural tasks that measure impulsivity have often fractioned the construct into two camps:

impulsive decision and impulsive action (i.e. motor impulsivity or inhibitory control) (Winstanley, Eagle et al. 2006). Inhibitory control may also be recruited in the regulation of compulsive behavior (Dalley, Everitt et al. 2011). Cognitive inflexibility, i.e. the inability to alter behavior in reaction to changing situational demands, is similarly hypothesized to contribute to compulsivity (Dalley, Everitt et al. 2011). If the substrate of compulsivity is partly habitual, the precise neural circuitry underpinning inhibitory control and cognitive flexibility is less clear (Everitt and Robbins 2016).

Addiction- Addiction involves an inability to control drug seeking behavior(Schoenbaum, Chang et al. 2016). It can be viewed as a shift from voluntary, recreational and impulsive drug use to compulsive-seeking habits (Everitt and Robbins 2016) and it is known to induce substance-related cognitive biases (Leung et al, 2017). The main neurobiological mechanism underlying this cognitive behavioural transition is a progression from cortical prefrontal regions(Balleine and O'Doherty 2010) to ventral striatum(Baler and Volkow 2006, Garavan and Hester 2007) to dorsal striatum(Corbit and Balleine 2016). While this hypothesis is constantly being updated and refined, efforts are made to identify targets that participate in the switch from ventral to dorsal striatum, from positive to negative reinforcement, from impulsivity to compulsivity.

MORs- Our previous study has already shown a decrease in motor impulsivity also called inhibitory control in MOR-/- mice as measured by premature responses in a signaled nose poke task(Olmstead, Ouagazzal et al. 2009) and the regulation of inhibitory control by MORs was attributed to the NAc(Wiskerke, Schetters et al. 2011). However, the role of MORs in inhibitory control and its potential contribution to other impulsivity/compulsivity was never further explored.

Here we used our widely characterized constitutive MOR knockout mouse line (Matthes, Maldonado et al. 1996) to explore the role of MORs in reward-related higher order cognitive functions. To this aim, we used a high-tech TouchScreen-based apparatus that allows improved, standardized and automated behavioral testing. We performed a series of tasks to specifically target (i) associative learning, (ii) reward value of naturally highly appetitive stimuli (autoshaping task), (iii) attention, (vi) motivation and (v)

impulsivity/compulsivity (5-Choice Serial Reaction Time Task). Emphasis was thus put on functions that involve reward sensitivity and contribute to SUDs and other self-control conditions using MOR-/- mice and their controls. Because all behavioural experiments were carried out in the same testing environment, using the same types of stimuli, responses and reward, a given task yielded results that could be useful not only in its own but also in the interpretation of other tasks in the battery(Mar, Horner et al. 2013), thus providing us with a solid cognitive phenotype for MOR-/- mice.

Cognition in mu opioid receptors knockout mice

Boulos LJ.[1,2], McNicholas M.[1], Kieffer BL.[1], Ben Hamida S.[2]

[1] McGill University, Faculty of Medicine, Douglas Research Centre, Montreal, Canada
[2] Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch-Graffenstaden, Strasbourg, France

Abstract
Mu opioid receptors (MORs), which are mainly known for their crucial role in pain control and reward processing, have also been implicated in motor impulsivity; their contribution to cognitive control remains however poorly explored. Here we examined MOR role in cognitive functions including attention, reward valuation, motivation and inhibitory control. We tested MOR knockout mice and their controls in a TouchScreen-based set of tests comprised of an autoshaping protocol and a 5-Choice Serial Reaction Time task (5-CSRTt). While discriminatory behavior and attention outcomes remained unchanged in MOR mutant mice, learning was delayed, acquisition of the stimulus-reward association was altered, and both motivation and impulsivity/compulsivity were remarkably decreased compared to controls. Collectively, our results confirm the previously reported implication of MORs in pavlovian learning and reward value, and further suggest that in addition to impulsivity, MOR activity in normal animals facilitates a compulsive-like behavior. In a broader context, these data suggest that alterations in MOR function may generally contribute to maladaptive self-control conditions such as substance use disorders, eating disorders and attention deficit hyperactivity disorders.

Keywords

- Mu opioid receptors
- Reward
- Cognition
- Impulsivity/compulsivity
- Addiction
- TouchScreen

Introduction

Endogenous and clinically administered opioids act on opioid receptors[1, 2] and are implicated in a wide variety of brain functions including reward mechanisms[3, 4]. Deletion of the mu-opioid receptor (MOR) gene in mice abolishes rewarding properties of opiates as well as non-opioid drugs[5, 6] and decreases consumption of highly palatable foods[7, 8], which could be due to an altered motivational state in these animals[9]. MOR knockout mice (MOR-/-) also exhibit diminished food anticipatory activity, a behavior linked to a deficit in motivational processes[10].

It is well established that dysregulation of reward processing leads to cognitive control deficits, at the heart of many psychiatric disorders including substance use disorders (SUDs)[11-14]. Our previous study has already demonstrated that MOR is

involved in motor impulsivity, as MOR-/- mice show reduced premature responses in a signaled nose poke task[15] and the regulation of inhibitory control by MORs was attributed to the NAc[16] in another study. Considering the well-documented self-control deficits linked to reward dysregulation in drug abusers[11, 17-19], the further investigation of MOR function in cognitive controls is worth pursuing.

Here we used our previously characterized MOR knockout mouse line[20] to explore the role of MORs in reward-related higher order cognitive functions. To this aim, we used a TouchScreen-based apparatus that allows improved, standardized and automated behavioral testing[21]. We performed a series of tasks to specifically investigate (i) associative learning, (ii) reward value of naturally highly appetitive stimuli (autoshaping task), (iii) attention, (vi) motivation and (v) impulsivity/compulsivity (5-Choice Serial Reaction Time Task). Our data show that deletion of MORs delays pavlovian learning and reduces motivation and inhibitory control.

Material and Methods

Animals

MOR-/- mice lacking mu opioid receptors were produced as previously described[22] under a mixed background (50% C57B1/6J:50% 129/SvPas) and compared to their wild type littermates (MOR+/+). All mice were bred at the Douglas Research Centre. Animals were group-housed (3-5 animals per cage) for all the experiments under a 12 h light/dark cycle and received water and food *ad libitum* until one week prior to experiments. During experiments, mice underwent a restriction diet, which consisted in providing restricted amounts of standard laboratory chow pellets daily at 6 p.m and maintain all animals at approximately 85% of respective baseline free feeding weight. Drinking water was available ad libitum. All animal procedures were conducted in accordance with the guidelines set forth by the Canadian Council of Animal Care and by the Animal Care Committees of McGill University/Douglas Mental Health University Institute.

Touchscreen Apparatus

All procedures were accomplished in standard mouse Bussey-Saksida Touchscreen system (Model 80614-20, Campden Instruments Ltd). For both experiments, mice are tested one session per day, 6 days per week. The test apparatus consists of fiberboard box (25 cm × 25 cm) individually housed within sound-attenuating cabinets and low-level noise fans. Each box comprises a standard operant chamber and a touchscreen (12.1-inch monitor). The ceiling of the chamber is made of clear Plexiglas and the floor of perforated stainless steel with a waste tray situated below. Within the chamber is a trapezoidal shape constructed from three black Plexiglas walls, which open to the touchscreen (Dimensions: 20x18cm screen-reward tray x 24cm at screen, or 6cm at reward tray). This shape is specially designed to direct the attention of the animal towards the touchscreen and reward tray (or food magazine). Located centrally either on the rear aluminum wall or attached to the wall opposite the touchscreen (depending on the test, see specific description of behavioural procedures below) is a food magazine linked to a liquid dispenser pump (Strawberry milkshake (Quebon) serves as

food reward). A light emitting diode illuminates the food magazine. Computer graphic white square stimuli are presented on the touchscreen. Depending on the task, different black aluminum masks are attached to the face of the screen approximately 1.5 cm from the surface of the display. These masks serve to restrict the mouse's access to the display except through response apertures. A miniature infrared camera is installed above the chamber to allow monitoring of the animals' behaviour. Animal activity is recorded via infrared photobeams traversing the sidewalls of the chamber at the front (screen) and rear (reward tray). The apparatus and online data collection for each chamber are controlled by a Dell computer connected to an Animal Behavior Environmental Test system (Lafayette Instruments) using the Whisker control system for research[23]. All experiments were performed with the houselight off.

Behavioural procedures

Autoshaping, apparatus- Autoshaping is a test that typically measures pavlovian approach learning. During Autoshaping, the chamber as well as the infrared photobeams must be in the autoshaping configuration. In this configuration the reward unit is fixed in the center on the same side as the touchscreen inside the chamber and the front beam is divided into two independent beams allowing for measurement of approaches to each side of the touchscreen separately. The autoshaping mask (24.3x28.0cm) is placed in front of the screen, providing two response windows (two white vertical rectangles of 6.5x14 cm) displayed on the left and right of the reward tray. The protocol used was adapted from the training schedule described in Horner et al, 2013.

Autoshaping, protocol- The protocol is described in detail in Figure 1A. *Training*. Mice were initially given one 30 minutes session per day for two consecutive days in which they were allowed to habituate to the testing chamber and collect the reward (strawberry milkshake) from the central food magazine. The house light was illuminated, and reward was delivered into the central magazine on a variable intertrial interval (ITI) 10-40 sec schedule. For the mice to familiarize with the floor panel, a bolus of milkshake was delivered at the beginning of the session. *Test.* One day after pre-training, mice began the training for the autoshaping task where they learn to associate one side of the screen as positive conditioned stimulus (CSp) and the other side as CSn. The CSp signals the delivery of 7ul milkshake immediately after the offset of the stimulus, associated with a tone and the illumination of the tray light. No tone or reward upon CSn display. One trial consists of the presentation of CSp or CSn in a randomized order during 10 seconds. After stimulus presentation (and entrance to the magazine for reward collection if CSp displayed), an inter-trial interval (ITI) variable begins (10-40s), after which the mouse is required to break the infrared beam at the rear of the chamber causing initiation of the next trial. This variable schedule ensures that the mice approach behavior is not temporally mediated and requiring the animal to go to the back of the chamber to initiate trials reduces chance approaches and ensures equal stimulus sampling. When a mouse breaks either the left or right infrared beam that runs either side of the central food magazine, it is scored as an approach to that stimulus, and no additional approaches are scored under "lit CS" during that stimulus presentation. If the first approach during a trial is to the unlit screen, it falls under the "total CS" category. All

additional approaches during before and after a trial fall under the “all CS” category (figures in supplementary). Stimulus presentation is performed in pairs, such that within a 40 trial session, there are 20 presentations of each CSp and CSn. CSp and CSn side selection is counterbalanced across subjects as are testing times across experimental groups. Mice were tested one session per day, 6 days per week.

5-Choice Reaction Time Task (5-CSRTt), apparatus- 5-CSRT is a test that typically measures attention and impulsivity. A photograph of the test apparatus for the autoshaping task is provided in Figure 1B. During 5-CSRT, the chamber as well as the infrared photobeams are set in the “normal” configuration. In this configuration, the reward dispenser is fixed on the opposite side to the touchscreen. The 5-CSRT mask (24.3x28.0cm) is placed in front of the screen, providing five response windows (small squares of 3x3 cm). Stimulus displays are represented as a white square in dimensions similar to the response windows. The protocol used was adapted from the training schedule described in Hoerner et al 2013[24].

5-CSRTt, protocol- The protocol is described in detail in Figure 1B. *Training*. Given the complexity of the task, a 4-step training is required before the mice can reach testing phase. Criteria are set for each step and must be reached for a mouse to be able to move to the next step. In step I, a phase that is similar to the habituation in Autoshaping, mice are initially given 2 sessions (30 min), in which they are allowed to habituate to the testing chamber and collect milkshake from the food magazine. Once mice are reliably retrieving and consuming the reward (30 trials in 30 minutes), they can move to the next training. In step II called “Initial Touch”, mice are trained to detect a brief visual stimulus presented randomly in 1 of the 5 spatial locations. After a delay, the image is removed and strawberry milkshake is delivered accompanied by illumination of the reward light. Collection of the reward turns off the reward light and the ITI begins (5s), after which another stimulus is presented. If the mouse touches the stimulus while it is being displayed, the image is removed and three times as much reward is delivered immediately in order to reinforce the mouse’s correct touching behavior. This is repeated until the 30 trials in 60 minutes criterion is reached. In step III or “Must Touch”, mice have to touch the stimulus in order to obtain reward delivery. The criterion is 30 trials per 60 minutes. In step IV or “Must Initiate”, mice are expected to initiate the next trial by entering and exiting the reward tray with their heads. The rest of the session was similar to step III. Once the criterion has been reached for two consecutive sessions, the animals are allowed to proceed to baseline 5-CSRT testing.

Test. In baseline testing, the first trial is initiated when the mouse collects the reward from the food magazine. After a fixed 5 s intertrial interval (ITI), the stimulus appears in one of the windows for a short period (32 s). Responses in this aperture during illumination and for 5 s afterwards (the limited hold period) are rewarded with the delivery of a single reward dose, and a correct response is recorded. Responses in a non-illuminated hole during the signal period (incorrect response) and failures to respond within the limited hold period (omission) result in a timeout period during which the house light is on for 5 s. Responses in the apertures during the ITI are recorded as premature responses and also result in a timeout period. Additional responses in the apertures during the limited hold period following a correct response are recorded as

perseverative responses. A response in the food magazine after the delivery of the reward, or after the timeout period, initiates the next trial. The 5-CSRT session is repeated until animals complete 40 trials/session and achieve >80% accuracy and <20% omissions on two consecutive days. After criteria are met, the animal is advanced to the next test phase which involves reduced stimulus duration to test for deficits in attentional accuracy.

Statistical analyses

Depending on the figure, data were analyzed using appropriate statistical test, including two-tailed unpaired t-test, two-way analysis of variance (ANOVA) or two-way repeated measures ANOVA. Significant main effects and/or interactions of the ANOVAs were further investigated with the Bonferroni's post hoc test or method of contrast analysis. Detailed statistics information related to all figures 2-5 and supplementary Figures S1-S3 are shown in supplementary Table 1. Data are expressed as mean±s.e.m. Statistical significance was set at $p<0.05$.

Results

Experiment 1: Autoshaping

Inhibition of MOR activity, whether genetic or pharmacological, results in the alteration of reward processing with different drugs of abuse[4, 6, 22]. However, little is known about learning performance of the stimulus-reward association in MOR-/- mice, and even less so in the context of natural rewards such as palatable food. In order to explore the effect of MOR knockout on stimulus-reward learning, we first conducted an autoshaping task, which is a discriminative conditioning procedure based on pavlovian approach learning of visual stimuli associated or not with the delivery of a reward. This task can be fractioned into two periods: a training phase and a testing phase (see Figure 1A. for detailed protocol and Supplementary Table 1 for detailed statistical analysis).

MOR knockout mice show delayed learning in the autoshaping task (training phase). The first step of this task is a training or habituation phase that consists in learning the association tray-visual cue-apparition of a reward. As shown in Figure 2A, MOR-/- mice spent a significantly higher number of sessions to reach criterion in this phase compared to MOR+/+ mice ($t_{(21)} = 3.64$, $p < 0.01$). Two-way ANOVA for trials per first vs. last session indicated significant effects of session (Figure 2B.left, $F_{(1.21)} = 1.62$, $p < 0.001$) and genotype ($F_{(1.21)} = 20.92$, $p < 0.001$) but no interaction. Subsequent analysis using method of contrast revealed an effect for first ($p<0.01$) but not last session, suggesting that MOR-/- mice learn, but slowly compared to their controls. Consequently, the average number of trials over the training phase is significantly reduced in MOR-/- (Figure 2B.right, $t_{(21)} = 4.05$, $p < 0.001$). At the end of the training phase, all animals of both genotypes reached criteria (40 trials per session of 40 minutes) and were able to move to the testing phase.

During this phase, mice have to learn the stimulus-reward association. As shown in Figure 2C, two-way ANOVA for session length for first vs. last session revealed significant effects of genotype ($F_{(1.21)} = 15.42$, $p < 0.001$), session ($F_{(1.21)} = 10.79$, $p <$

0.01) and interaction ($F_{(1.21)}$ = 8.01, p <0.05). Bonferroni post-hoc analysis showed a significant genotype effect for last (p<0.001) but not first session. In addition, two-way ANOVA for trials numbers during first vs. last session revealed genotype (Figure 2D. $F_{(1.21)}$ = 15.42, p < 0.001) and session ($F_{(1.21)}$ = 15.6, p < 0.001) effect but no interaction. Subsequent analysis using method of contrast showed a significant decrease in number of trials for MOR-/- during the first (p < 0.05) and last session (p<0.001) compared to MOR+/+ mice. Altogether, all animals reached the test phase of the autoshaping task but a lower number of MOR-/- than MOR+/+ mice reached criterion during test phase (40 trials per session of one hour), indicating altogether that the learning performance is lower in mutant mice. Subsequent analysis only includes mice that have reached criterion during the autoshaping test phase.

MOR knockout mice show normal discriminatory behavior in the autoshaping task (test phase). During the test phase of an autoshaping task, mice usually escalate the number of approaches or touches in response to CSp while the number of approaches and touches associated with the CSn stabilizes. In order to evaluate the effect of the MOR gene knockout on discriminated behavior, we analyzed the number of approaches and touches for either CSp or CSn in mice that had reached the test criteria (40 trials per session of one hour during testing phase). Here, we show the results for all three as well as the average per block of ten trials during the first session. Both MOR+/+ and MOR-/- mice increased their CSp approaches across the three first sessions (Figure 3.I.A, B left) as well as across the blocks of the first session (Figure 3.I.C, D left). Two-way ANOVA with repeated measures (RM) for approaches per session revealed a significant session effect for total CSp approaches (defined as the total number of approaches towards the CSp when either the CSp or the CSn is lit, Figure 3.I.B left, $F_{(2,30)}$ = 3.24, p = 0.05) suggesting that mice from both groups reaching criterion during training phase learn to associate the CSp to the reward. No significant session effect on number of lit (defined as the number of approaches specifically towards the CSp when the CSp is lit Figure 3.I.A.left) and all CSp approaches were detected. Also, no genotype or interaction effects were found significant for any tested parameters. Two-way ANOVA with RM performed on number of approaches per block of 10 trails during the first session revealed a significant block effect for lit CSp (Figure 3.I.C left, $F_{(3,45)}$ = 3.54, p < 0.05) strengthening the idea that, when reached criterion, MOR+/+ and MOR-/- mice learned the stimulus-reward association similarly. No significant session effect on number of total (Figure 3.I.D left) and all CSp (supplementary Figure 1A, C) approaches were detected. Also, no genotype or interaction effects were found significant for any tested parameters and the average of lit and total CSp across sessions and blocks were similar in both genotypes (Figure 3.I.A, B, C, D right).

Equally, both MOR+/+ and MOR-/- mice showed a tendency to increase touches of the CSp across sessions while stabilizing touches of the CSn (Figure 3.II). The average of touches for Lit CSp, total CSp and all CSp were similar in both genotypes across the sessions (3.II.A, C, E right). Two-way ANOVA performed on number of touches over sessions showed a significant session effect for Lit CSp touches (Figure 3.II.A left, $F_{(2,30)}$ = 3.58, p < 0.05). No significant session effect on number of total (Figure 3.II.C left) and all CSp (Supplementary figure 1B) touches were detected. Also,

no significant effects for genotype or interaction for any of the three parameters were detected. MOR+/+ and MOR-/- also showed similar numbers of lit, total and all CSn touches (see table 1 and Figure 3.II.B, D right as well as Supplementary Figure 1D). Two-way ANOVA performed on touches per session showed no effect of time, genotype or interaction (Figure 3.II.B, D, F left). Our analysis therefore suggests that MOR+/+ and MOR-/- mice do not differ during the test phase.

In sum, data from the autoshaping task indicate that MOR-/- animals show a lower performance in the acquisition of stimulus-reward association compared to controls during the training phase. Mutant animals that reached criterion further escalated their behavior towards CSp while stabilizing behavior in response to CSn similarly to controls, indicating that learning is delayed but Cs discrimination is preserved in MOR-/- mice.

Experiment 2: 5 Choice Serial Reaction Time task

Next, we assessed whether the lower performance in the acquisition of stimulus-reward learning observed in MOR-/- mice during the autoshaping task was due to a decrease in specific cognitive functions. We thus performed a 5-CSRT task that mainly explores attention and impulsivity. This test can also be fractioned into training and testing phases (see Figure 1B. for detailed protocol and Supplementary Table 1 for detailed statistical analysis).

MOR knockout mice show delayed learning in the 5-CSRT task (training phase). The 5-CSRT, a test that mainly explores attention and impulsivity, can also provide measures of learning, as the cognitive complexity of the test requires a long training period before mice can undergo the test. This training is divided into four main steps and can last as many sessions at it takes (with one session per day) for the animal to reach the criteria (40 trials on two out of three consecutive days) and move to the next step.

In the habituation phase (step I), a first observation was that the total number of sessions required for training was significantly increased in MOR-/- compared to MOR+/+ mice (Supplementary Figure 3E. $t_{(11)} = 2.85$, $p < 0.05$), indicating that overall knockout animals take longer to reach criteria and move on to the test. The analysis of each training step, further indicates that the difference of total number of sessions is due to a significant genotype effect in the first step of the training (Figure 4A. $t_{(11)} = 6.5$, $p < 0.0001$). In this habituation phase, similar to the autoshaping training phase, mice learn to associate the tray to the apparition of a reward. A two-way ANOVA for number of trials during the first vs. last session revealed a significant main effect of sessions (Figure 4B left. $F_{(1,22)} = 7.82$, $p < 0.001$), no effect of genotype and a significant interaction ($F_{(1,22)} = 2.66$, $p < 0.05$). Bonferroni Post hoc analysis showed significant decrease of trial counters at first ($p < 0.01$) but not last session. However, there was no significant effect on the total number of trial counter over all sessions (Figure 4B right), suggesting that MOR-/- mice learn though at a slower rate.

Next, we tested whether the difference of trial counters impacts latency to collect reward. As shown in Figure 4C left, two-way ANOVA for reward latency during the first vs. last session of the training phase revealed significant effects of session ($F_{(1,11)} = 15.78$, $p < 0.01$), genotype ($F_{(1,11)} = 10.84$, $p < 0.01$) and interaction ($F_{(1,11)} = 7.44$, $p <$

0.05). Similar to trial counter, Bonferroni post hoc analysis showed significant decrease of trial counters at first ($p < 0.001$) but not last session. Interestingly in this case, the average total latency to collect reward was also significantly increased in MOR-/- mice (Figure 4C right, $t_{(37)} = 2.52$, $p < 0.05$) compared to MOR+/+ mice. As for the autoshaping task, therefore, MOR-/- mice show delayed learning during the habituation phase, and the higher latency to collect reward further suggests decreased reward value in these animals.

In the three other training steps, we observed no genotype difference, except for the number of trial counters in step III (Figure 4H right. $t_{(70)} = 2.76$, $p < 0.01$), the "Must Touch" phase in which mice have to touch the stimulus in order to obtain a reward. This could be due once again to a delay to reach criterion in a step that is complex and the acquisition of which takes up to 8 days (see Supplementary Figure 2). No significant genotype effect was observed for step II (Figure 4. D, E, F, see table 1) or step IV (Figure 4. J, K, L).

In fine therefore, all the mice learned the task and reached criteria to move to test phase. Similarly to autoshaping training, however, MOR-/- mice showed delay in learning during the habituation step, and this delay was associated with a reduction in reward value.

MOR knockout mice show intact attention in the 5-CSRT task (test phase). The 5-CSRT test delivers information on accuracy, a measure of attention. Accuracy of performance was measured as the proportion of correct responses (correct responses/total responses) expressed as a percentage, without including errors of omission. Because each mouse spends a specific number of sessions per interval, the results are presented per interval rather than per session, an interval being defined by the time of appearance of the stimulus (32, 16, 8, 4 or 2 seconds) during a given number of intervals until criteria (>80% accuracy, <20% omission) are reached.

Two-way ANOVA for the average of accuracy percentage per interval revealed a main effect of interval (Figure 5A. $F_{(4.55)} = 40.42$, $p < 0.0001$) but no effect of genotype and no interaction, meaning that MOR+/+ and MOR-/- mice display similar percentage of accuracy. Performing a two-way ANOVA on first (Figure 5B. $F_{(4.55)} = 33.66$, $p < 0.0001$) or last (Figure 5C. $F_{(4.55)} = 8.66$, $p < 0.0001$) session of each interval also showed a main effect of interval but no effect of genotype or interaction. Altogether therefore attention processes seem intact in MOR-/- mice, at least as measured by the 5-CSRT task.

MOR knockout mice show decreased motivation during first but not last trial of the 5-CSRT task (test phase). Errors of omission were defined as failures to make a response during the 5 s limited hold period, expressed as a percentage of the total number of trials. This measure reflects possible failures of detection as well as motivational/motor deficits, depending on the overall pattern of effects[25].

Two-way ANOVA for the average of omission percentage per interval revealed a significant effect of interval (Figure 5D. $F_{(4.55)} = 54.04$, $p < 0.0001$) and genotype ($F_{(4.55)} = 10.88$, $p < 0.01$) but no interaction. Performing a two-way ANOVA on first (Figure 5E. $F_{(4.55)} = 47.38$, $p < 0.0001$) or last (Figure 5F. $F_{(4.55)} = 33.43$, $p < 0.0001$) session of each interval confirmed the effect of interval. However, the genotype effect was only present

in the first session of each interval ($F_{(4.55)}$ = 16.03, $p < 0.001$), not the last. There was no interaction in either the first or the last sessions. MOR-/- mice thus show an increase of % omission in the first session of each interval compared to control mice and this is compensated at the last session of every phase.

An increase of response omissions in the absence of accuracy change is regarded as an indicator for decreased motivation and/or motor deficit i.e. a goal that stimulates an action[25]. As an indicator of both motor activity and goal-directed behavior, we measured the front and back beam breaks, the front being the screen beam (sign) and the back being the reward tray beam (goal). Two-way ANOVA showed a significant decrease of back beam breaks in MOR-/- compared to MOR+/+ mice with a main effect of genotype (supplementary Figure 3B. $F_{(1.55)}$ = 11.3, $p < 0.01$) but no effect of interval and no interaction. There was no genotype difference for the front beam breaks (supplementary Figure 3A). Together therefore, data showing increased omission with intact accuracy in the first session strongly suggest decreased motivation for the reward in MOR-/- mice, and is in line with decreased reward value detected in the autoshaping task.

MOR knockout mice show decreased perseverative responses in the 5-CSRT task (test phase). We have previously reported that MORs normally facilitate motor impulsivity, based on the observation that MOR-/- mice perform remarkably well in a signaled nose poke task[15]. 5-CSRTt also provides a measure of motor impulsivity (premature responses). This task additionally provides a measure of compulsivity (perseverative responses) that is relatively outcome-insensitive, as opposed to the habitual nature of relatively incentively motivated impulsive action[26].

Given the very low number of premature responses recorded for our control mice, we did not observe significant reduction of motor impulsivity in MOR-/- mice as reported in our previous study. However, there was a trend despite the limiting factor (see Figure 5 G to I). Interestingly, we observed a drastic decrease of perseverative responses in MOR-/- mice compared to MOR+/+ animals (Figure 5 J to L). Two-way ANOVA performed on genotype per interval revealed a main genotype effect in the average of sessions per interval (Figure 5J. $F_{(1.55)}$ = 79.67, $p < 0.0001$). This result was similarly strong for the first (Figure 5K. $F_{(1.55)}$ = 39.59, $p < 0.0001$) and last (Figure 5L. $F_{(1.55)}$ = 50.76, $p < 0.0001$) session of each interval. Two-way ANOVA also revealed an interval effect for the average of sessions per interval (Figure 5J. $F_{(4.55)}$ = 2.78, $p < 0.05$) but not for the first or last sessions of each interval. There was no genotype effect per interval interaction.

Altogether therefore, MOR-/- mice showed a trend for lower motor impulsivity, although this effect did not reach statistical significance under our experimental conditions, and also showed significantly reduced perseverative behavior in the 5-CSRT task, which can also be interpreted as lower compulsive behavior (see discussion).

Discussion

Here we investigated whether MORs regulate reward-related processing and cognitive functions using autoshaping and 5-CSRT tasks in the Touchscreen. Our results reveal that total deletion of the MOR gene modifies a subset of cognitive functions (delayed learning, lower motivation and increased perseveration) while others are preserved (discriminatory behavior and attention).

First, our study demonstrates that MOR activity promotes the acquisition of a stimulus/natural reward association. We found that MOR-/- mice exhibit delayed learning in training phases of both autoshaping and 5-CSRT tasks. During these phases, the number of sessions required to reach criterion was increased and trial counts per session was reduced in MOR-/- mice, while session lengths were increased. All these measures of poor learning performance converge to suggest that MORs normally facilitate the stimulus-reward learning rate.

One mechanism underlying this phenotype may be the known role of MORs in mediating palatable food reward. In fact, there is a large literature showing decreased reward value for palatable food in MOR-/- mice as measured by a decreased rate of licks with both sucrose and sucralose, a calorie-free substance that allows study of licking behavior independently from homeostatic variables, thus providing more direct evidence of MORs contribution to hedonic palatability[8]. Pharmacological investigation of brain structures responsible for this effect further demonstrates that MOR blockade both intraventricularly [27] or directly in the NAc[28, 29] decreases licking and consumption of palatable solutions whereas MORs agonists increase these behavior.

In accordance, MOR-/- mice in our study show higher latency to collect reward in the 5-CSRT training phase. Further, MOR-/- mice also show decreased response rates, as measured by increased omissions across trials during the 5-CSRT. This observation is reminiscent to data showing reduced response rate in MOR-/- mice self-administering palatable food[9, 30], and validates the role of MORs in palatable food-driven operant behavior. Together therefore, the data suggest that delayed learning observed throughout this study results from the lower reward value of palatable food in the touchscreen (strawberry milkshake). Our data and the literature thus point at a role of MORs at the intersection between hedonic palatability and motivational reward, both converging on a reduced licking and consumption behavior. This phenotype echoes the positive association between increased frequency of the A118G single nucleotide polymorphism of the MOR and binge eating in obese patients[31], and further justifies the clinical efficacy of MOR antagonists in reducing size and frequency of binging in bulimic patients[27, 32]. Future studies with a robust phenotype and genotype characterization in humans will help uncover bulimic patients that can benefit from treatment with drugs targeting the opioid system.

A second and main aspect of the study is that MOR activity facilitates compulsive-like behavior. Indeed, MOR-/- mice showed a drastic reduction of perseverative responses in the test phase of the 5CSRT task compared to their control animals. Our laboratory already showed decreased motor impulsivity in MOR-/- mice as measured by premature responses in a signaled nose poke task[15], suggesting that inhibitory control is

stronger in mutant mice. In the present study we were not able to demonstrate a similar reduction of premature responses, as the baseline was very low, however we found highly decreased perseverative responses. This phenotype can be interpreted as a reduced compulsive-like behavior. Impulsivity/compulsivity is an umbrella term that can be fractioned in different sub-cognitive functions[33, 34]. While impulsivity depends on goal-directed motivational systems that mediate voluntary reward seeking [26], compulsivity can be defined as the maladaptive persistence of responding to a stimulus despite the fact that it is no longer rewarded[35]. Behavioural tasks that measure impulsivity have often fractioned the construct into two camps: impulsive decision and impulsive action (i.e. motor impulsivity or inhibitory control)[36]. Inhibitory control may also be recruited in the regulation of compulsive behavior[37], the substrate of which is partly habitual but not entirely clear[38]. Here we show that, in addition to being implicated in goal-directed impulsive actions[7, 26], MORs may also play crucial role in outcome-insensitive compulsions.

Together therefore, our previous and current data suggest that MOR normally favors both impulsivity[15] and compulsivity (this study), a notion that is particularly critical for SUDs whose one of the hallmarks is the transition from impulsive to compulsive actions[39, 40].

Two responses remained unaltered in MOR-/- mice, namely the discriminatory behavior (when acquired) in the autoshaping task and attention in the 5-CSRT test. A previous study already reported normal pavlovian-instrumental transfer, where MOR-/- mice showed increased CSp (20% sucrose) approaches and stabilized behavior towards the CSn, comparably to their controls[7]. Here we reproduced this result using a different task, further strengthening the notion that early phase MOR-delayed pavlovian learning does not impair later associative functions.

In our 5-CSRT paradigm, MOR-/- mice also showed preserved attention, as measured by percentage of accuracy (number of correct responses per session). Pharmacological MOR blockade in the NAc was reported to facilitate the loss of attention during fear learning[41]. To our knowledge however, no other study has reported attentional deficits in MOR-/- mice. It is therefore likely that, in a context of reward rather than fear, MOR activity does not contribute strongly to this particular aspect of cognition.

The preserved attentional performance in MOR mutant mice is in striking parallel with a clinical observation. It is well known that atomoxetine, the main prescription drug in ADHD, has strong abuse potential[42]. Clinical trials have used opioid receptor antagonist naltrexone to reduce abuse liability of the treatment, and shown that opioid receptor blockade does not interfere with the clinical benefits of stimulants, i.e. does not alter attention[43]. Our preclinical results therefore support the notion that an adjunct MOR antagonist may increase the benefit/risk ratio of traditional ADHD treatments.

Brain sites or circuits for reduced motivation, delayed reward value-based learning and increased perseveration in the behavior of MOR mutant mice have not been identified in this study. However, abundant literature indicates that structures underpinning these behaviors are the basal ganglia[44, 45], particularly the ventral striatum[16, 46] and the basolateral amygdala[47] that both happen to be rich in MORs and that both showed

altered connectivity with other basal ganglia regions in total[48] or conditional[3] MOR knockout mouse models. Another potential underlying structure could be the habenula that is strongly associated with cognition and inhibitory control[49, 50] and that happens to express the highest density of MORs[51]. Future studies will identify the specific brain circuit and MOR population responsible for the perseverative compulsive-like phenotype of MOR mutant mice.

Given the major translational advantage offered by the touchscreen[21, 52] (similar to Cantab® in humans), it would be interesting to test similar paradigms in the clinic. One approach would be to compare SUD (opiate or poly-drug users), ED (bulimia) and ADHD patients in the CANTAB stop-signal test and the intra-extra dimensional set shift that both provide measures of inhibitory control and compulsivity[53]. The results could provide parameters for the classification of populations by symptoms rather than disorders in an RDOC approach, allowing the design of personalized opioid treatments to targeted populations.

In conclusion, the broad link between reward/motivation, cognitive functions and cognitive control is complex [54, 55] and our study definitely positions MORs at the center of these processes. Beyond the traditional view of a main role for MORs in mediating drug and natural rewards, our study reveals a role for the receptor in more integrated facets of behavior, including the facilitation of reward-based motivation and learning, as well as the promotion of compulsive behavior. Regarding the later, the notion that blocking MOR activity may not only to limit drug reward but also reduce maladaptive habit forming is highly novel in the area of SUDs.

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Figure legends

Figure 1 Touchscreen protocols- All procedures are accomplished in standard mouse Bussey-Saksida Touchscreen system. **A- Autoshaping protocol.** The autoshaping protocol consists of a training and a testing phase (top panel). *Training phase*- The training phase is composed of a pre-habituation phase "pre-hab" i.e. handling and food deprivation (5 days) and a habituation phase named "hab" i.e. learning to associate the food tray to an apparition of the stimulus. Once mice reach criterion, they move to test. *Testing phase*- The testing phase is detailed in the bottom panel, with the flowchart overview of associative learning. Trial is initiated when the mouse inserts its head in the food tray. A stimulus is presented on one of the two sides of the screen. One side (CSp) is always followed by the apparition of a reward in the tray whereas the other is not followed by a reward (CSn). If CSp is displayed and the mouse collects its reward, an inter-trial interval (ITI) starts, at the end of which another trial starts. This loops back until mice reach criterion. **B- 5-Choice Serial Reaction Time task (5-CSRTt) protocol.** The 5-CSRTt protocol consists of a training and a testing phase. *Training phase*- The training phase is composed of 4 steps. Step I named "habituation" is similar to autoshaping training (pre-hab + hab). Step II named "Initial Touch", Step III named "Must Touch" and finally in Step IV name "Must Initiate". *Testing phase*- Once mice reach criterion, they move to the 5-CSRTt test phase. During test phase, mice have to initiate trial and touch the correct screen in order to obtain a reward, wait during an ITI interval then loop back. However, premature, incorrect and omission responses are punished by a time out (5-second time out during which house light is on and nothing can happen). See Material and Methods section for additional details.

Figure 2- MOR-/- mice show impaired acquisition of stimulus-reward association in the autoshaping task. Mice were tested in the TouchScreen autoshaping paradigm composed of a training and a testing phase. **A-B.** *Training phase*. The average number of sessions is higher (**A**) and the average number of trials per session for first and last session (**B** left panel) and the average number of trials per session across all the training sessions (**B** right panel) are lower in mutant mice**. C- D.** *Testing phase.* Session length (**C**) is reduced during the last session of this phase in control animals but not in MOR-/- and number of trials (**D**) is diminished in MOR-/- during the first and last session compared to MOR+/+ mice. Data are expressed as mean±s.e.m. N=10-13, * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Figure 3- MOR-/- mice show no alteration of discriminatory behavior in the autoshaping task. MOR-/- and their corresponding control animals that reached criterion (40 trials per session) during the autoshaping test phase exhibited comparable discriminatory behavior i.e. increased behavior towards CSp while CSn touches and approaches were stabilized. **I.AB –** Evolution (A and B left panel) and average (A and B left panel) of lit CSp (A) and CSp (A) approaches across sessions were similar for both genotype. **I.CD-** Evolution (C and D left panel) and average (C and D left panel) of lit CSp (C) and CSp (D) approaches across 10-trial blocks during the first session were similar for both genotype. **II.AB-** Evolution (A and B left panel) and average (A and B left panel) of lit CSp (A) and CSp (A) touches across sessions were similar for both

genotype. **II.CD-** Evolution (C and D left panel) and average (C and D left panel) of lit CSp (C) and CSp (D) approaches across 10-trial blocks during the first session were similar for both genotype. Data are expressed as mean±s.e.m. N=5-12, *p<0.05.

Figure 4- Learning is delayed and reward value is decreased for MOR-/- mice in the 5-Choice Serial Reaction Time task (5-CSRT). MOR-/- showed impaired acquisition of stimulus-reward association during the habituation part of the training phase. *Step I.* **A-** Average of number of sessions to reach criterion is higher in mutant mice. **B-** Number of trials per session is reduced in MOR-/- during the first session (left panel). No difference observed between both genotypes on number of trials during last session and on the average of trials (right panel) per session across all sessions. **C-** Reward latency for first session (left panel) is reduced in MOR-/- mice leading to a decrease in the average of reward latency per session across all step I sessions (right panel). *Step II-IV.* **DGJ-** Average of number of sessions is similar for MOR-/- and control animals for Initial touch (**D**), Must touch (**G**) and must initiate (**J**). **EHK-** Number of trials per session for first and last session (left panels) as well as average of trials per session across all sessions (right panels) of Initial touch (**E**), Must touch (**H**) and must initiate (**K**) phases are not altered in mutant mice. **FIL-** Reward latency for the first and last session (left panels) in addition to average of reward latency per session across (right panels) of Initial touch (**F**), Must touch (**I**) and must initiate (**L**) phases are not altered in mutant mice. Data are expressed as mean±s.e.m. N=7-8,* p<0.05; ** p<0.01; *** p<0.001.

Figure 5- MOR-/- mice show intact attention but lower motivation and perseveration in a 5-CSRT test.
MOR-/- mice showed a preserved attention processes and significantly reduced compulsive-like behavior in the 5-CSRT task. **A-C-** % of accuracy across different intervals of stimulus is similar for both genotypes. Data represent average of % of accuracy for all (**A**), first (**B**) and last (**C**) session for each interval. **D-F-** MOR-/- mice show an increase of % omission in the first session of each interval compared to control mice and this effect disappear during the last session of every phase. Data represent average of % omission for all (**D**), first (**E**) and last (**F**) session for each interval. **G-I-** No difference between MOR-/- and their control mice on the number of premature responses. Data represent the average of the number of premature response during all (**G**), the first (**H**) the last (**I**) session for each interval. **J-L-** MOR-/- mice showed a drastic decrease of perseverative responses in comparison to MOR+/+ animals. Data represent the average of the number of perseverative response during all (**J**), the first (**K**) the last (**L**) session for each interval. Data are expressed as mean±s.e.m. N=7-8, * p<0.05; ** p<0.01; *** p<0.001

Figure 1

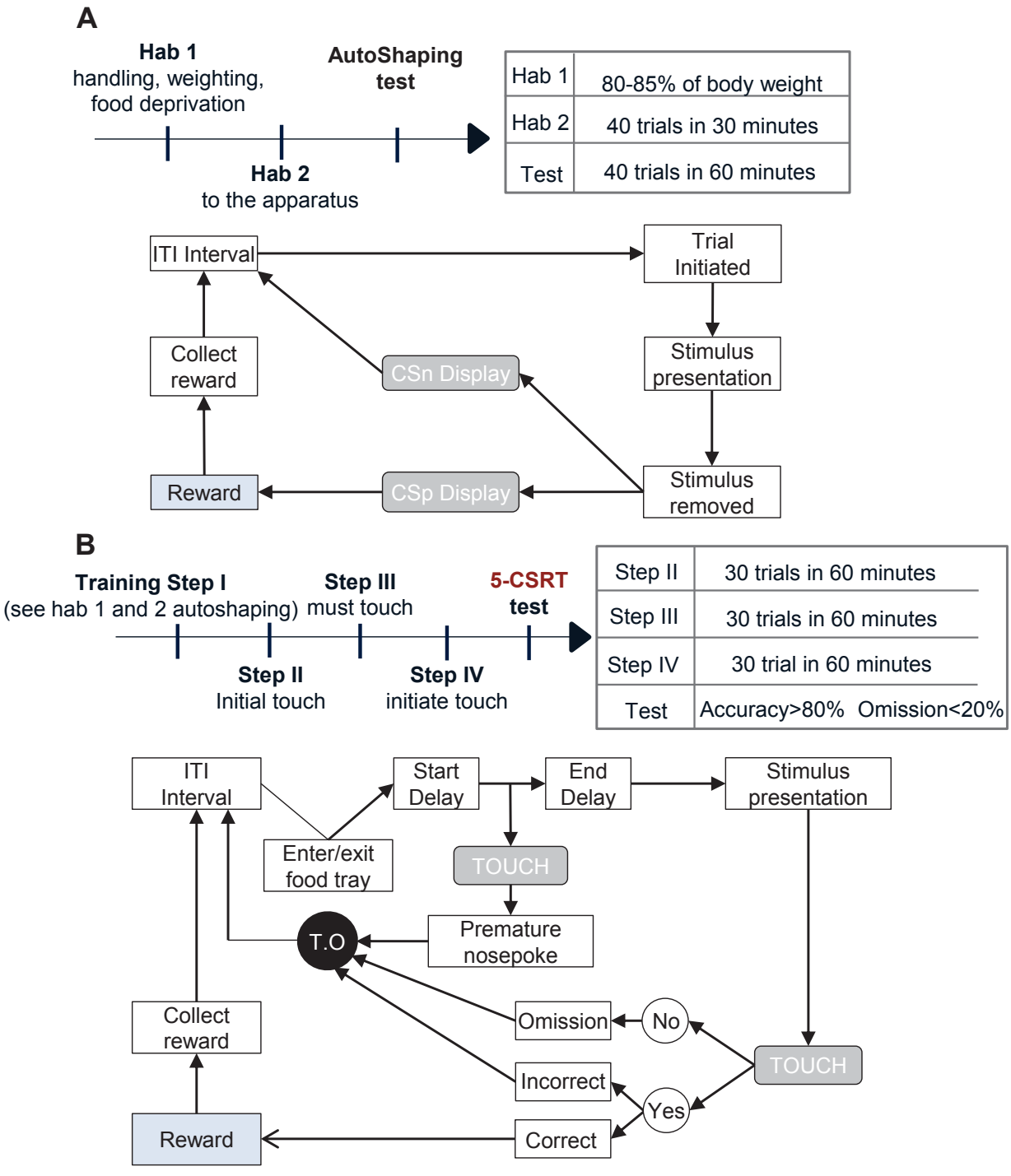

A
Hab 1
handling, weighting,
food deprivation
AutoShaping
test
Hab 2
to the apparatus
Hab 1
80-85% of body weight
Hab 2
40 trials in 30 minutes
Test
40 trials in 60 minutes
ITI Interval
Trial
Initiated
Collect
reward
CSn Display
Stimulus
presentation
Reward
CSp Display
Stimulus
removed
B
Training Step I
(see hab 1 and 2 autoshaping)
Step III
must touch
5-CSRT
test
Step II
Initial touch
Step IV
initiate touch
Step II
30 trials in 60 minutes
Step III
30 trials in 60 minutes
Step IV
30 trial in 60 minutes
Test
Accuracy>80% Omission<20%
ITI
Interval
Start
Delay
End
Delay
Stimulus
presentation
Enter/exit
food tray
TOUCH
T.O
Premature
nosepoke
Collect
reward
Omission
No
TOUCH
Incorrect
Yes
Reward
Correct

Figure 2

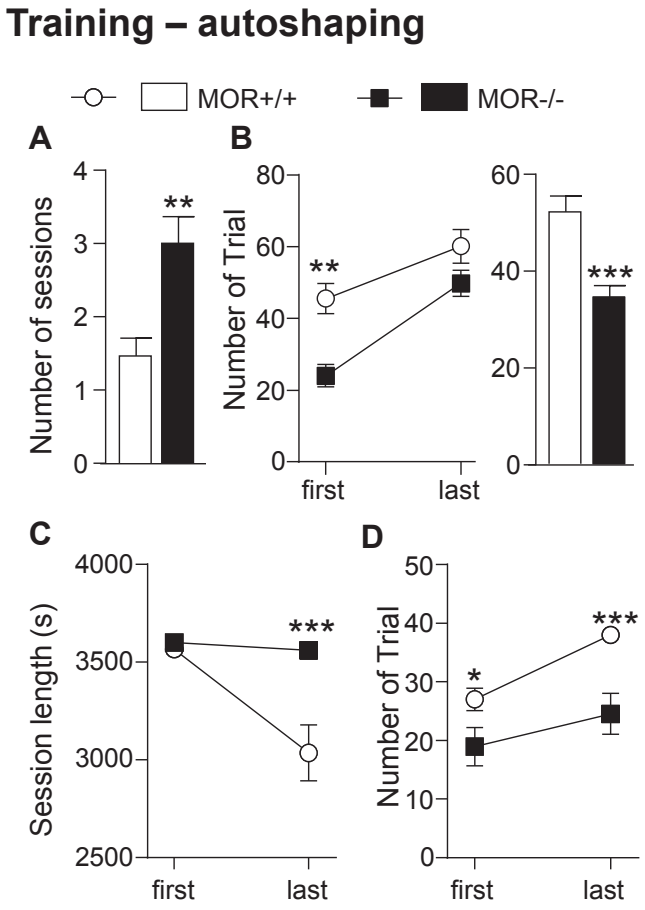

Training – autoshaping
MOR+/+
MOR-/-
A
B
Number of sessions
Number of Trial
first
last
C
D
Session length (s)
Number of Trial
first
last

Figure 3

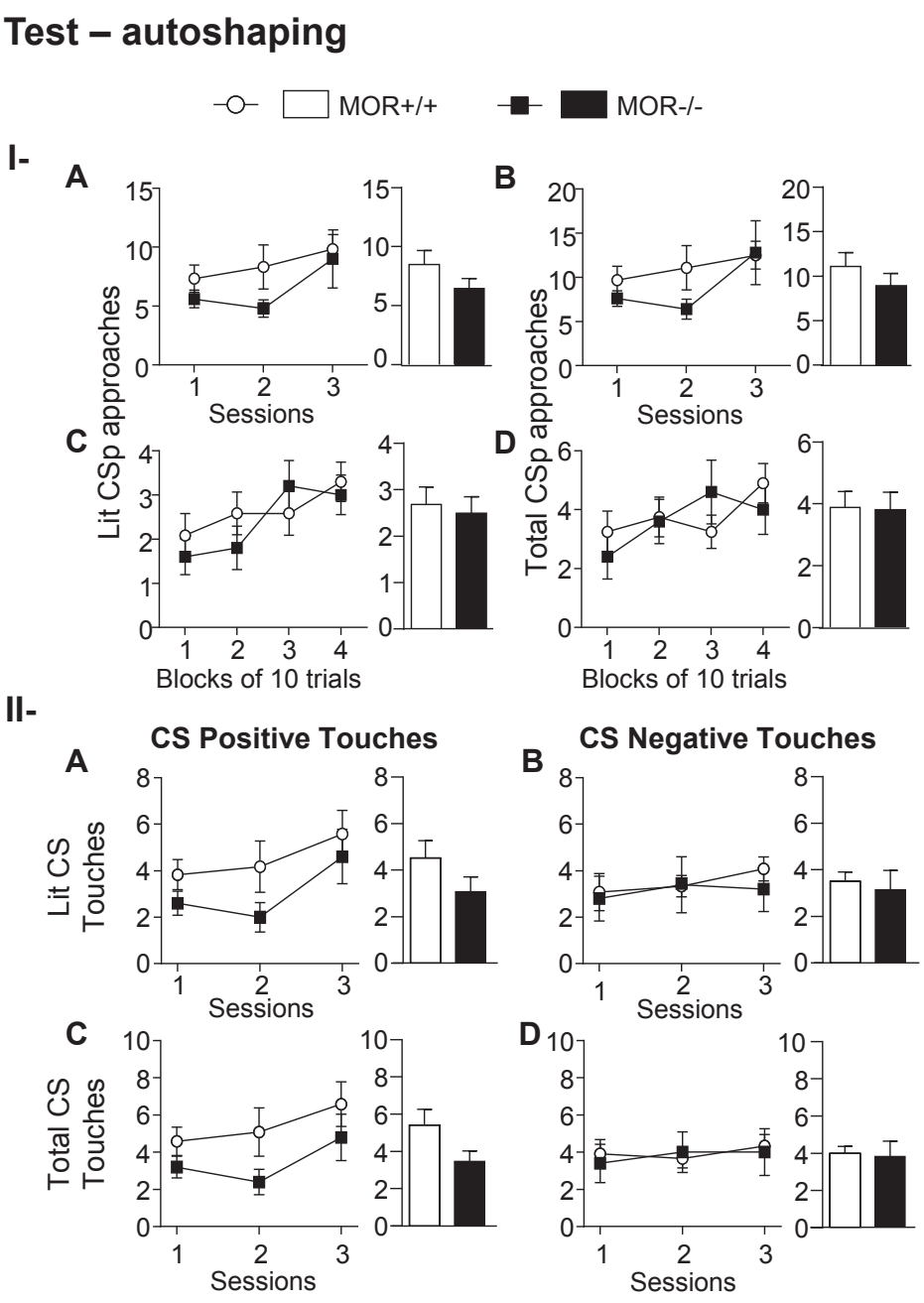

Test – autoshaping
MOR+/+
MOR-/-
I-
A
B
C
D
Lit CSp approaches
Total CSp approaches
Sessions
Blocks of 10 trials
II-
CS Positive Touches
CS Negative Touches
Lit CS Touches
Total CS Touches
Sessions

Figure 4

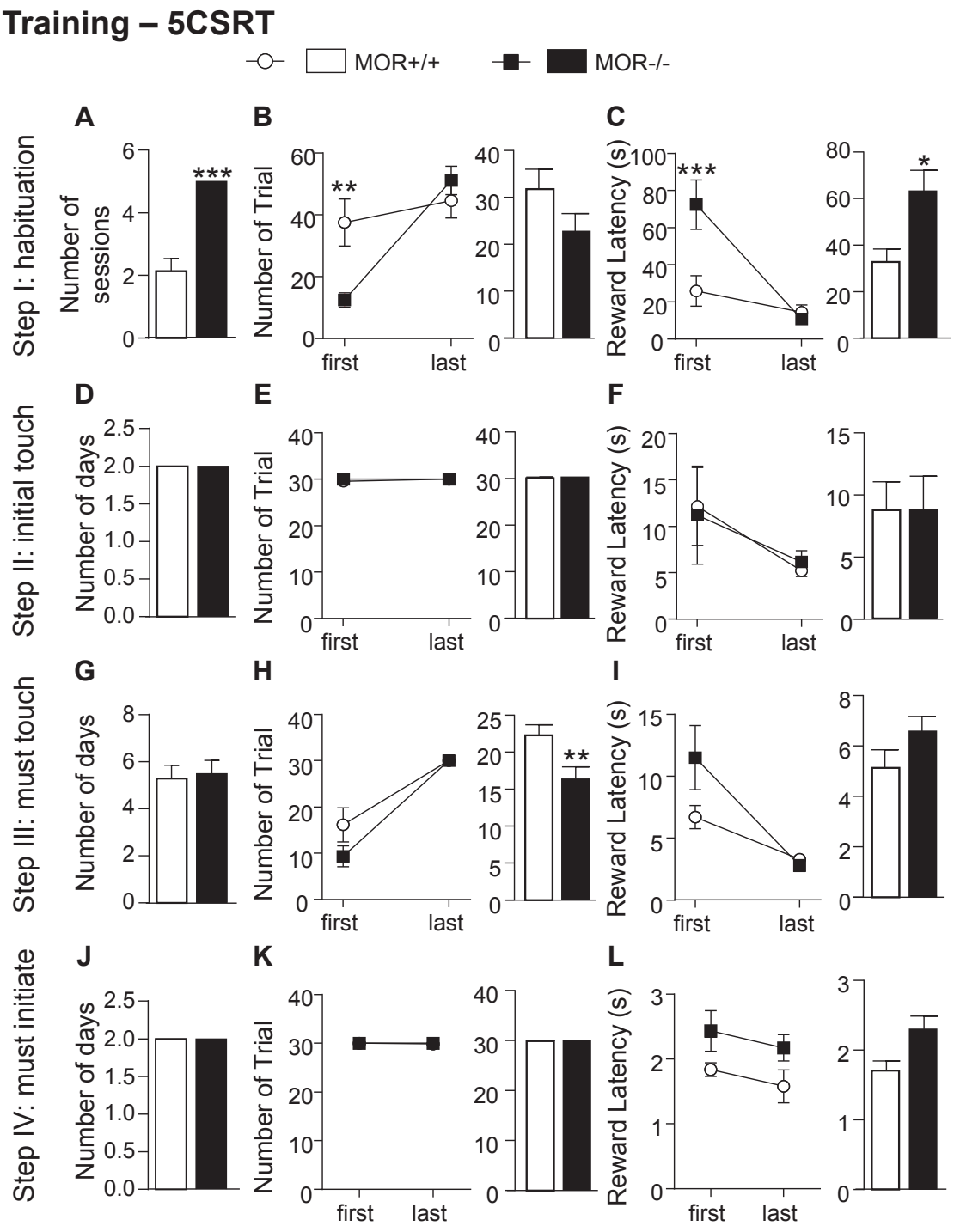
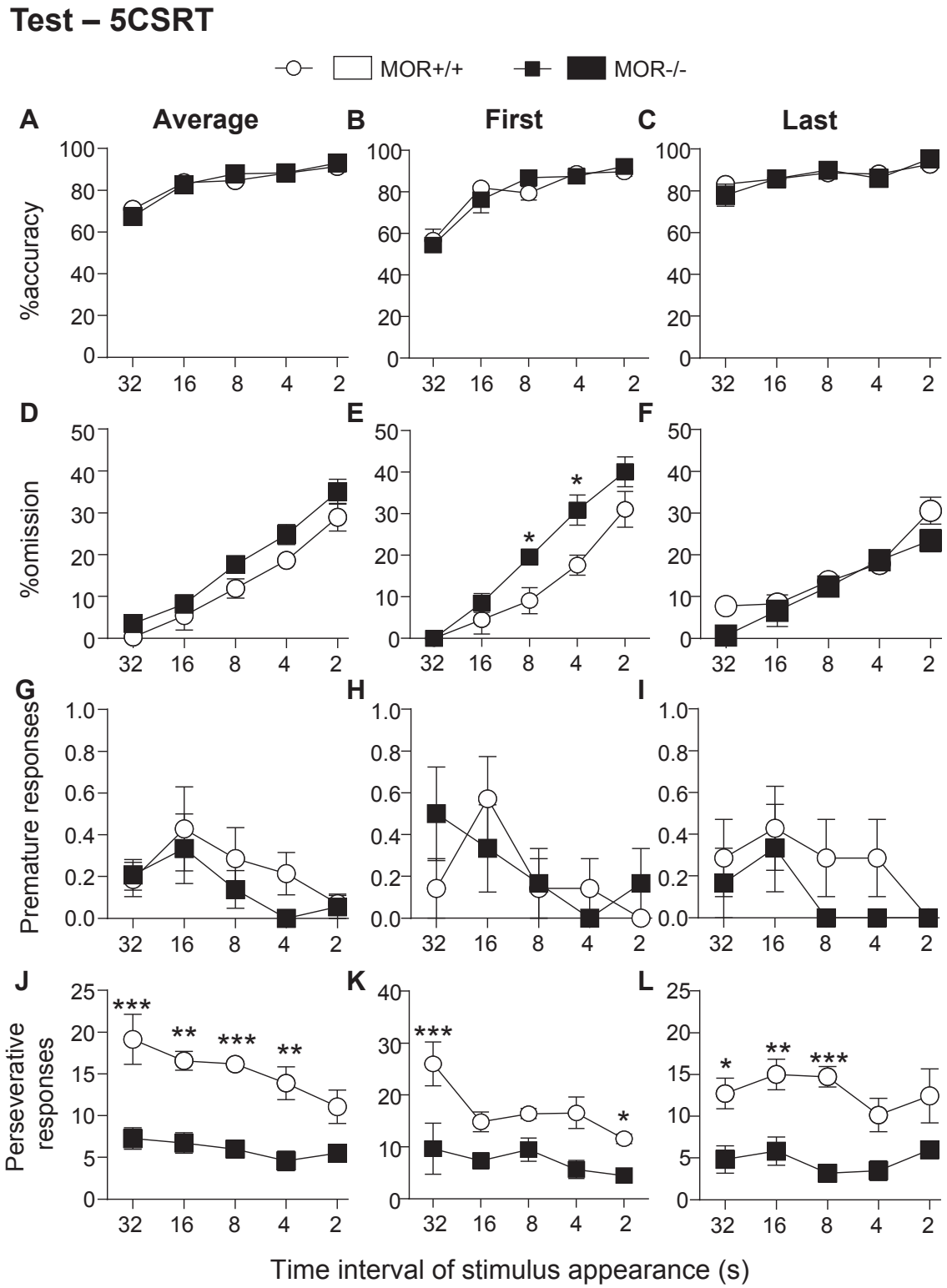
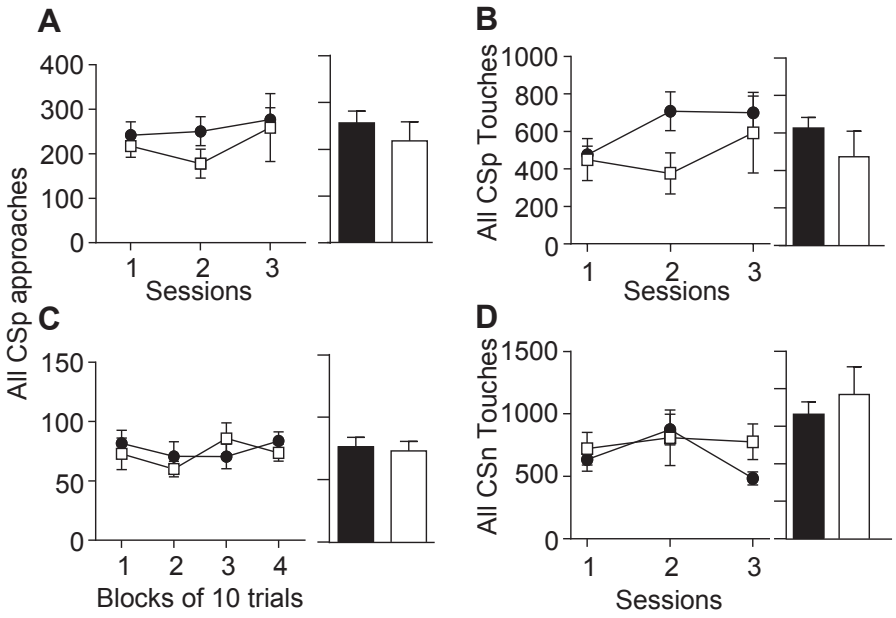

Training – 5CSRT
MOR+/+
MOR-/-
Step I: habituation
Step II: initial touch
Step III: must touch
Step IV: must initiate
A
B
C
D
E
F
G
H
I
J
K
L
Number of sessions
Number of days
Number of Trial
Reward Latency (s)
first
last
***
**
*

Figure 5


Test – 5CSRT
MOR+/+
MOR-/-
A
Average
B
First
C
Last
%accuracy
D
E
F
%omission
G
H
I
Premature responses
J
K
L
Perseverative responses
Time interval of stimulus appearance (s)

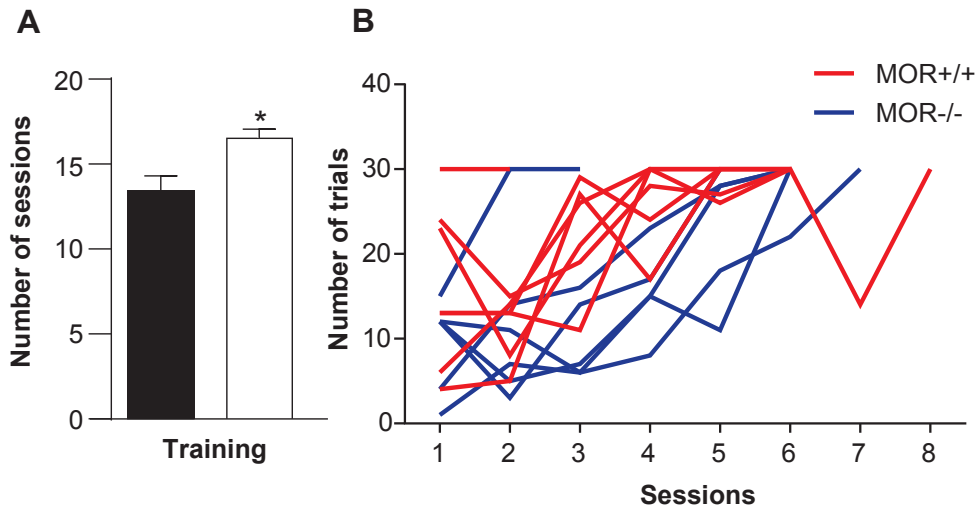
Supplementary

Supplementary Figure 1



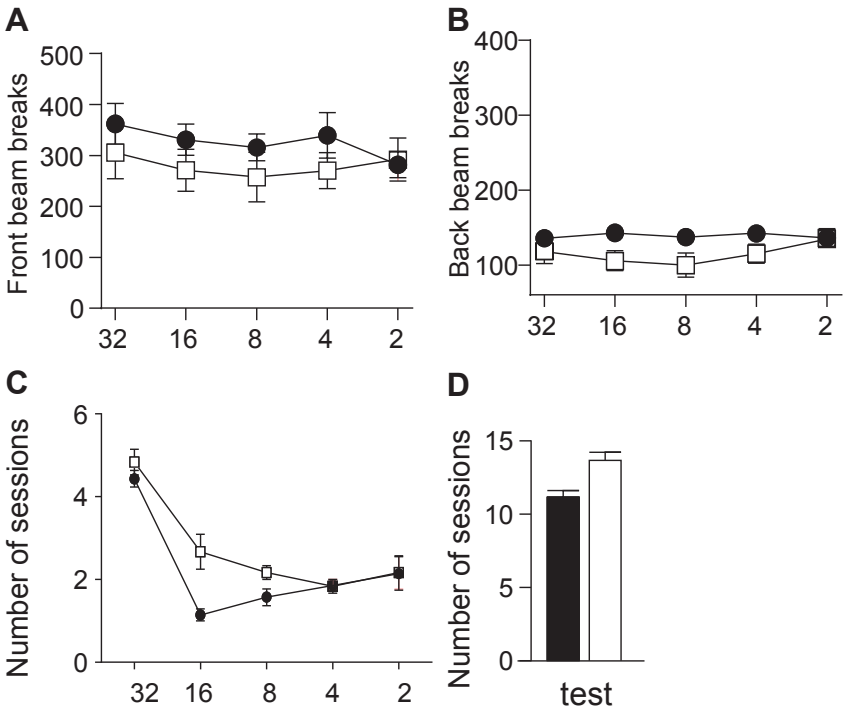
Supplementary Figure 1- MOR knockout does not impair discriminated behavior in an autoshaping test. All mice that reached criterion (40 trials per session) during the autoshaping test phase exhibited discriminated behavior i.e. increased behavior towards CSp while CSn touches and approaches were stabilized. **A. left-** Evolution of all CSp approaches across sessions. **A. right-** Average of all CSp approaches across sessions. **B. left-** Evolution of all CSp touches across sessions. **B. right-** Average of all CSp touches across sessions. **C. left-** Evolution of all CSp approaches across 10-trial blocks during the first session. **C. right-** Average of all CSp approaches across 10-trial blocks during the first session. **D. left-** Evolution of all CSn touches across 10-trial blocks during the first session. **D. right-** Average of all CSn touches across 10-trial blocks during the first session. Data are expressed as mean±s.e.m. N=5-12.

Supplementary Figure 2



Supplementary Figure 2- MOR knockout impairs acquisition in a 5-Choice Serial Reaction Time task (5-CSRTt) training. Mice were tested in the TouchScreen 5-CSRT paradigm composed of a training and a testing phase. MOR-/- show impaired acquisition of stimulus-reward association mainly during the habituation part of training. *Training phase*. A- Total number of sessions to reach criterion during training phases and move to test. B- *Step III.* Evolution of number of trials per session for each mouse during the Must Touch phase. N=7-8, * $p<0.05$.

Supplementary Figure 3



Supplementary Figure 3- MOR knockout decreases motivation and impulsivity/compulsivity but preserves attention in a 5-CSRTt test. MOR-/- mice showed preserved attention but decreased response rate and impulsivity compared to MOR+/+ mice. **A-** Evolution of front beam breaks across different intervals of stimulus appearance (32 seconds, 16 seconds, 8 seconds, 4 seconds and 2 seconds). **B-** Evolution of back beam breaks across different intervals. **C-** Number of sessions per interval to reach criteria. **D-** Average number of sessions to finish the 5-CSRT test. Data are expressed as mean±s.e.m. N=7-8, * $p<0.05$; ** $p<0.01$; *** $p<0.001$

Table 1

Figure	Statistical test	t or F	DFn	df or Dfd	p
Figure 2A	t-test	3.64		21	0.0015
Figure 2B.right	t-test	4.05		21	0.0006
Figure 2B.left	2way ANOVA Interaction	1.62	1	21	0.22
	2way ANOVA Session	20.92	1	21	0.0002
	2way ANOVA Genotype	16.29	1	21	0.0006
Figure 2C	2way ANOVA Interaction	1.65	1	21	0.21
	2way ANOVA Session	15.6	1	21	0.0007
	2way ANOVA Genotype	15.42	1	21	0.0008
Figure 2D	2way ANOVA Interaction	8.01	1	21	0.01
	2way ANOVA Session	10.79	1	21	0.0035
	2way ANOVA Genotype	11.24	1	21	0.003
Figure 3 I-A.right	t-test	1.06	15		0.3
Figure 3 I-B.right	t-test	0.81	15		0.42
Figure 3 I-C.right	t-test	0.29	15		0.77
Figure 3 I-D.right	t-test	0.09	15		0.93
Figure 3 II-A.right	t-test	1.18	15	0.26	
Figure 3 II-B.right	t-test	0.45	15	0.66	
Figure 3 II-C.right	t-test	1.4	15	0.18	
Figure 3 II-D.right	t-test	0.22	15	0.82	
Figure 3 I-A.left	2way ANOVA Interaction	0.5	2	30	0.61
	2way ANOVA Session	2.99	2	30	0.06
	2way ANOVA Genotype	1.12	1	15	0.31
Figure 3 I-B.left	2way ANOVA Interaction	0.96	2	30	0.39
	2way ANOVA Session	3.24	2	30	0.05
	2way ANOVA Genotype	0.67	1	15	0.43
Figure 3 I-C.left	2way ANOVA Interaction	0.87	3	45	0.4611
	2way ANOVA Block	3.54	3	45	0.02
	2way ANOVA Genotype	0.16	1	15	0.69
Figure 3 I-D.left	2way ANOVA Interaction	1.24	3	45	0.31
	2way ANOVA Block	2.07	3	45	0.12
	2way ANOVA Genotype	0.28	1	15	0.87
Figure 3 II-A.left	2way ANOVA Interaction	0.28	2	30	0.76
	2way ANOVA Session	3.58	2	30	0.04
	2way ANOVA Genotype	1.4	1	15	0.26
Figure 3 II-B.left	2way ANOVA Interaction	0.2	2	30	0.81
	2way ANOVA Session	2.17	2	30	0.13
	2way ANOVA Genotype	1.95	1	15	0.18
Figure 3 II-C.left	2way ANOVA Interaction	1.46	2	30	0.25
	2way ANOVA Session	1.98	2	30	0.15
	2way ANOVA Genotype	1.61	1	15	0.22
Figure 3 II-D.left	2way ANOVA Interaction	0.17	2	45	0.84
	2way ANOVA Session	0.37	2	45	0.69
	2way ANOVA Genotype	0.3	1	45	0.59

Table 1

Figure 4A	t-test	6.5	11	< 0.0001	
Figure 4D	t-test	NA	NA	NA	
Figure 4G	t-test	0.27	11	0.79	
Figure 4J	t-test	NA	NA	NA	
Figure 4B.right	t-test	1.55	37	0.13	
Figure 4E.right	t-test	NA	NA	NA	
Figure 4H.right	t-test	2.76	70	0.007	
Figure 4K.right	t-test	NA	NA	NA	
Figure 4C.right	t-test	2.52	37	0.016	
Figure 4F.right	t-test	0.0015	24	0.99	
Figure 4I.right	t-test	1.54	70	0.13	
Figure 4L.right	t-test	1.49	11	0.16	
Figure 4B.left	2way ANOVA Interaction	7.8	1	22	0.01
	2way ANOVA Session	16.25	1	22	0.0006
	2way ANOVA Genotype	2.66	1	22	0.12
Figure 4E.left	2way ANOVA Interaction	0.85	1	11	0.38
	2way ANOVA Session	0.85	1	11	0.38
	2way ANOVA Genotype	0.85	1	11	0.38
Figure 4H.left	2way ANOVA Interaction	2.82	1	11	0.16
	2way ANOVA Session	58.65	1	11	< 0.0001
	2way ANOVA Genotype	2.82	1	11	0.16
Figure 4.Kleft	2way ANOVA Interaction	0.85	1	11	0.38
	2way ANOVA Session	0.85	1	11	0.38
	2way ANOVA Genotype	0.85	1	11	0.38
Figure 4C.left	2way ANOVA Interaction	7.44	1	11	0.02
	2way ANOVA Session	15.78	1	11	0.002
	2way ANOVA Genotype	10.84	1	11	0.007
Figure 4F.left	2way ANOVA Interaction	0.06	1	11	0.81
	2way ANOVA Session	2.6	1	11	0.14
	2way ANOVA Genotype	3.072	1	11	0.99
Figure 4I.left	2way ANOVA Interaction	3.42	1	11	0.09
	2way ANOVA Session	17.87	1	11	0.0014
	2way ANOVA Genotype	1.18	1	11	0.19
Figure 4.Lleft	2way ANOVA Interaction	0.0005	1	22	0.99
	2way ANOVA Session	1.3	1	22	0.27
	2way ANOVA Genotype	6.75	1	22	0.16

Table 1

Figure 5A left	2way ANOVA Interaction	0.8	4	55	0.53
	2way ANOVA Session	40.42	4	55	< 0.0001
	2way ANOVA Genotype	0.02	1	55	0.9
Figure 5A middle	2way ANOVA Interaction	1.06	4	55	0.3
	2way ANOVA Session	33.66	4	55	< 0.0001
	2way ANOVA Genotype	0.003	1	55	0.96
Figure 5A right	2way ANOVA Interaction	0.84	4	55	0.5
	2way ANOVA Session	8.66	4	55	< 0.0001
	2way ANOVA Genotype	0.19	1	55	0.67
Figure 5B left	2way ANOVA Interaction	0.27	4	55	0.9
	2way ANOVA Session	54.04	4	55	< 0.0001
	2way ANOVA Genotype	10.88	1	55	0.0017
Figure 5B middle	2way ANOVA Interaction	1.667	4	55	0.17
	2way ANOVA Session	47.38	4	55	< 0.0001
	2way ANOVA Genotype	16.03	1	55	0.0002
Figure 5B right	2way ANOVA Interaction	1.34	4	55	0.27
	2way ANOVA Session	33.43	4	55	< 0.0001
	2way ANOVA Genotype	5.4	1	55	0.24
Figure 5C left	2way ANOVA Interaction	0.34	4	55	0.85
	2way ANOVA Session	2.23	4	55	0.08
	2way ANOVA Genotype	1.5	1	55	0.23
Figure 5C middle	2way ANOVA Interaction	1.15	4	55	0.34
	2way ANOVA Session	2.21	4	55	0.08
	2way ANOVA Genotype	0.11	1	55	0.74
Figure 5C right	2way ANOVA Interaction	0.34	4	55	0.85
	2way ANOVA Session	1.7	4	55	0.16
	2way ANOVA Genotype	2.69	1	55	0.11
Figure 5D left	2way ANOVA Interaction	0.98	4	55	0.43
	2way ANOVA Session	2.78	4	55	0.04
	2way ANOVA Genotype	79.67	1	55	< 0.0001
Figure 5D middle	2way ANOVA Interaction	1.15	4	55	0.35
	2way ANOVA Session	3.69	4	55	0.11
	2way ANOVA Genotype	39.69	1	55	< 0.0001
Figure 5D right	2way ANOVA Interaction	0.65	4	55	0.63
	2way ANOVA Session	0.98	4	55	0.43
	2way ANOVA Genotype	50.76	1	55	< 0.0001

Table 2

Figure	Statistical test	t or F	DFn	df or Dfd	p
Supp 1A.right	t-test				
Supp 1B.right	t-test	1.27	15	0.22	
Supp 1C.right	t-test				
Supp 1D.right	t-test	0.75	15	0.46	
Supp 1A.left	2way ANOVA Interaction	0.58	2	30	0.57
	2way ANOVA Session	2.01	2	30	0.15
	2way ANOVA Genotype	0.68	1	15	0.42
Supp 1B.left	2way ANOVA Interaction	0.15	2	30	0.86
	2way ANOVA Block	0.2	2	30	0.82
	2way ANOVA Genotype	0.05	1	15	0.83
Supp 1C.left	2way ANOVA Interaction	0.76	3	45	0.52
	2way ANOVA Session	0.79	3	45	0.5
	2way ANOVA Genotype	0.07	1	15	0.79
Supp 1D.left	2way ANOVA Interaction	1.59	2	30	0.22
	2way ANOVA Block	2.42	2	30	0.11
	2way ANOVA Genotype	0.56	1	15	0.47
Supp Fig. 2A	t-test	2.85	11		0.015
Supp Fig. 3A	2way ANOVA Interaction	0.34	4	55	0.85
	2way ANOVA Session	0.49	4	55	0.75
	2way ANOVA Genotype	3.62	1	55	0.06
Supp Fig. 3B	2way ANOVA Interaction	0.89	4	55	0.48
	2way ANOVA Session	0.59	4	55	0.67
	2way ANOVA Genotype	11.3	1	55	0.0014
Supp Fig. 3C	2way ANOVA Interaction	2.42	4	44	0.06
	2way ANOVA Session	36.05	4	44	<0.0001
	2way ANOVA Genotype	12.44	1	11	0.0047
Supp Fig. 3D	t-test	3.53	11		0.0047

Study II- Mu opioid receptors in the habenula: aversion in addiction

Aim of the study

Given the high density of MORs in the MHb and important shared functions between the receptor and the structure, we explored the contribution of habenular MORs to morphine responses. We further focused on the dual role of the structure in reward/aversion, a balance that is particularly salient in the context of addiction to which MORs and the MHb have been extensively associated.

Why did we look at mu opioid receptors in the habenula?

The habenula (Hb) is a highly conserved small brain structure located near the pineal gland and third ventricle (Aizawa, Kobayashi et al. 2012). Despite its small size, it is divided into two anatomically and transcriptionally distinct structures: the medial (MHb) and the lateral habenula (LHb) (Klemm 2004, Matsumoto and Hikosaka 2009). While we commonly read that the Hb has been neglected in the scientific literature, this is no longer true for its lateral subdivision that has attracted most of the neuroscientific and psychological interest in the past decade (Geisler and Trimble 2008). The LHb has specifically been shown to play a critical role in the brain's response to reward prediction (Matsumoto and Hikosaka 2007) and has additionally been linked to major depression (Proulx, Hikosaka et al. 2014). Meanwhile, the MHb remains much less studied (Viswanath, Carter et al. 2013) apart for one extensive association of the structure with aversive effects of nicotine (Frahm, Slimak et al. 2011, Fowler and Kenny 2014, Zhao-Shea, DeGroot et al. 2015). Taken together, this body of studies confers to the habenula as a whole a fascinating dual role in reward and aversion that is of particular interest in the context of addiction.

Notably the MHb, that is emerging both preclinically (Frahm, Slimak et al. 2011, Fowler and Kenny 2014, Soria-Gomez, Busquets-Garcia et al. 2015, Zhao-Shea, DeGroot et al. 2015, McLaughlin, Dani et al. 2017) and in human studies (Bierut 2009, Erlich, Hoffman et al. 2010, Curtis, Viswanath et al. 2017) as a center for aversion processing, is one of the strongest expression sites for mu opioid receptors (MORs), the main target for opiates (Kitchen, Slowe et al. 1997, Lutz and Kieffer 2013). A study describing MOR expression in a MORmCherry knock-in mouse showed a strong MOR

expression in the MHb, fasciculus retroflexus and interpeduncular nucleus (IPN) (Gardon, Faget et al. 2014), suggesting that habenular MORs are in a unique position to contribute to morphine and other opiates rewarding and/or aversive effects. In the light of this anatomical position, it is surprising to note how poor the literature is when it comes to MORs in the habenula.

In the herein presented study therefore, our goal was to characterize behaviors/functions that are specifically regulated by MORs in the MHb.

How did we look at mu opioid receptor function in the habenula?

The tool: To explore habenular MORs function, we generated a conditional knockout mouse model targeting MORs in the MHb. We used the Cre/Lox recombination system (Galli-Taliadoros, Sedgwick et al. 1995) with which two mouse lines are needed: one carries the Cre recombinase driver and the other one carries the floxed gene. By breeding these two lines, the Cre specifically expressed under a promoter gene permanently excises the floxed gene segment leading to specific gene inactivation (Gaveriaux-Ruff and Kieffer 2007). Here we crossed our MOR-floxed mouse with a B4Cre line that expresses the Cre under the B4 gene promoter, as this nicotinic receptor subunit is expressed solely at the level of the MHb. Hence we deleted MORs specifically in B4 positive neurons of the MHb. Notably, B4 contributes to nicotine addiction and in particular to somatic and affective aversion.

The behaviors: Both MORs (Charbogne, Kieffer et al. 2014) and the Hb(Boulos, Darcq et al. 2017) have been extensively implicated in a variety of brain functions that alter behavior, most of which focus on and reward processes (Fields and Margolis 2015) and aversive states (McLaughlin, Dani et al. 2017). In this work, we will measure several behavioral outcomes, and the rationale for each testing set is detailed below:

Pain and morphine analgesia.

MORs- Pain transcends the boundaries of all medical specialties and impacts almost everyone at some stage of their life (Pasternak and Pan 2013). While there are many

classes of drugs used to relieve different degrees of pain, the backbone of pain management for severe pain has been for thousands of years and remains opiates. As for the modern use of opiates, it starts with the isolation of morphine, continues with the cloning of delta (Kieffer, Befort et al. 1992) and mu (Chen, Mestek et al. 1993) opioid receptors and development of specific MORs antagonists, and is still ongoing as recent works search for non-addictive drugs (Pasternak 2014). Effort has been particularly devoted to identifying specific neuronal populations controlling morphine analgesia and defining the circuits and interactions among different populations using genetic and pharmacological approaches.

MHb- Preclinical data implicate the habenula in multiple behaviors that may be considered part of the pain experience including a putative role in pain modulation, affective, and motivational processes (Shelton, Becerra et al. 2012). Moreover, morphine acts directly on habenula neurons to produce analgesia(Cohen and Melzack 1993). Opioids may thus modulate habenula function potentially via MORs. It has been established that MORs activate the ERK1/2 signaling cascade of which the Ser/Thr kinase RSK2 is a direct downstream effector. Using specific downregulation of RSK2 in the MHb, our lab has determined a role for RSK2 in nociception (Darcq, Befort et al. 2012), which suggests a potential MORs-RSK2 signaling mechanism contributing to morphine analgesia at the level of the medial habenula.

Locomotion and morphine hyperlocomotion.

MORs- In addition to its anti-nociceptive properties, morphine is also addictive, thereby complicating its usage (von Zastrow 2004). The addictive properties of morphine has majorly been attributed to its ability to activate MORs on GABAergic neurons, leading to a disinhibition of VTA dopaminergic neurons and resulting in an increased dopamine release in the striatum (Di Chiara and Imperato 1988). Dopamine release in the striatum regulates many behaviors among which locomotion. Morphine injections in rodents thus enhance locomotor activity in a dose-dependent manner (Bohn, Gainetdinov et al. 2003), a behavior that is abolished in constitutive MOR knockouts (Matthes, Maldonado et al. 1996).

MHb- Drug-induced changes in locomotion are not solely driven by mesolimbic circuitry. Habenular B4 subunits were shown to be necessary for nicotine-induced hypolocomotion (Salas, Pieri et al. 2004). Additionally, mice with dorsal MHb lesions perform poorly in motivation-based locomotor behaviors (Hsu, Gile et al. 2017), and immunireactive c-fos expression in the MHb is related to motor activity (Paul, Indic et al. 2011), indicating a role for the region in basal and drug-induced locomotion.

Reward and morphine conditioned place preference.

MORs- One of the most widely studied manifestations of morphine addictive properties associated with dopamine release in the striatum is the conditioned place preference (CPP) that indicates rewarding properties of the conditioning drug, i.e. morphine (Sanchis-Segura and Spanagel 2006, Urs, Daigle et al. 2011). Morphine CPP is abolished in constitutive MOR knockouts (Matthes, Maldonado et al. 1996) but the exact anatomical structures and mechanisms underlying this effect haven't been elucidated (Charbogne, Gardon et al. 2017).

MHb- While nicotine CPP is complex given the dose-dependent aversive/appetitive properties of this drug of abuse in rodents (Le Foll and Goldberg 2005), the MHb has been extensively associated with rewarding properties of nicotine (Fowler et al, 2009). Blocking habenular a3b4 dose-dependently attenuated nicotine reward (Jackson, Muldoon et al. 2015) and habenular a5 subunit controls nicotine intake (Fowler et al, 2011). Independently from nicotine, optical activation of the dorsal MHb demonstrates that the region is involved in regulation of hedonic states and is part of an intrinsic reinforcement circuit (Hsu et al, 2014). Altogether, these data strongly suggest a role for the MHb in reward and potentially in rewarding properties of drugs of abuse.

Withdrawal and morphine somatic aversion.

Withdrawal syndrome manifests as a collection of affective and physical symptoms that largely prevent success in quitting (Changeux 2010). In humans, this includes affective symptoms such as irritability, anxiety, depressed mood, difficulty concentrating,

disrupted cognition and craving; and physical symptoms such as bradycardia, gastrointestinal discomfort and increased appetite accompanied by weight gain (Dani and De Biasi 2001, De Biasi and Dani 2011). In rodent models, physical signs (often called “somatic signs”) include scratching, rearing, jumping, head nods, and body shakes (Damaj, Kao et al. 2003); whereas affective signs include anxiety-like behaviors (Damaj, Kao et al. 2003), elevated reward thresholds (Kenny and Markou 2001), and withdrawal-induced conditioned place aversion (CPA) (Jackson, Martin et al. 2008).

MORs- Spontaneous withdrawal from opiates induces physical symptoms that can also be precipitated by naloxone, a MOR antagonist. While this syndrome is abolished in constitutive MOR knockouts (Matthes, Maldonado et al. 1996), little is known about the mechanisms underlying it or the brain regions and pathways responsible for it.

MHb- Symptoms of nicotine withdrawal can also be induced in mice using chronic nicotine treatment followed by either sudden treatment cessation or mecamylamine (a broad spectrum nAChR antagonist) injection (De Biasi and Salas 2008). Using this methodology, a role for α5 and β4 subunits in the MHb during nicotine withdrawal was determined (Salas, Sturm et al. 2009, Viswanath, Carter et al. 2013). Furthermore, specific blocking of habenular B4 subunit with 18-MC compound blocks nicotinic withdrawal (Glick, Ramirez et al. 2006). Although the molecular mechanisms mediating the effect of the MHb on nicotine withdrawal are not well understood, it was shown that the pacemaker activity of cholinergic (but not peptidergic) neurons in the MHb is critical for withdrawal (Gorlich, Antolin-Fontes et al. 2013). Interestingly, 18-MC also effectively reduces the overall intensity of morphine withdrawal symptoms in rats (Panchal, Taraschenko et al. 2005), indicating the importance of the MHb and B4 in somatic withdrawal and suggesting the potential occurrence of an opiate-nicotine cross-withdrawal induction in the MHb.

Withdrawal and morphine affective aversion.

MORs- In relation to withdrawal, morphine-dependent mice show negative affective symptoms associated with protracted abstinence (Goeldner, Lutz et al. 2011). Naloxone conditioned place avoidance, that is known to provide measuring of the aversive

properties of conditioning drug i.e. naloxone, is abolished in constitutive MORs knockouts (Skoubis, Matthes et al. 2001) but MORs have not been associated with aversion in general.

MHb- On the other hand, growing evidence is accumulating to define the MHb as a center for aversion with emphasis on nicotinic aversion and the B4 subunit. While elevated expression of the B4 subunit increases nicotine aversion in mice by enhancing activity of the MHb to the IPN, nicotine aversion is reversed by increasing α5 expression in the MHb (Frahm, Slimak et al. 2011). This literature has been excellently reviewed by Fowler et al, 2014 (Fowler and Kenny 2014).

Medial habenular mu opioid receptors reduce aversive states

Boulos LJ[1, 2], Ben Hamida S[1, 2], Bailly J[1], Darcq E[1], Gavériaux-Ruff C[2], Kieffer BL[1, 2]

[1] McGill University, Faculty of Medicine, Douglas Research Centre, Montreal, Canada

[2] Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch-Graffenstaden, Strasbourg, France

Abstract

Recent preclinical data describe the medial habenula (MHb) as a brain center for aversive states. Focus was on nicotine addiction, and particularly the β4 nicotinic receptor subunit (β4) that shows restricted expression in the MHb and is key to nicotine withdrawal and aversion. The MHb also expresses highest densities of mu opioid receptors (MORs), a receptor classically associated to drug and natural rewards while little is known about MOR contribution to aversive processing. Here we deleted the *Oprm1* gene specifically in neurons expressing β4 and test the hypothesis that the encoded receptor population (β4MORs) are involved in aversion. Conditional mutants were made morphine dependent, and showed reduced somatic withdrawal in response to both MOR (high naloxone) and nicotinic (mecamylamine) antagonists. Dependent mutant animals also showed reduced conditioned place aversion (CPA) to a low naloxone dose, indicating together that β4MORs contribute to both physical and emotional aversive signs of withdrawal, and cross-talk with the nicotinic system. Further, and contrary to control animals, naïve mutant mice failed to express CPA to naloxone, demonstrating that a MOR-mediated opioid tone normally reduces place aversion at the level of MHb β4 neurons. Finally, CPA to lithium was also absent in mutant mice, confirming a role for these neurons in aversion. Locomotor, analgesic, and rewarding effects of morphine were otherwise intact. This study uncovers a novel role for MORs, and demonstrates for the first time that MORs expressed in β4 neurons of the MHb specifically regulate, and limit, aversive responses. In addition to facilitating reward at the level of mesocorticolimbic network, therefore, MORs limit aversion within the MHb-IPN circuitry, and the two mechanisms together contribute to increase approach and decrease avoidance.

Key words

mu opioid receptors; medial habenula; β4 nicotinic receptors subunit; morphine withdrawal; avoidance behavior; aversive states

Intro

The habenula (Hb) is a small epithalamic structure connecting forebrain to midbrain regions [1, 2]. It is composed of two sub-structures, the lateral (LHb) and the medial habenula (MHb) [3]. The MHb receives input mainly from the septum through the stria medularis and projects massively to the interpeduncular nucleus (IPN) through the fasciculus retroflexus[1].

Recent preclinical data describe the MHb as a brain center for aversion [4] and evidence suggests that the MHb encodes negative affective states and aversive memories[5, 6]. In addiction research, most studies have focused on nicotine [7] with emphasis on the α3β4 nicotinic receptor, as the β4 subunit shows an expression

pattern mostly restricted to the MHb. Among key findings, the overexpression of β4 single nucleotide polymorphisms associated to nicotine dependence in the mouse MHb altered nicotine consumption [8], and subunit rescue in the IPN of β4 knockout animals restored intravenous nicotine self-administration [9, 10]. β4 knockout animals also showed milder physical withdrawal symptoms precipitated by mecamylamine, a nicotine receptors antagonist, after chronic nicotine [11, 12]. Further, transgenic mice with targeted overexpression of β4 displayed strong aversion to nicotine, a phenotype that was reversed by expressing the variant of another nicotine subunit (α5) in the MHb [13, 14]. All these data thus converge towards a role for medial habenular β4 subunits in both nicotine reinforcement, as well as somatic and affective withdrawal from nicotine [15-17]. These data also suggest a key role for MHb β4-expressing neurons in addiction-related behaviors, at least with regards to nicotine.

Interestingly, the MHb also expresses the highest density of mu opioid receptors (MORs) [2, 18] in the brain. MORs mediate both strong analgesic and addictive properties of opiates [19] and also contribute to rewarding effects of other drugs of abuse [20] and natural reward [21-23]. Brain sites for MOR-mediated reward have been extensively investigated both genetically [20] and pharmacologically [24, 25], and the implication of MORs in dopaminergic mesolimbic circuitry is largely demonstrated. Because MORs are typically associated to reward processing and hedonic states, much less is know about the contribution of MORs in regulating negative affect. Aversive states can be somatic or affective [4]. Preclinical studies have shown that pharmacological blockade of MORs produces highly aversive states, either emotional in naïve animals [26, 27] or even somatic in opioid-dependent animals [25], and a study also suggested a role for MORs in emotional deficits that develop upon protracted abstinence to opiates [28]. Circuit mechanisms underlying the potential contribution of MORs in negative affect-related behaviors have not been investigated. Here we hypothesized that MORs expressed in MHb neurons, particularly in β4-expressing neurons, may contribute to control aversive responses.

To test this, we genetically inactivated MORs specifically in MHb β4-expressing neurons and characterized both rewarding and aversive effects of opiate. Our data demonstrate that this targeted MOR knockout (KO) reduces aversive states and context-dependent avoidance behavior in both naïve and morphine-dependent mice, whereas reward-related behaviors are unchanged. We therefore demonstrate for the first time that this small population of MORs is strategically located to act as a break on aversive processes.

Materials and methods

Animals- In this paper, we first crossed β4-Cre mice and MORmCherry mice to verify with viral injections that habenular β4 and MOR projections overlapped. For all other experiments we used conditional knockout males β4MOR-/- and β4MOR+/+ mice obtained by crossing β4-Cre mice with MOR-floxed mice in-house on a hybrid 50% 129SvPas - 50% C57Bl/6J background. All animals were group-housed (3-5 animals per cage) under a 12 h light/dark cycle except for animals studied in conditioned taste aversion that were housed individually under a 12 h reversed

light/dark cycle. Temperature and humidity were controlled and food and water were available *ad libitum* except for touchscreen experiments (food restriction to 85% of bodyweight). All mice were 3-5 months old and weighted 25-35 g at the time of the experiments. All animals procedures in this report were conducted in accordance with the guidelines set forth by the Canadian Council of Animal Care and by the Animal Care Committees of McGill University/Douglas Mental Health University Institute.

Genotyping- PCR analysis on genomic DNA was performed in order to genotype mice for presence of 1) Cre recombinase, 2) loxP sites and 3) excision of *Oprm1*. See Supplementary Text for full protocol.

Drugs- Morphine was dissolved in physiological saline (0.9%) and administered by intraperitoneal route in a volume of 10ml/kg. Naloxone and mecamylamine hydrochloride (Sigma, Madrid, Spain) were dissolved in physiological saline (0.9%) and administered by subcutaneous route in a volume of 10 ml/kg.

Viral injections- β4Cre-MORmCherry mice under isoflurane anesthesia were injected with purified AAV2-FlexGFP into the medial habenula using stereotaxic apparatus (Kopf instruments). The virus was infused unilaterally (0.4 µl / 4 min ; n = 3) using a 10- µl microsyringe (Hamilton). After 5 minutes of waiting, the needle was up of 0.05 mm and then held in place for an additional 10 min before removed. The coordinates for the injections were AP, −1.34 mm from bregma; ML, −0.25 mm from midline; DV, −2.8 mm from dura skull surface (adapted from Darcq et al, 2011). Following surgery, mice were placed back in their home cage, monitored for 3 days and sacrificed 5 weeks after injection.

Quantitative real-time PCR (qPCR)- qPCR was performed on 7 to 9 brains of β4MOR-/- conditional knockouts, 6 to 7 β4MOR+/+ positive control brains and 4 CMVMOR total knockout negative control brains. See Supplementary Text for full protocol.

mRNA analyses- In situ Hybridization- Two in situ hybridization RNAscope experiments were performed. The first one was to simultaneously assess MOR and β4 mRNAs in the MHb of β4MOR mice and visualize the colocalization of these mRNAs. In the second experiment, we used probes to label MOR as well as SP and ChAT to characterize the specific cell population that we target in our conditional knockout. Quantification was done on three mice per genotype and per experiment, see Supplementary Text for full protocol.

Behavioral procedures

Naloxone-precipitated morphine withdrawal- While there are no perfect animal models of protracted withdrawal, precipitated opioid withdrawal in rodents serves as an animal model of acute withdrawal (Wills and Parker, 2016). In this protocol, mice were injected intraperitoneally with escalating doses of morphine sulfate (20, 40, 60, 80, 100 mg/kg; Sigma-Aldrich, St-Louis, USA) or saline (0.9% sodium chloride) as control, twice daily for five days and received a single 100 mg/kg injection on day 6. Morphine was administered twice a day as, unlike constant drug delivery devices, it allows partial opiate withdrawal between drug injections, a condition encountered in the clinical setting. To measure physical dependence, withdrawal was precipitated by naloxone (1 mg/kg, subcutaneously) 2 h after the last morphine injection on day 6

[29]. Mice were individually placed in Plexiglas cages immediately after the injection and somatic signs of withdrawal were scored per blocks of 5 minutes across a 20 minutes session. The somatic signs were also recorded during 5 minutes prior to naloxone injection, as a control. A global withdrawal score was calculated for each animal giving each individual sign a relative weight: 0.5 for each episode of jump, paw tremor, body tremor, sniffing and wet dog shake; and 1 for the presence of ptosis, piloerection, and teeth chattering during each 5 min observation period. Results were reported as the average of the total signs per group. The experience was repeated and the spontaneous withdrawal symptoms scored after respectively 1 and 4 weeks of withdrawal. All testing was conducted in a blind manner.

Mecamylamine-precipitated morphine withdrawal- Antagonism of nicotinic receptors has already been shown to potentiate opiate withdrawal in rats [30]. We also know that knockout of β4 in the MHb attenuates cfos activation during morphine withdrawal [31]. To test the effects of mecamylamine on morphine withdrawal, we repeated the same experiment but this time morphine-withdrawal was precipitated with mecamylamine (1 mg/kg, subcutaneously) instead of naloxone, 2 hours after the last morphine injection on day 6.

Conditioned Place Aversion in morphine dependent mice- Negative affective states associated with morphine withdrawal were examined by using conditioned place avoidance test (CPA), a behavioral technique commonly used to evaluate the emotional consequences of drug withdrawal in rodents by measuring avoidance behavior [32]. This test was conducted in an apparatus (Imétronic, Pessac, France) composed of two compartments (15.5 x 15.5 x 20 cm each) that only differ by their shapes and floor. A central compartment (6 x 16.5 x 20 cm) separates the two others. The test lasted 6 days. Mice were injected intraperitoneally with escalating doses of morphine sulfate (20, 40, 60, 80 mg/kg; Sigma-Aldrich, St-Louis, USA) or saline (0.9% sodium chloride) as control, twice daily for four days and received a single 100 mg/kg injection on day 5); the injections took place in the homecage. 5 hours after the morning injection on day 3, mice were allowed free exploration of the CPA apparatus for 15 minutes (preconditioning). For the conditioning phase we used a biased CPA design in which naloxone treatment was associated with the more preferred compartment during the preconditioning test. Conditioning lasted two days with one session of 30 minutes each day. On day 4, mice were administered saline solution and confined immediately to one of the compartments and on day 5 mice were administered naloxone (0.25 mg/Kg s.c.) or saline solution and were confined to the other compartment. Post-conditioning test took place on day 6, 24 hours after the naloxone injection. CPA score was calculated as the % of time spent in the drug-paired compartment on the test day minus the % of time spent in the same compartment on the preconditioning day.

Naloxone conditioned place avoidance in naïve mice- Naloxone has been shown to induce place avoidance in naïve mice too but at higher doses. In this experiment, we used the same apparatus to test the effect of naloxone in mice that hadn't received chronic morphine. The test lasted 5 days. *Pre-conditioning*- On day 1, animals were placed in the middle compartment and permitted free access to the entire apparatus for 15 min. For the conditioning phase we used a biased CPA design in which morphine treatment was associated with the most preferred compartment during the preconditioning test. *Conditioning*- On day 2, conditioning training started with two conditioning trial per day for 3 days as follows: mice were administered (i.p.) saline solution and confined immediately to one of the

compartments for 30 min in the morning session. During the afternoon session, mice were administered saline solution or naloxone (10 mg/Kg) and were confined to the other (drug-paired) compartment. This schedule was repeated twice more till day 4. *Postconditioning (test)*- On day 5, 24 h after the last drug treatment, animals were allowed to explore the entire apparatus for 15 min. The time spent in each chamber was recorded. CPA score was calculated as the % of time spent in the drug-paired compartment on the test day minus the % of time spent in the same compartment on the preconditioning day.

Lithium conditioned place avoidance in naïve mice- Lithium has been shown to induce place avoidance in naïve mice too. In this experiment, we used the same apparatus to test the effect of lithium in mice that hadn't received chronic morphine and see whether avoidance behavior could be generalized with non-opioid compounds. The protocol used was similar to the naloxone CPA except that lithium (3 mEq, ip) was injected instead of naloxone during conditioning sessions in the drug-paired compartments (days 2, 3, 4 in the afternoon).

Conditioned taste aversion- To test the ability of our knockout mice to form taste aversions, knockouts and wild-type littermates were conditioned against sucrose paired with high and low doses of LiCl. Mice were placed on a water deprivation schedule that restricted access to water overnight for 4 days, with water returned each morning for 6 h (n = 6–7 for each LiCl dose). On the conditioning day, a 5% sucrose solution was available for 30 min. Drinking tubes were weighed before and after test periods to measure fluid intake. At the end of the 30 min access to sucrose, mice were injected with LiCl at one of two doses (15 mg/ml). Drinking water was returned after the injection for 6 h. The deprivation schedule resumed until 48 h post-pairing when mice were given 5% sucrose for 30 min and then water for the remainder of the day. Sucrose consumption during 30 min access after overnight water deprivation was measured daily for 4–5 consecutive testing days. After another week of nightly water deprivation, mice were given access to a 75 mM NaCl solution for 30 min. At the end of the 30 min access to the NaCl solution, mice were injected with LiCl (0.15 M, 20 mL/kg or 40 mL/kg, i.p.). Drinking water was returned after the injection for 6 h. The deprivation schedule resumed until 48 h post-pairing when mice were given 75 mM NaCl for 30 min and then water for the remainder of the day. NaCl consumption during 30 min access after overnight water deprivation was measured daily for 4–5 consecutive testing days. Because no significant difference was found in intakes between taste solutions, the results from sucrose and NaCl were pooled.

Morphine conditioned place preference- CPP classically measures the rewarding properties of the conditioning substance as well as the acquisition of the reward-context association. Here our protocol was conducted in the same apparatus that the one used for CPA protocols. The 5-day protocol was also similar except that, instead of receiving aversive naloxone or lithium injections on conditioning sessions to the drug-paired compartment (days 2, 3, 4 in the afternoon), mice received a supposedly appetitive dose of morphine (10 mg/kg i.p.).

Touchsceen-based battery of tests- All procedures were accomplished in standard mouse Bussey-Saksida Touchscreen system (Model 80614-20, Campden Instruments Ltd). We used two different tests: autoshaping (see Figure 4-I-A for protocol), that mainly measures pavlovian learning, and 5-Choice Serial Reaction Time task (see Supplementary Figure 5 for protocol), that allows measuring of

attention and impulsivity/compulsivity. Given their cognitive complexity, both tasks consisted in a training and a testing phase.

Statistical analysis- All data are presented as mean ± SEM. Data were analyzed with (GraphPad Prism) unpaired t test or two-way ANOVA with or without repeated measures (RM-ANOVA). Significant main effects and interactions of the ANOVAs were further investigated with the Bonferroni *post-hoc* test or method of contrast analysis. Statistical significance was set at $p<0.05$. Detailed statistics information related to all figures and supplementary figures are shown in supplementary Table 1.

Results

β4MOR-/- mice show MOR deletion specifically in β4 neurons of the MHb

We first determined whether MOR and β4 are co-localized in MHb neurons using neuronal tracing. We crossed a transgenic mouse line expressing the Cre recombinase (Cre) under the β4 promoter [33] with knock-in MORmCherry mouse, which express the MOR protein in fusion with the red fluorescent protein mcherry at the C terminus and is visible in tissues [34, 35]. We injected a Cre-dependent anterograde eGFP viral tracer (AAV2-FlexGFP) in the resulting β4Cre-MORmCherry mice, and found co-localization of MOR-mcherry and eGFP fluorescence at the level of the MHb, and also along tracks forming the IPN (Figure 1A). Observation of both sagittal and coronal sections showed a clear, although partial, signal overlap. To further quantify co-expression of MOR and β4 in the same cells, we used in situ hybridization in wild type mice [36]. Visualization of coronal sections revealed, as expected, distribution of the *Oprm1* transcript encoding MOR throughout the brain[35] with prominent expression in the MHb and IPN. In the MHb, *Oprm1* was more specifically expressed in both the apical and lateral part of the MHb, in accordance with previously described MORs distribution in the habenula [18]. In contrast, β4 transcript showed a limited pattern of distribution mainly in the MHb, and more specifically in the ventromedial and ventrolateral part of the structure. β4 and MORs mainly overlapped in the lateral part of the MHb, in both substance P and acetylcholine transferase positive neurons (Supplementary Figure 1). Our data confirm that the two transcripts overlap in 53,08% MHb MOR-positive neurons, suggesting that the β4-Cre line should allow producing a significant decrease of MORs in the MHb.

Next, we inactivated the *Oprm1* gene in the MHb by crossing MOR floxed mice [37] with the β4-Cre driver mouse line. To assess the extend of *Oprm1* deletion in the MHb, we first conducted relative quantitative real-time polymerase chain reaction and quantified *Oprm1* mRNA levels in microdissected MHb samples from β4MOR conditional KO mice (β4MOR-/-) and their control littermates (β4MOR+/+). As shown in Figure 1C, *Oprm1* expression was significantly decreased in the habenula of β4MOR-/- (left panel) compared to β4MOR+/+ mice (right panel) (45.57%; $t_{(16)}= 2.98$, $p < 0.01$), a ratio consistent with the *Oprm1-β4* transcript overlap.

To further examine spatial distribution of the *Oprm1* deletion in the MHb, we conducted in situ hybridization analysis of *Oprm1* and *β4* transcripts in β4MOR-/- mice compared to β4MOR+/+ controls. In β4MOR-/- mice, the *Oprm1* mRNA was deleted mainly in the lateral part of ventral and apical MHb (Figure 1B right). Semi-

quantification using the VS_DESKTOP measurement tools revealed an overall 49.85% decrease of *Oprm1*-positive neurons (Figure 1C left; $t_{(28)} = 6.12$, $p < 0.001$). In addition, there was a 99.39% decrease of double positive Oprm1-β4 neurons in conditional mutants compared to control animals (Figure 1C middle; $t_{(28)} = 10.11$, $p < 0.001$), demonstrating that the targeting strategy led to a complete loss of MOR expression in β4 positive neurons.

Finally, we evaluated MOR protein activity in the MHb of mutant mice. To this aim, we tested MOR-mediated G protein activation in β4MOR KO and control mice. We used the [^{35}S]-GTPγS binding assay on MHb membrane preparations, and our data show that the selective MOR agonist DAMGO increased [35S]-GTPγS binding in samples from both genotypes, but the response was significantly lower for mutant mice (Figure 1D; $t_{(4)} = 14.29$, $p < 0.001$). This result confirms that the gene KO effectively reduced receptor activity levels and the reduction of Emax value (20%), which reflects receptor density, is consistent with the *Oprm1*-*β4* overlap ratio in the MHb.

Overall, the data indicate that β4-MOR mice lack approximately half of MORs in the MHb, and that the receptors are deleted specifically in β4+ neurons.

Targeted MOR deletion in β4 neurons does not alter analgesic and locomotor response to morphine

To explore phenotypic consequences of the MOR loss in β4MHb KO mice, we first tested basal and morphine-induced locomotor activity. Analysis of horizontal spontaneous activity in the open field revealed no significant difference between β4MOR-/- and control mice. Similarly, the stimulant activity of morphine on locomotor activity did not differ between genotypes (Figure 1E). Next, we tested whether the opioid regulation of pain perception was altered in the conditional KO mice. Our results first show similar basal response latencies across genotypes using both 48 (Figure 1F-left) and 52 (Figure 1F-right) temperatures in the tail immersion test, indicating that basal pain perception is unaltered in mutant mice. We further found that both acute morphine analgesia (day 1) and the reduction of this effect upon repeated morphine treatment (day 1 to 5) were intact in mutant mice, indicating that the β4-specific MOR KO does not modify morphine analgesia and analgesic tolerance, at least in this test.

Targeted MOR deletion in β4 neurons reduces physical symptoms of morphine withdrawal

The MHb seems to contribute both to naloxone-precipitated morphine withdrawal [38] format and to mecamylamine-precipitated nicotine withdrawal [11]. Recent clinical and preclinical studies have demonstrated the occurrence of an opioid/nicotinic cross-talk [39]. Specifically, antagonism of nicotinic receptors potentiates opiate withdrawal in rats [30] a mechanism that could be β4-dependent since β4 antagonism reduces naloxone-precipitated somatic withdrawal in morphine-dependent animals [40] and knockdown of β4 in the MHb attenuates cfos activation during morphine withdrawal in mice [31]. Thus, to address the potential implication of targeted habenular MORs in physical signs of withdrawal, we tested both naloxone-

and mecamylamine-precipitated somatic withdrawal in morphine-dependent β4MOR mutant mice.

Reduced naloxone-precipitated somatic withdrawal- Precipitated withdrawal in rodents serves as an animal model of acute withdrawal [41]. In this protocol, β4MOR-/- and their control littermates were chronically injected intraperitoneally with escalating doses of morphine and withdrawal was precipitated by naloxone two hours after the last injection of morphine [29]. Withdrawal signs (body tremor, paw tremor, wet dog shake, sniffing, ptosis, activity, jumps, teeth chattering and piloerection) were scored 5 minutes before and immediately after naloxone injection every 5 minutes for 20 minutes and a global withdrawal score was calculated as described in Le Merrer et al, 2012 [29]. As shown in Figure 2-I-A left panel, we observed a significant difference in the evolution of the global score over time between both genotypes. Two-way RM ANOVA showed significant effect of genotype ($F_{(1, 30)} = 27.5$, $p < 0.0001$), block ($F_{(4, 120)} = 38.25$, $p < 0.0001$) and genotype x block interaction ($F_{(4, 120)} = 4.42$, $p < 0.001$). Subsequent Bonferroni post-hoc analysis showed a significant reduction of global score in conditional knockouts compared to controls at block 5 ($p<0.01$) and 10 ($p<0.001$). Overall, the global score (Figure 2-I-A right panel) was significantly decreased in β4MOR-/- mice compared to control littermates ($t_{(30)} = 4.45$, $p < 0.001$).

Next, we tested whether this decrease in global withdrawal score was due to the decrease of one or more specific withdrawal symptoms. Statistical analysis on the evolution of each physical withdrawal symptom using two-way ANOVA is detailed in Supplementary Text. Overall, for both genotypes, naloxone significantly increased all behaviors that were monitored during the 20-min test sessions compared to baseline (Figure 2.I.B to J) or saline-treated mice (Supplementary Figure 2). We found that the decreased global withdrawal score observed in β4MOR-/- following naloxone treatment is mainly due to reduced expression of–body tremors, paw tremors, wet dog shakes and sniffing compared to control animals.

Together, these data suggest that MORs expressed in β4 neurons of the MHb significantly contribute to the expression of several signs, which all contribute to the aversive state of physical withdrawal from morphine (Wils and Parker, 2016).

Reduced mecamylamine-precipitated somatic withdrawal- We repeated the somatic withdrawal experiment but this time withdrawal was precipitated by mecamylamine, a nicotinic receptor antagonist. As shown in Figure 2-II-A left panel, we observed a significant difference in the evolution of the global score over time between both genotypes. Two-way ANOVA on genotype per 5-minute blocks showed significant genotype (Figure 2-II-A left; $F_{(1, 65)} = 20.70$, $p <0.0001$), block ($F_{(4, 65)} = 13.77$, $p <0.0001$) and genotype x block interaction ($F_{(4, 65)} = 13.77$, $p <0.05$). Subsequent Bonferroni post-hoc analysis showed a significant reduction of global score in β4MOR-/- compared to controls at block 5 ($p<0.05$), 10 ($p<0.01$) and 15 ($p<0.05$) indicating that the major genotype difference happens 5 to 20 minutes after mecamylamine injection. Overall, the global score was significantly decreased in conditional knockouts compared to control mice (Figure 2-II-A right; $t_{(13)} = 3.48$, $p < 0.01$) and Next, we tested whether this decrease in global withdrawal score was due to a difference in the expression of specific somatic signs of morphine withdrawal between mutant mice and their control littermates. Statistical analysis on the evolution of each physical withdrawal symptom using two-way ANOVA RM is detailed in Supplementary Text. Overall, for both genotypes, mecamylamine

significantly increased all behaviors that were monitored during the 20-min test sessions compared to baseline (Figure 2) or saline-treated mice (Supplementary Figure 2). Moreover, we found that decreased global withdrawal score in β4MOR-/- mice following mecamylamine injection is mainly due to reduced expression of body tremor and ptosis compared to control animals.

This result confirms the contribution of β4MORs to the expression of physical withdrawal in morphine-dependent animals, and further supports the notion opioid-nicotine cross-talk that takes place in the MHb.

Targeted MOR deletion in β4 neurons reduces conditioned place aversion

Physical withdrawal symptoms are one component of what is now recognized as a more complex opiate dependence syndrome that also includes an affective aversion [4, 42]. Affective or emotional aversion is most commonly measured using conditioned Place Aversion (CPA). In this paradigm, very low doses of naloxone are used that elicit place aversion, yet fail to produce physical withdrawal signs in morphine-dependent mice [43]. Further CPA can also be produced in naïve animals using high doses of naloxone, and aversion in this case results from blockade of an opioid endogenous tone controlled by MORs. We therefore examined whether β4MORs contribute to the expression of naloxone-induced CPA in both morphine-dependent and naive animals.

Reduced naloxone-conditioned aversion in morphine-dependent mice- Animals were first chronically treated with morphine, using the ascending doses protocol that produces severe physical dependence, as above. 5 hours after the last morphine injection, animals were trained to associate the effect of a low naloxone dose (0.25 mg/kg) with the apparatus context, resulting in place conditioning. Naloxone doses were chosen as to avoid both aversion in saline-treated mice and somatic withdrawal signs in morphine-treated mice [44], and data are shown in Figure 3A. Three-way ANOVA conducted on genotype per drug (morphine or saline) per treatment (naloxone or saline) revealed significant treatment ($F_{(1, 85)} = 18.83$, $p < 0.001$), drug ($F_{(1, 85)} = 17.69$, $p < 0.001$), as well as significant treatment x genotype interaction ($F_{(1, 85)} = 11.81$, $p < 0.001$) and drug x treatment interaction effect ($F_{(1, 85)} = 4.54$, $p < 0.05$) but no significant difference for genotype ($p = 0.07$), genotype x drug interaction effect or genotype x acute x chronic treatment interaction ($p = 0.09$). Subsequent analysis using method of contrasts showed that only morphine-dependent control mice show significant aversion ($p < 0.001$). All other groups (saline-naloxone, morphine-saline and saline-saline) showed similar results across genotypes with no significant preference or avoidance for the drug-associated compartment.

This result demonstrates that MORs expressed in β4 neurons of the MHb play a key role in aversive memories associated with drug withdrawal in morphine-dependent mice.

Reduced naloxone-precipitated avoidance in naïve mice- We next performed a CPA experiment using a higher dose of naloxone that is known to induce place avoidance in naïve mice[45], and data are shown in Figure 3B. Two-way ANOVA performed on CPA score revealed significant genotype ($F_{(1, 47)} = 5.45$, $p < 0.05$), treatment ($F_{(1, 47)} = 54.7$, $p < 0.001$) and compartment x treatment interaction effect ($F_{(1, 47)} = 9.57$, $p < 0.01$). Subsequent post-hoc analysis showed significant increase of CPA in naloxone-treated control mice compared to saline-treated control mice

(p<0.001) but no CPA was detected in conditional mutant mice when compared to wild-type naloxone-treated mice.

This result first demonstrates a role for habenular β4MORs in the expression of a context-dependent aversion even in the absence of morphine dependence. Also, the data suggest that the previously reported tonic activity of endogenous opioids, which regulates the basal affective state, is mainly controlled at the level of MHb, and that this effect is mediated by MORs in MHb β4 neurons.

Reduced lithium-precipitated avoidance in naïve mice- As β4MORs in the habenula play a role in aversion induced by opioid transmission blockade, this receptor population may also contribute to the expression of other aversive states, arising for instance from a visceral malaise. In order to test whether β4MORs contribute to an avoidance behavior induced by another mechanism, we performed a CPA experiment with lithium chloride (LiCl) injections that classically induces a physical malaise. Data are shown in Figure 3C. Two-way ANOVA performed on CPA score revealed significant genotype x treatment interaction ($F_{(1, 51)}$ = 5.5, p <0.05). Subsequent Bonferroni post-hoc analysis showed a significant place aversion for β4MOR+/+ treated with lithium compared to saline group (p<0.05). No significant lithium effect was observed in β4MOR-/- mice.

These data indicate that habenular β4MORs also mediate non-opioid dependent CPA as well. This result also suggests that β4-positive medial habenular neurons are implicated in the association between contextual cues and lithium-induced aversive state.

Targeted MOR deletion in β4 neurons does not alter reward-related mechanisms

MORs are most widely associated with reward-processes [23], which classically involve the dopaminergic mesolimbic circuit but may also involve the habenula [2]. Thus, we finally investigated whether the β4MORs population in the MHb also contributes to reward processes. To address this question, we tested reward-dependent pavlovian learning with both morphine and highly palatable food.

Preserved conditioned place preference to morphine- Since β4MOR-/- mice show decreased acquisition of context-dependent aversion, we first tested whether mutant mice also show alterations of context-dependent reward. To do so, we used the widely characterized Conditioned Place Preference (CPP) paradigm that classically measures rewarding properties of the conditioning substance as well as the acquisition of the reward-context association [46]. The protocol is similar to the CPA protocols except that, instead of receiving aversive naloxone or lithium injections in the drug-paired compartment for conditioning, mice received an appetitive dose of morphine (10 mg/kg) [47]. Data are shown in Figure 4A. Three-way ANOVA performed on compartment per treatment (morphine or saline) per genotype revealed significant compartment effect ($F_{(1, 83)}$ = 9.94, p <0.01) as well as compartment x treatment interaction effect ($F_{(1, 83)}$ = 6.29, p <0.05) but no significant effect of genotype, compartment, genotype x compartment interaction, treatment x genotype x treatment interaction and genotype x treatment x compartment interaction effects. Subsequent post-hoc analysis showed significant increase of % of time spent in post- compared to pre-compartment in both control and conditional knockout mice

(p<0.01). This result shows that CPP is preserved in mutant mice suggesting that habenular β4MORs do not contribute to rewarding properties of morphine.

Preserved stimulus-reward association and discriminatory behavior in a touchscreen-based autoshaping task- We also used another reward-procedure based on Pavlovian conditioning called autoshaping, using the touchscreen-based approach[48]. In contrast to the CPP procedure, autoshaping provides approach measures towards discrete stimulus rather than diffuse context [49], pairings are shorter and repeated over a large number of trials per session (vs. long and single daily pairings in CPP), and the reward in this case is highly palatable food (strawberry milkshake). The task is conducted in two phases: a training phase and a testing phase (see Figure 4B for detailed protocol and). The training or habituation phase consists in learning the association tray-visual cue presentation of the reward. β4MOR+/+ and β4MOR-/- mice spent a similar number of sessions to reach criterion in this phase (data not shown) and both genotypes also spent similar number of sessions to reach criterion during testing phase (Figure 4C) indicating that targeted habenular MORs are not necessary for the acquisition of cue-reward association.

During the test phase (Figure 4D) mice escalate the number of approaches or touches in response to positive conditioned stimulus (CSp) while the number of approaches and touches associated with the negative conditioned stimulus (CSn) stabilizes. In order to evaluate the effect of habenular MOR deletion on discriminatory behavior, we analyzed the number of approaches of CSp or CSn in mice that had reached the test criteria (40 trials per session of one hour during testing phase). Two-way ANOVA revealed significant session effect for CSp (Figure 4E; $F_{(3, 42)} = 17.08$, $p < 0.0001$) but not CSn (Figure 4F). No genotype effect or genotype x session interaction were observed in either CSp or CSn, indicating that β4MOR+/+ and β4MOR-/- mice similarly increased their CSp touches across the four sessions while stabilizing their CSn approaches. Altogether, the data show similar performance in β4MOR-/- and β4MOR+/+ animals, indicating that both acquisition of discrete stimulus-reward association and discriminatory behavior are preserved in conditional knockout mice. MORs in MHb β4 neurons, therefore, are not necessary for reward-related learning in the autoshaping task.

We also tested our mutant mice in a 5-CSRTt and results confirmed preserved stimulus-reward association learning and further revealed intact attention and inhibitory control (see Supplementary Text and Supplementary Figure 6).

In conclusion, this last set of results strongly suggests that β4MORs do not contribute to context-dependent reward learning as measured by morphine CPP or to stimulus-reward association learning as measured by autoshaping with highly palatable food.

Discussion

MOR function has been extensively associated with reward processes, but the potential role of MOR in regulating aversive states has been poorly studied. Our data indicate that deletion of the *Oprm1* gene in MHb-β4 neurons reduces physical withdrawal from chronic morphine and abolishes context-dependent avoidance behavior to naloxone in both morphine-dependent and naive states, whereas reward-related processes including context-dependent reward learning are preserved. This is the first report demonstrating that MOR controls both somatic and affective aversion specifically at the level of β4 neurons of the MHb.

β4, a nicotinic receptor subunit, shows a specific pattern of expression mainly in the MHb [50]. We used β4-Cre mice to target the MOR gene neurons, and obtained conditional knockout mice with an almost 50% deletion of MORs in the MHb, while the receptor was preserved elsewhere in the brain. Our prior tracing and in situ hybridization experiments indeed showed that β4 and MORs colocalize in 50% neurons projecting from the MHb to the IPN via the fasciculus retroflexus mainly in the lateral and apical part of the MHb. The conditional targeting strategy used here, therefore, led to a complete deletion of MORs specifically in β4-positive neurons. Important to note, all the subsequent analysis addresses this particular MHb MOR population, and we cannot exclude that the 50% MORs expressed in non-β4 neurons of the MHb also contribute to behavioral processes that are undetected in our study.

Behavioral analysis of mutant mice suggests that targeted MORs expressed in this brain structure are not implicated in spontaneous locomotor activity and morphine-induced hyperlocomotion. The data further suggest that β4MORs are not involved in pain perception and morphine analgesia indicating that remained opioid system is potentially intact in β4MOR-/- mice. These data are consistent with the intact MOR expression in basal ganglia and nociceptive pathways in the conditional mutant mice. Most importantly, our study uncovered the key role of β4MORs in the regulation of aversion-related behaviors.

Based on the growing literature showing the role of habenular β4 in somatic aversive processes associated with nicotine withdrawal syndrome [8, 11, 12], we wanted to test whether deletion of habenular β4MORs would alter somatic aversion related to morphine withdrawal. Interestingly, deletion of β4MORs in the MHb diminished the expression of aversive states measured by physical withdrawal in morphine-dependent mice. It is also interesting to note that shakes and sniffing but not jumps were abolished in mutant mice, indicating that β4MORs do not mediate the full spectrum of somatic withdrawal signs but rather specific symptoms that could be underpinned by distinct mechanisms.

The mechanisms by which β4MOR activity in the MHb contributes to somatic opioid withdrawal are not known, but evidence highlights the importance of cholinergic transmission in the MHb-IPN pathway for withdrawal from nicotine and potentially under drugs [14]. Indeed, chronic morphine administration was shown to reduce acetylcholinesterase activity in the MHb, an activity that returns to baseline during withdrawal, suggesting that chronic opiates alter cholinergic homeostasis [31]. As the MOR deletion occurs in significantly more in cholinergic neurons (Supplementary Figure 1), which compose a large part of MHb output neurons, it is possible that MORs normally regulate cholinergic transmission and that deregulation of MOR activity in these neurons under chronic opiates normally contribute to somatic withdrawal signs including particularly the sign subset identified in our study. Targeting MORs specifically in MHb cholinergic neurons may in the future confirm this hypothesis.

Our data extend a growing body of evidence supporting that nicotinic receptors play an important role in opiate withdrawal [12]. Indeed, we demonstrated decreased mecamylamine-precipitated morphine withdrawal symptoms in our mutant model compared to control littermates, suggesting that opioid and nicotine systems interact in the MHb and contribute to the aversive properties of morphine abstinence. Clinical and preclinical studies have already explored the existence of an opioid-nicotinic

cross-induction of withdrawal [30] with emphasis on α3β4 nicotinic receptors [51]. Specifically, knockdown of β4 in the MHb attenuates cfos activation during morphine withdrawal in mice [31] indicating a role for β4 subunit in morphine withdrawal. β4 is an interesting candidate since the CHRNA5/CHRNA3/CHRN4 gene cluster coding for the α5, α3 and β4 nACh receptor subunits has emerged as a candidate region contributing to risk of heavy smoking and nicotine dependence in humans [52, 53]. In addition, polymorphism of α3β4 gene cluster increases risk and severity of opiate dependence in humans [54]. A recent resting state fMRI study also reveals increased habenular connectivity in opiate users and further demonstrates that this change in connectivity is associated with subunit nicotinic gene variants [55]. This cross-talk is likely to take place in a brain region that contains high densities of receptors responsive to both nicotine and opiates. Given the specific habenular pattern of expression of MORs and β4, the MHb represents the ideal brain site for the interaction. Our result, if further investigated, could position the MHb not only as a center for aversion but also as a center for cross-talk aversion, thus shedding new light on the high comorbidity of nicotine and opiate use disorder and the possible consumption of cigarettes as a self-medication in heroin users.

Another important finding here is that, in addition to somatic aversion, β4MHb neurons also regulate negative affective states and, in particular, control their association to a context whether or not animals are morphine-dependent. Our data in CPA, a recognized paradigm of aversive learning, showed that deletion of β4MOR alters context-dependent avoidance behavior in both morphine-dependent and naive mice, consistent with increased CPA in Tabac mice, a mutant model overexpressing β4 in endogenous sites [7, 13]. Interestingly, abolished CPA is also observed in a context associated with a malaise provoked by lithium, a non-opioid compound known to induce aversion. This indicates that, beyond aversive states associated with pharmacological blockade of the opioid system, deletion of medial habenular β4MORs minimize avoidance behavior without requirement of the endogenous opioid system. It is therefore likely that MOR-β4 double positive neurons mediate avoidance behaviour in the CPA paradigm independently from any upstream opioid tone, a hypothesis that may be further tested using opto or chemogenetic manipulation of this particular neuron population. The extent to which these neurons require contextual cues and the circuit mechanisms underlying the association deserves further investigation.

An important aspect of the study also is the apparent lack of reward-related phenotype in mutant mice, suggesting that reward processing is preserved. Mu opioid receptors largely contribute to drug reward as classically measured by CPP and self-administration [23, 56], as well as to more complex reward-related behavior such as discrete stimulus-reward learning [57] and impulsivity [58]. Given the high density of habenular MORs and the role of the Hb in reward processes [59, 60] we explored the contribution of MHb β4 MORs in reward processes. Our negative results in context-dependent morphine-reward as classically measured by CPP or in responses to discrete stimulus-reward association in an autoshaping task with palatable food, lead us to conclude that MORs in the β4 neurons of the MHb are not in involved in traditionally measured reward-related behaviors. This is also consistent with preserved MORs populations in the mesocorticolimbic system that have been widely established to be main contributors to reward processing and motivation [23, 56]. Further self-administration studies are needed to provide information on the potential contribution of habenular MORs in motivational reward.

In summary, our results strongly suggest that, rather than controlling motivational/rewarding states, MORs in β4 neurons of the MHb contribute to the aversion states and avoidance behaviors. This novel result extends the literature highlighting the MHb as a center for aversion, and involves MORs in this MHb function. In addition to β4, other habenular nicotinic subunits also contribute to aversion behaviors in rodents [8, 9], an effect that depends on nicotine evoked current amplitudes as well as cholinergic transmission in the IPN [13]. Beyond nicotine aversion, conditional deletion of CB1 receptors in MHb neurons also abolishes conditioned aversion without affecting appetitive memories and associations, a contribution that depends on cholinergic transmission into the IPN [6]. Finally, human studies using fMRI as participants experience rewarding/aversive/neutral stimuli are also starting to point at an aversive role for the habenula (medial and lateral inclusively) [61]. All these data converge towards a major role of the MHb in coding aversion states and a potential involvement of MHb-IPN cholinergic, peptidergic and glutamatergic transmissions in this role. Our novel finding of a role for MORs in this process now positions the opioid system as another important player in MHb-based aversion processing.

In fine, a fundamental task of the brain is to assign affective valence to environmental stimuli by determining whether they are rewarding and should be approached, or are aversive and should be avoided [62]. Our work showing reduced aversive responses in mutant mice demonstrates that MORs in β4 neurons of the MHb are involved in avoidance processes, and indeed contributes to these by limiting the extent of aversive responses. In addition to facilitating reward at the level of mesocorticolimbic network, therefore, MORs limit aversion within the MHb-IPN circuitry, and the two mechanisms together contribute to increase approach and decrease avoidance. Further analyses of the total habenular MORs population, as well as neurons on which they are located and their associated circuitry, will be required to fully understand the aversive properties of this neuronal population and its balanced contribution to approach/avoidance behaviors.

Clinically, given the fact that negative memories of drug withdrawal are critical for the induction of a motivational state that leads to drug seeking and consumption, developing a therapeutic strategy that specifically targets the negative symptoms of withdrawal can have a profound impact on opiate cessation.

Figure Legends

Figure 1- Deletion of habenular Mu Opioid Receptors in β4 positive neurons. We generated β4MOR-/-, a genetic conditional knockout mouse model lacking Mu Opioid Receptors (MORs) specifically in the medial habenula (MHb). **A-** β4Cre-MORmCherry mice were obtained by crossing β4Cre with MORmCherry mice. Viral injections of AAV2-FlexGFP in the MHb of these mutant mice reveal that β4 (green) and MOR (red) overlap in the MHb and fasciculus retroflexus (FR) projections; n=4. **B-** β4MOR-/- mice were obtained by crossing β4Cre with MORflox mice. Quantification of MOR revealed a significant decrease in β4MOR-/- (blue) compared to β4MOR+/+ (black), when using qPCR to assess relative *Oprm1* mRNA quantities in the MHb of β4MOR mice (right panel; n=7-9). **C-** Left panel: RNAscope probes targeting MORs and β4 in the MHb show a colocalization of MORs and β4 in the lateral part of the MHb in β4MOR+/+ control mice (left image). This colocalization is

not present in β4MOR-/- conditional knockout mice (right image); n=10. Right panel: Quantification of MOR revealed a significant decrease in β4MOR-/- (blue) compared to β4MOR+/+ (black), when using VS_DESKTOP measurement tools or counting RNAscope results; Similarly, colocalization of MOR+β4 as counted in RNAscope images was abolished in β4MOR-/- mice compared to β4MOR+/+ mice. n=3. **D-** [^{35}S]-GTPγS binding assay on MHb membrane preparations using selective MOR agonist DAMGO. For β4MOR+/+ (black): EC50= 9.74 and Emax= 197. For β4MOR-/- (blue): EC50=28.89 nM and Emax= 197;164. n=2 pools of 5 mice. **E-** Mice underwent a test of locomotion in an open field. Results are expressed as total distance. Intact basal locomotor activity (first 30 minutes) in β4MOR+/+ (black) and β4MOR-/- (blue) mice, as well as similar locomotor activity after saline injection (next 30 minutes) and similar hyperlocomotion after 40 mg/kg morphine injection (last 120 minutes). n=18-21 **F-** Mice were tested in a tail immersion test (TI) to assess morphine analgesia. Pain responses were similar in basal and saline groups across genotypes (histograms "B" and "S"). Morphine analgesia was also intact in a chronic TI in β4MOR-/- (blue) compared to β4MOR+/+ (black) (Evolution above the histograms). B=basal, S=saline n=7-9; Data are expressed as mean±s.e.m; * p<0.05; ** p<0.01; *** p<0.001.

Figure 2- Reduced morphine somatic withdrawal in β4MOR-/- mice. Morphine-dependent mice were tested in a precipitated withdrawal protocol that provides measures of somatic withdrawal. **I-** Withdrawal was first precipitated with naloxone, a MOR antagonist. **A-** The global withdrawal score calculated was significantly lower in β4MOR-/- (blue) compared to β4MOR+/+ control mice (black) both across blocks of five minutes (left panel) and in the total score of the session (right panel). We then looked at withdrawal symptoms individually to see whether global withdrawal score decrease was due to one or more changes. **B-J-** Detailed evolution of each withdrawal symptom monitored during the 25 min session. **B-C-D-E-** Paw tremor, body tremor, wet dog shakes and sniffing were significantly reduced in conditional knockouts compared to controls. **F-G-H-I-J-** Activity, ptosis, jumps, teeth chattering, and piloerection were not significantly different across genotypes. n=17-18.**II-** Instead of using naloxone, morphine-dependent mice were injected with mecamylamine, a nicotinic antagonist that has also been shown to induce opiate withdrawal symptoms. **A-** The global withdrawal score calculated was significantly lower in β4MOR-/- (blue) compared to β4MOR+/+ control mice (black) both across blocks of five minutes (left panel) and in the total score of the session (right panel). We looked at withdrawal symptoms individually to see whether global withdrawal score decrease was due to one or more changes. **B-J-** Detailed evolution of each withdrawal symptom monitored during the 25 min session. **D-H-** Body tremor and ptosis were significantly reduced in conditional knockouts compared to controls. **A-B-C-E-F-G-I-** Paw tremor, wet dog shakes, sniffing, activity, jumps, teeth chattering, and piloerection were not significantly different across genotypes. n=13-14. Data are expressed as mean±s.e.m. * p<0.05; ** p<0.01; *** p<0.001.

Figure 3- Reduced context aversion in morphine-dependent and naive β4MOR-/- mice. I- *Conditioned place aversion:* Naïve and morphine-dependent mice underwent different CPA paradigms that provide measures of avoidance classically attributed to a context-aversive state association. **A-** Results are expressed as % of

time spent in drug-paired compartment during post-conditioning - % of time spent in drug-paired compartment during pre-conditioning (named “CPA score”). Morphine-dependent β4MOR+/+ mice conditioned with naloxone (M.N) express a significant lower score than morphine-dependent β4MOR+/+ mice conditioned with naloxone. Saline-saline (S.S), saline-naloxone (S.N) and morphine saline (M.S) group show similar scores across genotypes. **B, C-** β4MOR+/+ mice (black) conditioned with naloxone (**B**) or LiCl (**C**) spend a significant lower % of time in drug-paired compartment in post than in pre-conditioning whereas β4MOR-/- mice (blue) conditioned with naloxone are not different from β4MOR-/- mice treated with saline. **II-** *Conditioned taste aversion:* Naïve β4MOR mice were conditioned with lithium (dose, i.p) immediately after a 30 min session of sucrose or NaCl consumption. n=5-9. **A, C** Results are expressed in g/kg. They show similar initial sucrose (**A**) or NaCl (**C**) intake across genotypes (white bars) and similar significant decrease of sucrose consumption compared to initial drinking in both controls (black) and knockouts (blue). **B, D-** Results are expressed in % of sucrose (**B**) or NaCl (**D**) consumption relative to basal level. Mice from both genotypes having received lithium (gray and green) show similar extinction profile and, 96 hour after lithium conditioning, they ingest similar amounts of sucrose or NaCl than β4MOR+/+ and β4MOR-/- control groups having received saline (black and blue). Data are expressed as mean±s.e.m.; * $p<0.05$; ** $p<0.01$

Figure 4- Preserved reward-related mechanisms in β4MOR-/- mice- I. Mice were tested in a morphine-conditioned place preference paradigm. Results are expressed as % of time spent per drug-paired compartment. Both β4MOR+/+ mice (black) and β4MOR-/- knockouts (blue) conditioned with morphine spend a significant higher % of time in drug-paired compartment in post than in pre-conditioning. Saline-saline groups of both genotypes did not show difference in post compared to preconditioning. **II-** Mice were tested in an autoshaping test in standard mouse Bussey-Saksida Touchscreen system. **A-** The autoshaping protocol consists of a training and a testing phase. Top panel represents the different phases of the protocol that is, the training phase and the testing phase. The training phase is composed of a pre-habituation phase “pre-hab” i.e. handling + food deprivation (5 days) and a habituation phase named “hab” i.e. learning to associate the food tray to an apparition of the stimulus. The testing phase is detailed in the bottom panel i.e. a flowchart overview of associative learning. Once mice reach criterion, they move to test. Trial is initiated when the mouse inserts its head in the food tray. A stimulus is presented on one of the two sides of the screen. One side (CSp) is always followed by the apparition of a reward in the tray whereas the other is never followed by a reward (CSn). If CSp is displayed and the mouse collects its reward, an inter-trial interval (ITI) starts, at the end of which another trial starts. This loops back until mice reach criterion. **B-** Results from the training phase are presented in this figure. Similar number of trials for both β4MOR-/- (blue) and β4MOR+/+ (black) during training phase; both groups reach criterion (40 trials per session of one hour) and move to test. **C-** Similar number of trials across test sessions for both genotypes. **D-** Autoshaping testing phase results are expressed in number of stimulus approaches per session. Similar CSp approaches increase across sessions in both genotypes. E- Similar stabilization of approaches towards the CSn across sessions in both genotypes. n=8; data are expressed as mean±s.e.m; * $p<0.05$; ** $p<0.01$

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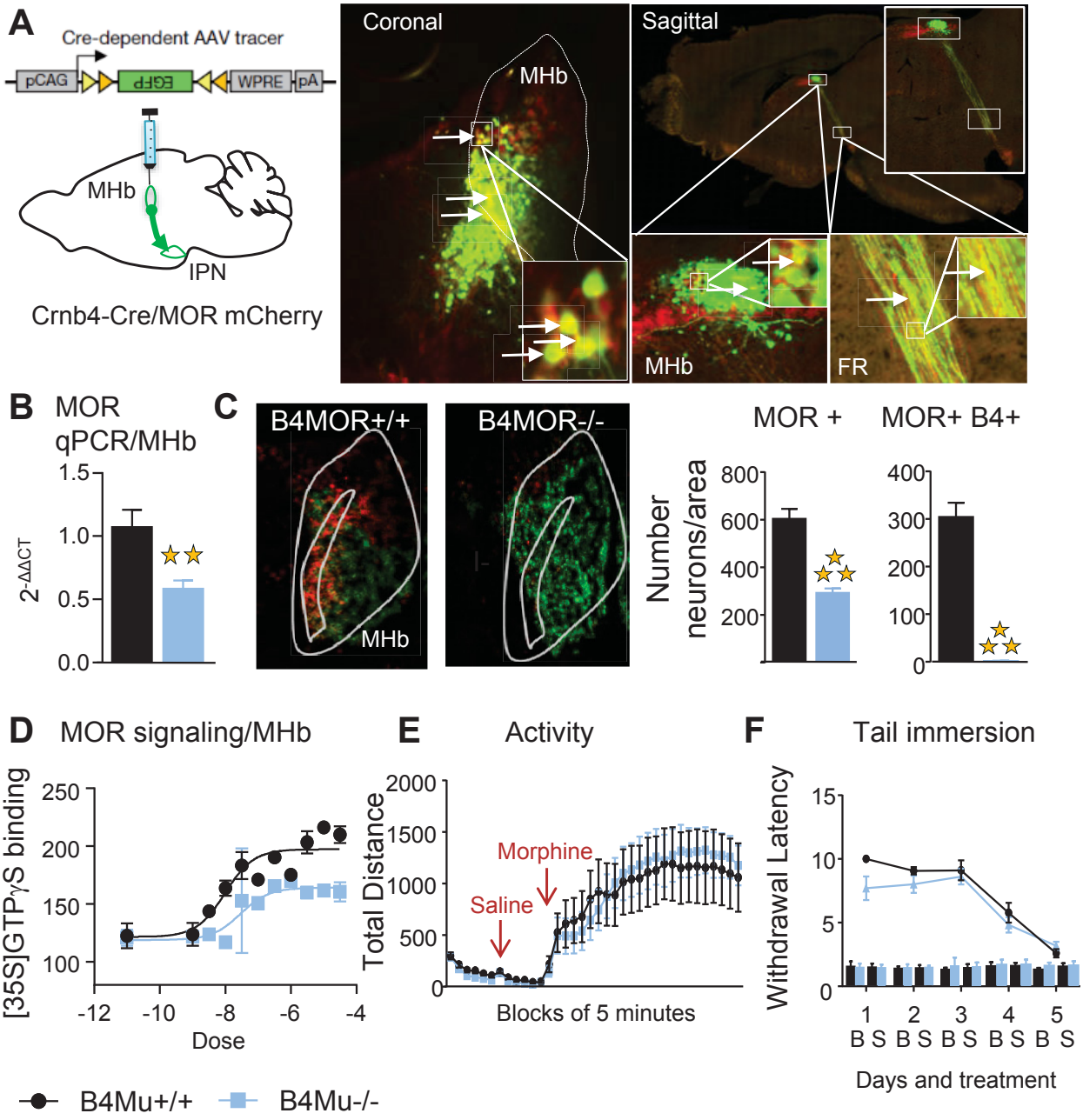
Figure 1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Cre-dependent AAV tracer
pCAG
EGFP
WPRE
pA
MHb
IPN
Crnb4-Cre/MOR mCherry
Coronal
MHb
Sagittal
MHb
FR
B
MOR qPCR/MHb
2-ΔΔCT
1.5
1.0
0.5
0.0
C
B4MOR+/+
B4MOR-/-
MHb
MOR +
MOR+ B4+
Number neurons/area
800
600
400
200
400
300
200
100
0
D
MOR signaling/MHb
[35S]GTPγS binding
250
200
150
100
-12
-10
-8
-6
-4
Dose
E
Activity
Total Distance
2000
1500
1000
500
0
Morphine
Saline
Blocks of 5 minutes
F
Tail immersion
Withdrawal Latency
15
10
5
0
1 2 3 4 5
B S B S B S B S B S
Days and treatment
B4Mu+/+
B4Mu-/-

Figure 2

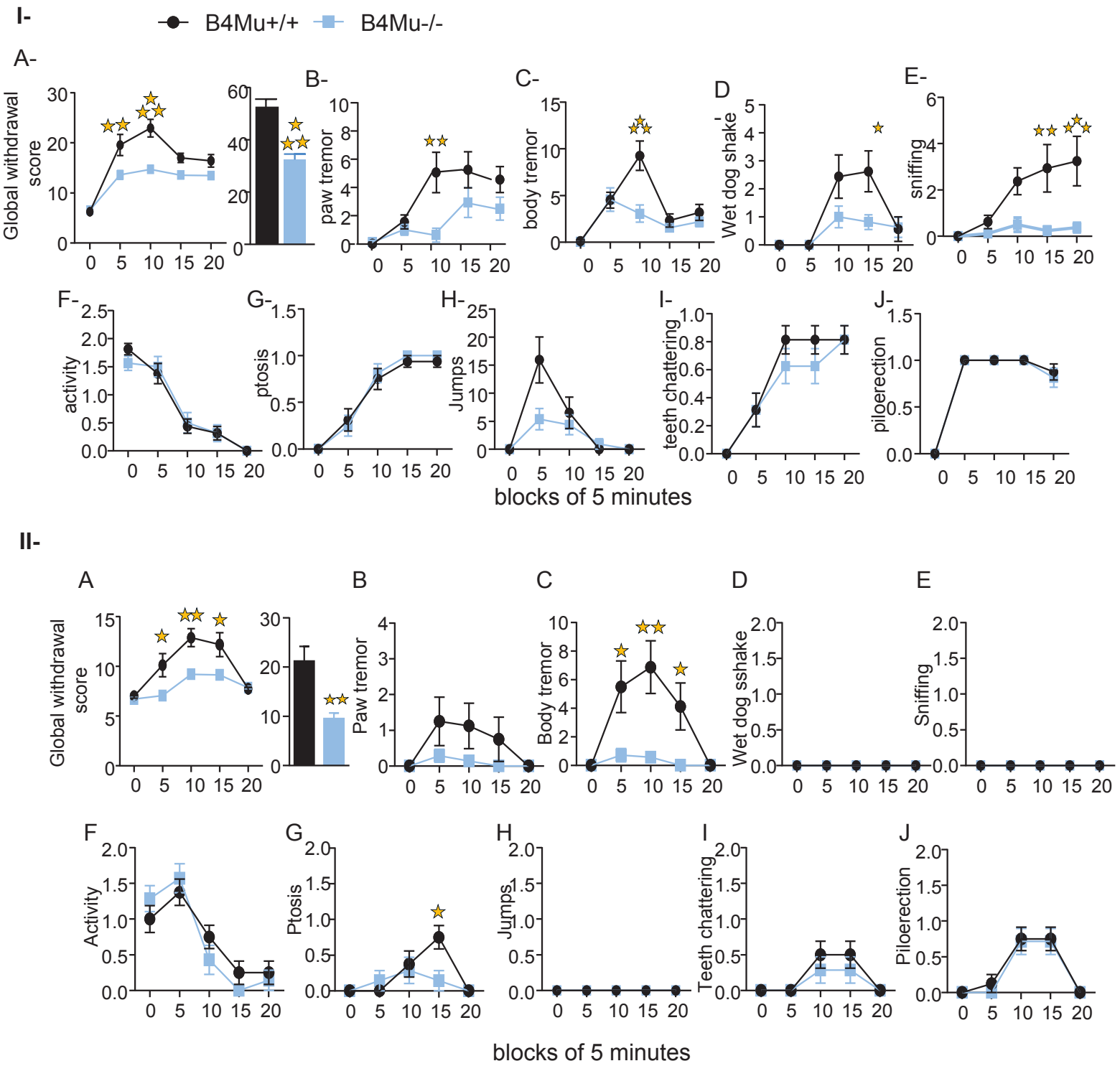

I-
B4Mu+/+
B4Mu-/-
A-
Global withdrawal score
B-
paw tremor
C-
body tremor
D
Wet dog shake
E-
sniffing
F-
activity
G-
ptosis
H-
Jumps
I-
teeth chattering
J-
piloerection
blocks of 5 minutes
II-
A
Global withdrawal score
B
Paw tremor
C
Body tremor
D
Wet dog sshake
E
Sniffing
F
Activity
G
Ptosis
H
Jumps
I
Teeth chattering
J
Piloerection
blocks of 5 minutes

Figure 3

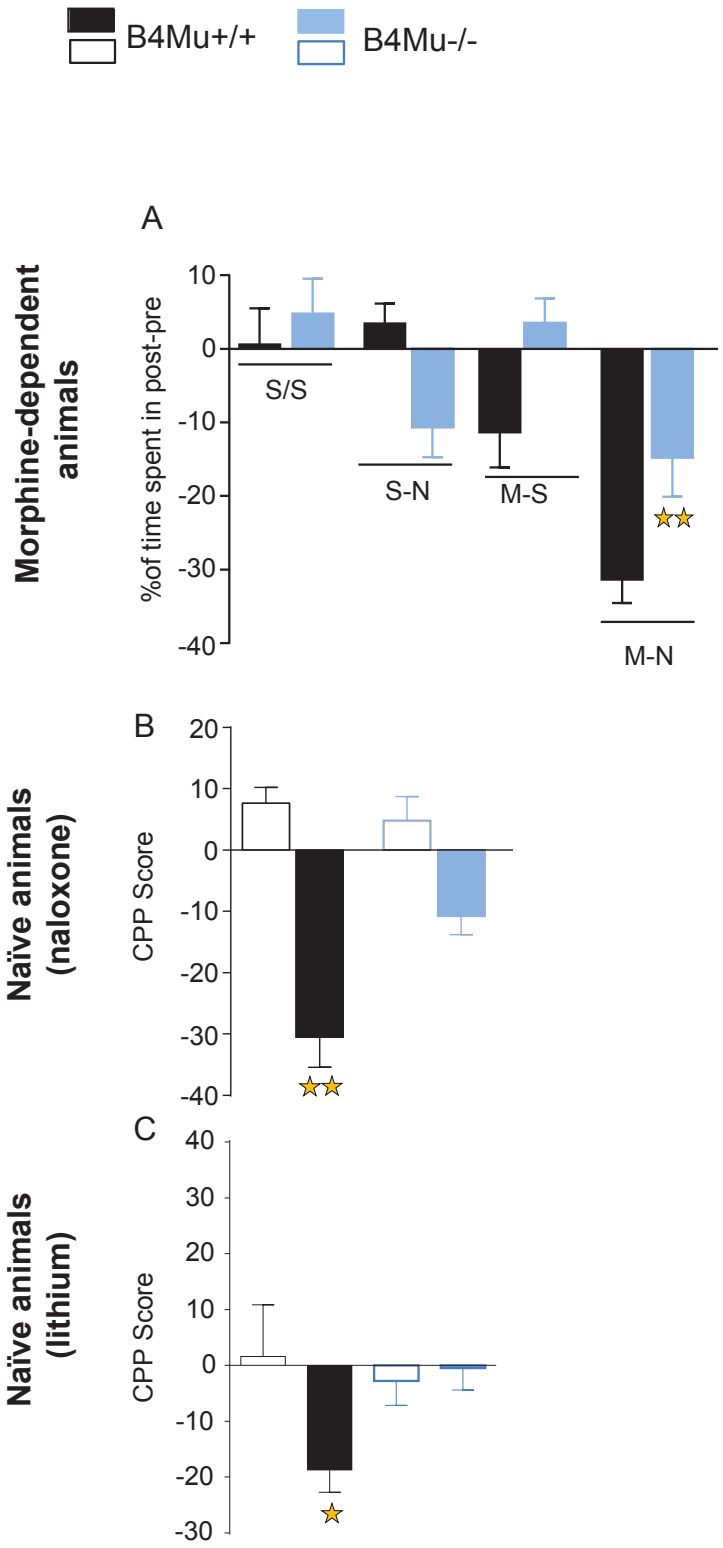
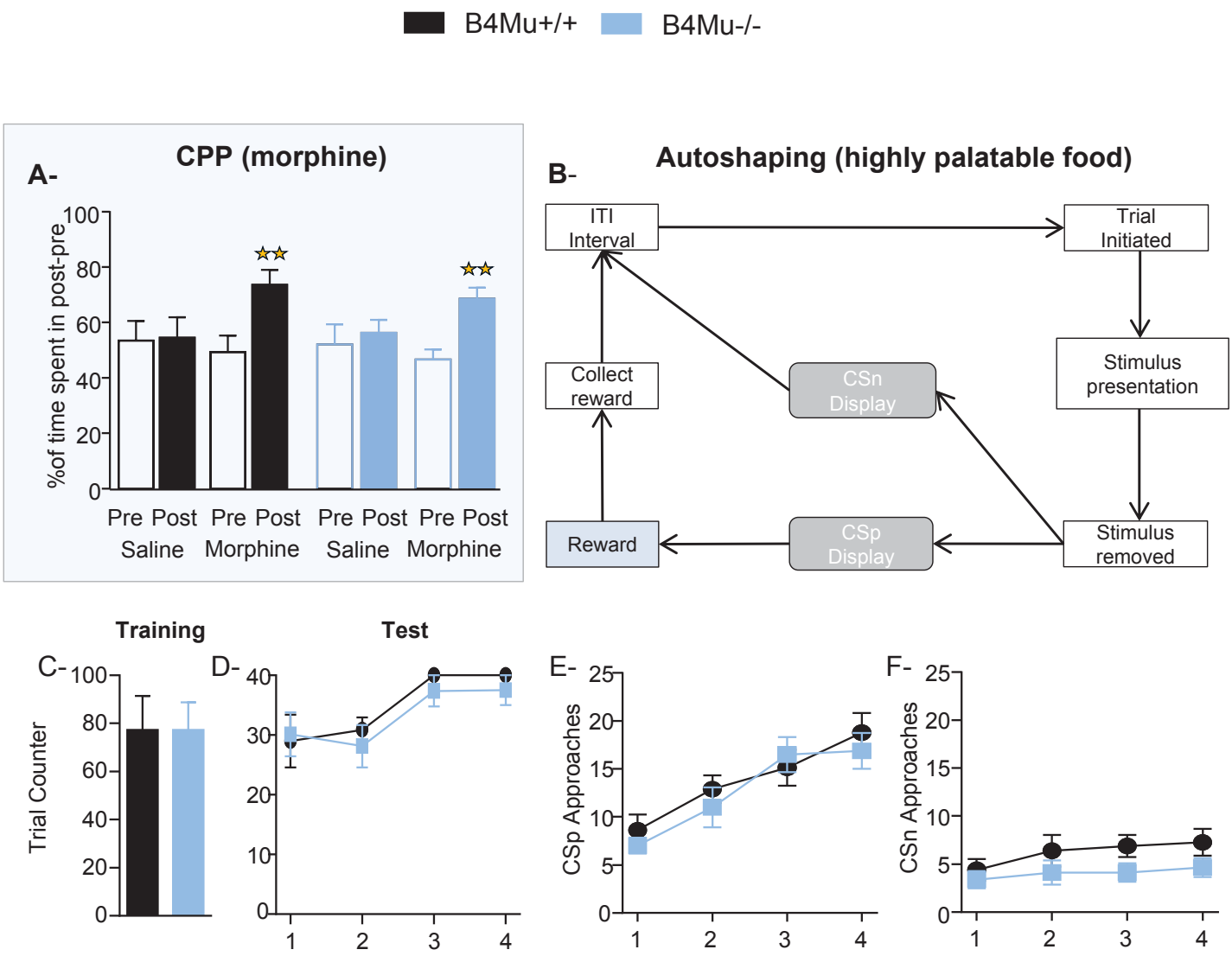

B4Mu+/+
B4Mu-/-
A
Morphine-dependent animals
%of time spent in post-pre
10
0
-10
-20
-30
-40
S/S
S-N
M-S
M-N
B
Naïve animals (naloxone)
CPP Score
20
10
0
-10
-20
-30
-40
C
Naïve animals (lithium)
CPP Score
40
30
20
10
0
-10
-20
-30

Figure 4


B4Mu+/+
B4Mu-/-
A-
CPP (morphine)
%of time spent in post-pre
100
80
60
40
20
0
Pre Post Pre Post Pre Post Pre Post
Saline Morphine Saline Morphine
B-
Autoshaping (highly palatable food)
ITI Interval
Trial Initiated
Stimulus presentation
Stimulus removed
CSp Display
CSn Display
Reward
Collect reward
C-
Training
Trial Counter
D-
Test
E-
CSp Approaches
F-
CSn Approaches

Supplementary

Supplementary Material and Methods

Genotyping- PCR was achieved on DNA from mouse digested digit sample (NaCl 0.2M; Tris-HCl 100 mM pH8.5; EDTA 5mM; SDS 0.2%; proteinase K (Sigma) 10 mg/mL; overnight at 55°C). The Cre PCR reaction was performed by adding 0.5 µL lysate to 49.5 µL reaction mix (1X PCR buffer (Sigma); MgCl2 (Sigma) 2.5 mM; dNTPs 0.2 mM (Thermo Scientific); TAQ DNA polymerase 2.5 U (Sigma); forward *Cre* primer (5'-GAT CGC TGC CAG GAT ATA CG- 3'), reverse *Cre* primer (5'-CAT CGC CAT CTT CCA GCA G-3'), forward *myosin* gene primer (5'-TTA CGT CCA TCG TGG ACA GC-3'), reverse *myosin* gene primer (5'-TGG GCT GGG TGT TAG CCT TA-3') 0.5 µM). PCR temperature cycling parameters were: 94°C 5 min, 30x (94°C 1 min, 62°C 1 min, 72°C 1 min), and 72°C 10 min. The loxP sites PCR reaction was performed by adding 0.5 µL lysate to 49.5 µL reaction mix (1X PCR buffer GoTaq (Promega); MgCl2 (Sigma) 1 mM; dNTPs 0.4 mM (Thermo Scientific); TAQ DNA polymerase 2.5 U (Promega); forward *mu floxed* gene primer (5'-GTT ACT GGA GAA TCC AGG CCA AGC-3'), reverse *mu floxed* gene primer (5'-TGC TAG AAC CTG CGG AGC CAC A-3') 1 µM). PCR temperature cycling parameters were: 94°C 5 min, 30x (95°C 1 min, 60°C 1 min, 72°C 1 min), and 72°C 10 min. The *Oprm1* gene excision PCR reaction was performed by adding 0.2 µL lysate to 49.8 µL reaction mix (1X PCR buffer (Sigma); MgCl2 (Sigma) 2.5 mM; dNTPs 0.2 mM (Thermo Scientific); TAQ DNA polymerase 2.5 U (Sigma); forward excision primer (5'-ACC AGT ACA TGG ACT GGA TGT GCC-3'), reverse excision primer (5'-GAG ACA AGG CTC TGA GGA TAG TAA C-3'), forward *myosin* gene primer (5'-TTA CGT CCA TCG TGG ACA GC-3'), reverse *myosin* gene primer (5'-TGG GCT GGG TGT TAG CCT TA-3') 0.5 µM). PCR temperature cycling parameters were: 94°C 5 min, 35x (94°C 30 sec, 61°C 30 sec, 72°C 30 sec), and 72°C 10 min.

qPCR- Mice were sacrificed by cervical dislocation. Brains were extracted, rinsed in cold 1X PBS (phosphate-buffered saline solution, Sigma) and 1-mm thick slices were cut with a stainless steel coronal brain matrix chilled on ice (Harvard apparatus, Holliston, MA, USA). The different brain regions were collected from 4 to 9 mice per genotype according to the stereotaxic atlas of mouse brain. The dorsal striatum the habenula and the cerebellum were dissected manually. Samples were immediately frozen on dry ice and kept at -80°C until processing. Samples were processed to extract total RNA, using TRIzol reagent (Invitrogen, Cergy Pontoise, France) according to manufacturer's instructions. The quality and quantity of RNA was measured with ND-1000 NanoDrop (Thermo Fisher Scientific, Wilmington, USA) spectrophotometer. Reverse transcription of 800 ng to 1 µg total RNA was performed on bilateral pooled brain samples in triplicate, in a 20 µL final volume, with Superscript II kit (Superscript II RT, Invitrogen). Real-time PCR was performed on the resulting cDNA using a Light Cycler 480 apparatus (Roche, Meylan, France) and iQ SYBR Green supermix (Biorad, Marnes-la-Coquette, France). Primers sequences were:
CCGAAATGCCAAAATTGTCA (*Oprm1* forward),
GGACCCCTGCCTGTATTTTGT (*Oprm1* reverse),
TGAGATTCGGGATATGCTGTTG (*arbp* gene "36B4" forward),
TTCAATGGTGCCTCTGGAGAT (*arbp* gene "36B4" reverse),
TGACACTGGTAAAACAATGCA (*HPRT* forward),
GGTCCTTTTCACCAGCAAGCT (*HPRT* reverse),

Thermal cycling parameters were 1 min at 95°C followed by 40 amplification cycles of 15 sec at 95°C, 15 sec at 60°C and 30 sec at 72°C. Expression levels were normalized to *β-actin* housekeeping gene levels. Two reference genes (*HPRT*, *arbp*) were tested in each run as an internal control. The 2-ΔΔCt method was used to evaluate differential expression levels (3) of Ctl, B4MOR and CMVMOR mice. Ctl animals were used as baseline to normalize. We used 6 to 7 Ctl animals, 7 to 8 B4MOR mice, and 4 CMV-MOR (total KO for the negative control). The qRT-PCR experiments were done in triplicate, and were run three times. Gene expression results are expressed relative to Ctl.

mRNA analyses- In situ Hybridization- B4MOR-/-, B4MOR+/+ and MOR-/- male mice were sacrificed and fresh brains were included into OCT and cut in a cryostat. The 20-µm thick coronal sections were directly mounted on superfrost slides and kept at -80°C till processing. In situ hybridization probes were designed by Advanced Cell Diagnostics, Inc. (Hayward, CA, USA) to detect mRNA encoding MORs (*Oprm1*), B4 (*ChrnB4*), substance P (*Tac1*), and acetylcholine transferase (*ChAT*). Experiments were conducted using the RNAscope® 2.0 Assay as previously described (Wang et al. 2012). Probes for *Oprm1*, *ChrnB4*, *ChAT* and *Tac1* were revealed using Alexa 488- Atto 550- and Atto-647-labeled probes respectively, in addition to DAPI staining. Probes were designed by ACD as follows: *Tac1*-C1 target region 20-1034, *ChAT-C2* target region 1090-1952, ChrnB4-C2 target region 1188-2482 and *Oprm1*-C3 target region 1135-2162. Briefly, tissue sections were incubated in a protease treatment, and then the probes were hybridized to their target mRNAs for 2 h at 40 °C before being exposed to a series of incubations that amplified the target probes. The images were acquired using an Olympus slide scanner (Center Valley, PA, USA) at 40X magnification and analyzed with VS-Desktop software. A 1-mm2 zone in each hemisphere was selected in one section per animal (n=3-4) of the habenula. The number of *Oprm1* positive neurons was manually counted using VS-Desktop counter, and colocalization with ChrnB4 on one hand and ChAT or Tac1 on the other was evaluated bilaterally. Results are represented as a percentage of *Oprm1* mRNA positive cells.

Two experiments were performed. The first one was to simultaneously assess MOR and B4 mRNAs in the MHb of B4MOR-/- mice and their controls and visualize the colocalization of these mRNAs. In the second experiment, we used probes to label MOR as well as SP and ChAT to characterize the specific cell population that we target in our conditional knockout.

Touchscreen Apparatus- All procedures were accomplished in standard mouse Bussey-Saksida Touchscreen system (Model 80614-20, Campden Instruments Ltd). For both experiments, mice are tested one session per day, 6 days per week. The test apparatus consists of fiberboard box (25 cm × 25 cm) individually housed within sound-attenuating cabinets and low-level noise fans. Each box comprises a standard operant chamber and a touchscreen (12.1-inch monitor). The ceiling of the chamber is made of clear Plexiglas and the floor of perforated stainless steel with a waste tray situated below. Within the chamber is a trapezoidal shape constructed from three black Plexiglas walls which open to the touchscreen (Dimensions: 20x18cm screen-reward tray x 24cm at screen, or 6cm at reward tray). This shape is specially designed to direct the attention

of the animal towards the touchscreen and reward tray (or food magazine). Located centrally either on the rear aluminum wall or attached to the wall opposite the touchscreen (depending on the test, see specific description of behavioural procedures below) is a food magazine linked to a liquid dispenser pump (Strawberry milkshake (Quebon) serves as food reward). A light emitting diode illuminates the food magazine. Computer graphic white square stimuli are presented on the touchscreen. Depending on the task, different black aluminum masks are attached to the face of the screen approximately 1.5 cm from the surface of the display. These masks serve to restrict the mouse's access to the display except through response apertures. A miniature infrared camera is installed above the chamber to allow monitoring of the animals' behaviour. Animal activity is recorded via infrared photobeams traversing the side walls of the chamber at the front (screen) and rear (reward tray). The apparatus and online data collection for each chamber are controlled by a Dell computer connected to an Animal Behavior Environmental Test system (Lafayette Instruments) using the Whisker control system for research[19]. All experiments were performed with the houselight off.

Autoshaping, apparatus- Autoshaping is a test that typically measures pavlovian approach learning. During Autoshaping, the chamber as well as the infrared photobeams must be in the autoshaping configuration. In this configuration the reward unit is fixed in the center on the same side as the touchscreen inside the chamber and the front beam is divided into two independent beams allowing for measurement of approaches to each side of the touchscreen separately. The autoshaping mask (24.3x28.0cm) is placed in front of the screen, providing two response windows (two white vertical rectangles of 6.5x14 cm) displayed on the left and right of the reward tray. The protocol used was adapted from the training schedule described in Horner et al, 2013.

Autoshaping, protocol- The protocol is described in detail in Supplementary Figure 7A. *Training*. Mice were initially given one 30 minutes session per day for two consecutive days in which they were allowed to habituate to the testing chamber and collect the reward (strawberry milkshake) from the central food magazine. The house light was illuminated, and reward was delivered into the central magazine on a variable intertrial interval (ITI) 10-40 sec schedule. For the mice to familiarize with the floor panel, a bolus of milkshake was delivered at the beginning of the session. *Test.* One day after pre-training, mice began the training for the autoshaping task where they learn to associate one side of the screen as positive conditioned stimulus (CSp) and the other side as CSn. The CSp signals the delivery of 7 ul milkshake immediately after the offset of the stimulus, associated with a tone and the illumination of the tray light. No tone or reward upon CSn display. One trial consists of the presentation of CSp or CSn in a randomized order during 10 seconds. After stimulus presentation (and entrance to the magazine for reward collection if CSp displayed), an inter-trial interval (ITI) variable begins (10-40s), after which the mouse is required to break the infrared beam at the rear of the chamber causing initiation of the next trial. This variable schedule ensures that the mice approach behavior is not temporally mediated and requiring the animal to go to the back of the chamber to initiate trials reduces chance approaches and ensures equal stimulus sampling. When a mouse breaks either the left or right infrared beam that runs either side of the central food magazine, it is scored as an approach to that stimulus, and no

additional approaches are scored under “lit CS” during that stimulus presentation. If the first approach during a trial is to the unlit screen, it falls under the “total CS” category. All additional approaches during before and after a trial fall under the “all CS” category (figures in supplementary). Stimulus presentation is performed in pairs, such that within a 40 trial session, there are 20 presentations of each CSp and CSn. CSp and CSn side selection is counterbalanced across subjects as are testing times across experimental groups. Mice were tested one session per day, 6 days per week.

5-Choice Reaction Time Task (5-CSRTt), apparatus- 5-CSRT is a test that typically measures attention and impulsivity. During 5-CSRT, the chamber as well as the infrared photobeams are set in the “normal” configuration. In this configuration, the reward dispenser is fixed on the opposite side to the touchscreen. The 5-CSRT mask (24.3x28.0cm) is placed in front of the screen, providing five response windows (small squares of 3x3 cm). Stimulus displays are represented as a white square in dimensions similar to the response windows. The protocol used was adapted from the training schedule described in Hoerner et al 2013[20].

5-CSRTt, protocol- The protocol is described in detail in Supplementary Figure 7B. *Training*. Given the complexity of the task, a 4-step training is required before the mice can reach testing phase. Criteria are set for each step and must be reached for a mouse to be able to move to the next step. In step I, a phase that is similar to the habituation in Autoshaping, mice are initially given 2 sessions (30 min), in which they are allowed to habituate to the testing chamber and collect milkshake from the food magazine. Once mice are reliably retrieving and consuming the reward (30 trials in 30 minutes), they can move to the next training. In step II called “Initial Touch”, mice are trained to detect a brief visual stimulus presented randomly in 1 of the 5 spatial locations. After a delay, the image is removed and strawberry milkshake is delivered accompanied by illumination of the reward light. Collection of the reward turns off the reward light and the ITI begins (5s), after which another stimulus is presented. If the mouse touches the stimulus while it is being displayed, the image is removed and three times as much reward is delivered immediately in order to reinforce the mouse’s correct touching behavior. This is repeated until the 30 trials in 60 minutes criterion is reached. In step III or “Must Touch”, mice have to touch the stimulus in order to obtain reward delivery. The criterion is 30 trials per 60 minutes. In step IV or “Must Initiate”, mice are expected to initiate the next trial by entering and exiting the reward tray with their heads. The rest of the session was similar to step III. Once the criterion has been reached for two consecutive sessions, the animals are allowed to proceed to baseline 5-CSRT testing. *Test*. In baseline testing, the first trial is initiated when the mouse collects the reward from the food magazine. After a fixed 5 s intertrial interval (ITI), the stimulus appears in one of the windows for a short period (32 s). Responses in this aperture during illumination and for 5 s afterwards (the limited hold period) are rewarded with the delivery of a single reward dose, and a correct response is recorded. Responses in a non-illuminated hole during the signal period (incorrect response) and failures to respond within the limited hold period (omission) result in a timeout period during which the house light is on for 5 s. Responses in the apertures during the ITI are recorded as premature responses and also result in a timeout period. Additional responses in the apertures during the limited hold period following a correct response are recorded as

perseverative responses. A response in the food magazine after the delivery of the reward, or after the timeout period, initiates the next trial. The 5-CSRT session is repeated until animals complete 40 trials/session and achieve >80% accuracy and <20% omissions on two consecutive days. After criteria are met, the animal is advanced to the next test phase which involves reduced stimulus duration to test for deficits in attentional accuracy.

Quinine and saccharin consumption- 48 hours after the conditioned taste aversion study, mice were tested for saccharin (0.066%) (sweet) or quinine (0.06 mM) (bitter) intake in a two-bottle choice paradigm. Each solution was offered continuously for 3 days and the amount of fluid intake was recorded every day.

Morphine-induced locomotor activity in an open field- Locomotor activity was measured in open field boxes set (16" L x 8" H x 5" W) on an infrared-emitting floor (VersaMax) under a 40-lux indirect lighting, with horizontal movements monitored. Mice were placed in the activity cages for 30 minutes (basal exploration), injected with saline (1 ml/kg), and returned to the cages for 1 h. Mice were then injected with morphine (40 mg/kg) or saline and activity was recorded for 2 h. Distance traveled every 5-min was recorded over the whole session (VersaMax monitoring system).

Morphine-induced analgesia in a tail immersion protocol- Analgesic effects of morphine on thermal nociception were assessed using the tail immersion (TI) test in two different paradigms as previously described (Scherrer et al, 2004; Contet et al, 2008; Charbogne et al, 2017). *First protocol:* Briefly, mice received i.p. injections of 10 mg/kg and were tested 45 minutes later. Mouse tails were successively immersed in two different temperature baths (48°C then 52°C) with one-minute interval between each test to avoid tissue damage. Tail withdrawal latencies were measured with a cut-off time of 10 and 15 seconds respectively. Baseline responding was measured for 3 days before testing and just before the first injection. To test tolerance of morphine analgesia (10 mg/kg daily), latencies were measured once daily for 5 days, 45 min following the morning injection. *In the second protocol*, mice received i.p. injections of cumulative doses of morphine (0, 2, 4 and 6 mg/kg) every 45 minutes and two tests were done successively, with two different temperatures and one-minute interval between each test (TI 48°C then TI 52°C).

Supplementary Results

Reduced naloxone-precipitated morphine withdrawal somatic symptoms- Next, we tested whether the decrease in global withdrawal score was due to the decrease of one or more specific withdrawal symptoms. Statistical analysis on the evolution of each physical withdrawal symptom using two-way ANOVA RM Two-way ANOVAs is detailed in Supplementary Table 1. Two-way ANOVA was performed examining effects of genotype and block were performed for each measured behavior. For paw tremor, two-way ANOVA showed significant genotype (Figure 2-I-B; $F_{(1, 30)} = 10.11$, $p < 0.01$), block ($F_{(4, 120)} = 8.42$, $p < 0.001$) and interaction effect ($F_{(4, 120)} = 8.42$, $p = 0.05$) and subsequent post-hoc revealed significant decrease of paw tremor in conditional knockouts compared to controls at block 10 ($p<0.001$). For body tremor, two-way

ANOVA showed significant genotype (Figure 2-I-C; $F_{(1, 30)}$ = 6.36, p <0.05), block ($F_{(4, 120)}$ = 16.63, p < 0.0001) and interaction effect ($F_{(4, 120)}$ = 5.16, p < 0.001) and subsequent post-hoc revealed significant decrease of body tremor in conditional knockouts compared to controls at block 10 (p<0.01). For wet dog shake, two-way ANOVA showed significant genotype (Figure 2-I-D; $F_{(1, 30)}$ = 2.64, p <0.05), block ($F_{(4, 120)}$ = 9.55, p < 0.0001) and interaction effect ($F_{(4, 120)}$ = 5.02, p < 0.05) and subsequent post-hoc revealed significant decrease of body tremor in conditional knockouts compared to controls at block 15 (p<0.05). For sniffing, two-way ANOVA showed significant genotype (Figure 2-I-E; $F_{(1, 30)}$ = 10.05, p <0.01), block ($F_{(4, 120)}$ = 6.77, p < 0.0001) and interaction effect ($F_{(4, 120)}$ = 4.35, p < 0.01) subsequent post-hoc revealed significant decrease of sniffing in conditional knockouts compared to controls at blocks 15 (p<0.01) and 20 (p<0.001). No genotype differences were observed for other withdrawal signs (activity Figure 2-I-F, ptosis Figure 2-I-G, jumps Figure 2-I-H, teeth chattering Figure 2-I-I and piloerection Figure 2-I-J).

Reduced mecamylamine-precipitated morphine withdrawal somatic symptoms- Two-way ANOVAs examining effects of genotype and block were performed for each measured behavior. For body tremor, two-way ANOVA showed significant genotype (Figure 3-II-C; $F_{(1, 65)}$ = 20.85, p <0.0001), block ($F_{(4, 65)}$ = 5.38, p <0.001) and interaction effect ($F_{(4, 65)}$ = 3.76, p <0.01) and subsequent post-hoc revealed significant decrease of body tremor in conditional knockouts compared to controls at block 5 (p<0.05), 10 (p<0.01) and 15 (p<0.05). For ptosis, two-way ANOVA showed significant block (Figure 3-II-G; $F_{(4, 65)}$ = 7.49, p <0.0001) and genotype x block effect ($F_{(4, 65)}$ = 3.7, p <0.01) but no genotype effect. Subsequent post-hoc revealed significant decrease of ptosis in conditional knockouts compared to controls at block 15 (p<0.05). No genotype differences were observed for other withdrawal signs (paw tremor Figure 3-II-B, wet dog shake Figure 3-II-D, sniffing Figure 3-II-E, activity Figure 3-II-F, jumps Figure 3-II-H, teeth chattering Figure 3-II-I and piloerection Figure 3-II-J). All control groups having undergone chronic treatment of saline instead of morphine or having received saline instead of mecamylamine at the end of the treatment showed no significant genotype difference (supplementary fig and supplementary table).

Preserved palatability to sweetness and bitterness- Palatability has shown to be altered in total MORs knockout (REF). In order to evaluate palatability in our conditional knockout, we administered a two-bottle choice to our mice with access to water in one bottle and in turn saccharine (sweet) the quinine (bitter) in the other bottle. As shown in Supplementary Figure 3, results indicate similar intake of saccharine (Supplementary Figure 3A) and quinine (Supplementary Figure 3C) as well as similar saccharine (Supplementary Figure 3B) and quinine (Supplementary Figure 3D) preference in B4MOR-/- and their controls, suggesting that targeted habenular MORs population is not necessary to palatability.

Preserved lithium-conditioned taste aversion in naïve mice- Next, we examined whether the lack of lithium CPA observed in mutant animals was limited to context-state association or could also occur during the formation of conditioned taste aversion (CTA), the other most widely used paradigm to evaluate aversive properties of a given drug{Gore-Langton, 2015 #70}. We first investigated whether taste perception was

altered in B4MOR-/- mice (Supplementary Figure 3 A to D) and results showed preserved sweet (saccharine) and bitter (quinine) taste palatability in mutant mice. Next, we tested B4MOR-/- and their controls in a series of CTA tests in which sweet (sucrose) or salty tastes (NaCl) as the conditioned flavour were paired with toxic injections of LiCl. Results show similar initial sucrose (Supplementary Figure 4A) and NaCl (Supplementary 4C) intake across genotypes, indicating that B4MOR-/- mice are capable of finding and ingesting sweet and salty solutions similarly to B4MOR+/+. Then both genotypes demonstrated neophobia towards the sucrose or NaCl solution on the first drinking session following the pairing with LiCl. Two-way ANOVA with RM revealed main days effect (Supplementary Figure 4B; $F_{(3,\ 63)sucrose}$ =11.75, $p < 0.001$; Supplementary Figure 4D; $F_{(3,\ 63)NaCl}$ =9.76, $p < 0.001$) but no genotype effect and no genotype x days interaction. Both groups reached the equivalence of their initial consumption 120 hours after LiCl injection indicating similar CTA extinction patterns in both groups. These results demonstrate that habenular MORs expressed in B4 neurons do not contribute to the formation of taste aversions.

Preserved attention and unchanged impulsivity/compulsivity in a 5-Choice Serial Reaction Time task- Next, we assessed whether other reward-related functions were altered in these mice. We thus performed a 5-CSRT task that mainly explores attention and impulsivity. This test can also be fractioned into training and testing phases (see Supplementary Figure 5B. for detailed protocol and Supplementary Table 1 for detailed statistical analysis).

The 5-CSRT, a test that mainly explores attention and impulsivity, can also provide measures of reward-related learning. The cognitive complexity of the test requires a long training before mice can reach the actual task. This training is divided into four main steps and can last as many sessions at it takes (with one session per day) for the animal to reach the criteria (40 trials on two out of three consecutive days) and move to the next step. The number of sessions spent in training for each of the four steps as well as the total number of all four steps is similar across genotypes (see Supplementary figure 4-I-A, B, C, D and E), and the number of trials during each phase is also similar (see Supplementary Figure 4-I-G, H, I and J) thus confirming the results observed in training phase of Autoshaping (Figure 4-II-A and B).

The 5-CSRT task further delivers information on accuracy, a measure of attention. Accuracy of performance was measured as the proportion of correct responses (correct responses/total responses) expressed as a percentage, without including errors of omission. Because each mouse spends a specific number of sessions per interval, the results are presented per interval rather than per session, an interval being defined by the time of appearance of the stimulus (32, 16, 8, 4 or 2 seconds) during a given number of interval until criteria (>80% accuracy, <20% omission) are reached. Two-way ANOVA for the average of accuracy percentage per interval revealed a main effect of interval (Supplementary Figure 4-II-A; $F_{(4,\ 65)}$ = 51,2, $p < 0.0001$) but no effect of genotype and no genotype x interval interaction, meaning that B4MOR+/+ and B4MOR-/- mice display similar percentage of accuracy and suggesting that targeted habenular MORs are not implicated in attention processes as measured by a 5-CSRT task.

Errors of omission are defined as failures to make a response during the 5 s limited hold period, expressed as a percentage of the total number of trials. This measure reflects possible failures of detection as well as motivational/motor deficits, depending on the overall pattern of effects[36]. Two-way ANOVA for the average of omission percentage per interval revealed a significant effect of interval (Supplementary Figure 4-II-B; $F_{(4.65)}$ = 65.23, $p < 0.0001$) but no genotype effect and no interaction indicating similar % of omissions across intervals for both genotypes. Targeted habenular MORs population does not seem to be necessary for failure of detection or motivational omissions.

5-CSRTt also provides a measure of motor impulsivity (premature responses) as well as a measure of compulsivity (perseverative responses). There were no genotype difference in either of these two measures, indicating similar impulsivity/compulsivity actions in our conditional knockouts compared to controls (Supplementary Figure 4-II-C; $F_{(4.65)}$ = 20.8, $p < 0.0001$), indicating that targeted habenular MORs population does not seem to modulate impulsivity/compulsivity.

Supplementary Figure Legends

Supplementary Figure 1- Habenular MORs are present and deleted in both substance P and acetylcholine transferase positive neurons- RNAscope probes targeting MORs (red), substance P (green) and acetylcholine transferase (blue) reveal that MORs colocalize in the two main neuronal subpopulations of the MHb. **A-** Visualization of MORs and acetylcholine transferase in β4MOR+/+ mice. **B-** Visualization of MORs and substance P in β4MOR+/+ mice. **C-** Left panel: Quantification of MOR in β4MOR+/+ mice reveals a 40.5% colocalization in substance p positive neurons (green) vs. a 36.7% of colocalization with acetylcholine transferase positive neurons (blue). Right panel: Quantification of MOR in β4MOR-/- reveals 55.6% colocalization in substance p positive neurons (green) vs. a 31.3% of colocalization with acetylcholine transferase positive neurons, when using VS_DESKTOP measurement tools for counting of RNAscope results; Data are expressed as mean±s.e.m. n=3.

Supplementary Figure 2- No withdrawal syndrome in B4MOR control groups- Four control groups per genotype were tested in the precipitated withdrawal syndrome experiment. Results are expressed as global withdrawal score per blocks of 5 minutes. **A-** B4MOR+/+ and B4MOR-/- groups having received chronic saline treatment and an injection of saline on day 6 two hours after the last treatment injection and right before scoring session showed similar absence of withdrawal syndrome. Similarly, there were no genotype difference in saline-naloxone groups (**B**), saline-mecamylamine groupe (**C**) and morphine-saline group (**D**). Data are expressed as mean±s.e.m. n=5-6

Supplementary Figure 3- Intact palatability to sweetness and bitterness in B4MOR-/- mice- Mice palatability was evaluated in a two-bottle choice with access to water and saccharine (sweet) or water and quinine (bitter). **A-** Results show similar saccharine intake in controls (black) and conditional knockouts (blue) in g/kg. **B-** Results show similar saccharine preference (saccharine consumption / water consumption)

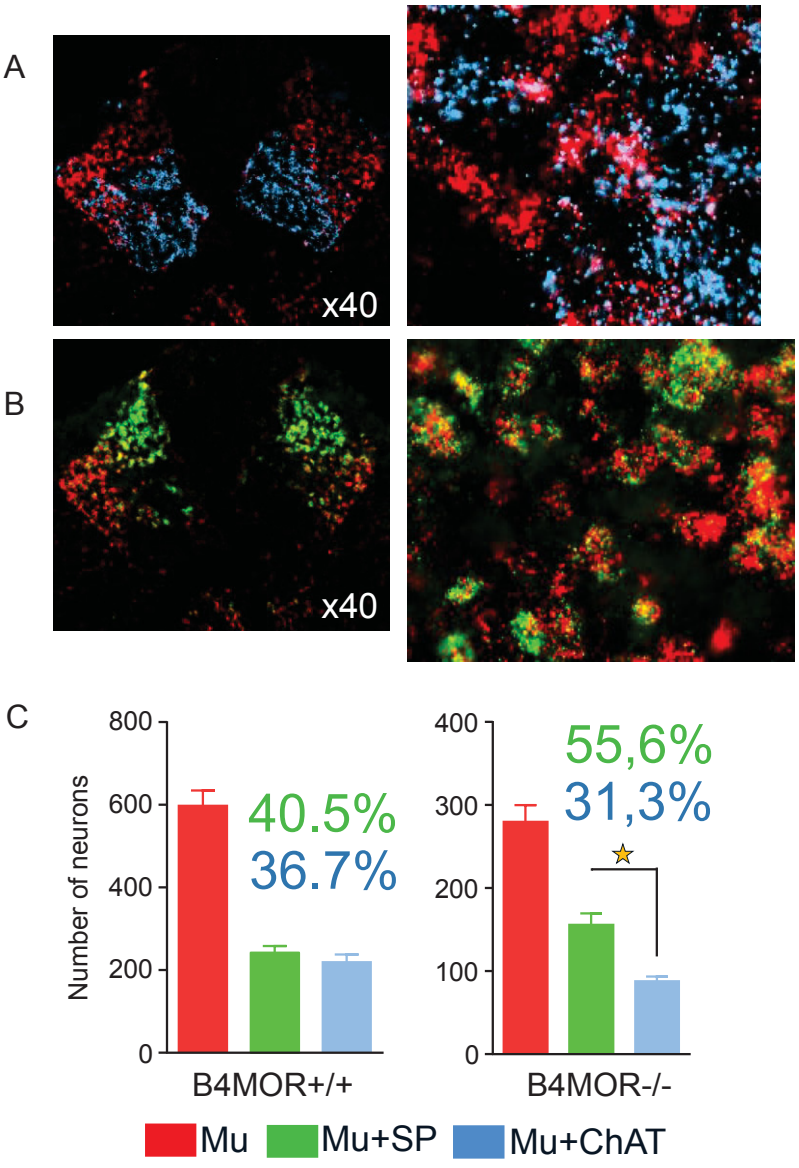
across genotypes in g/kg. **C-** Results show similar quinine intake across genotypes in g/kg. **D-** Results show similar quinine preference (quinine consumption / water consumption) across genotypes in g/kg. Data are expressed as mean±s.e.m. n=5-9

Supplementary Figure 5- 5-Choice Serial Reaction Time task (5-CSRTt) protocol- The 5-CSRTt protocol consists of a training and a testing phase. *Training phase*- The training phase is composed of 4 steps. Step I named "habituation" that is similar to autoshaping training (pre-hab + hab), Step II named "Initial Touch", Step III named "Must Touch" and Step IV named "Must Initiate". *Testing phase*- Once mice reach criterion, they move to the 5-CSRTt test phase. During test phase, mice have to initiate trial and touch the correct screen in order to obtain a reward, wait during an ITI interval then loop back. However, premature, incorrect and omission responses are punished by a time out (5-second time out during which house light is on and nothing can happen). Please refer to protocol in material and methods for additional details.

Supplementary Figure 6- Preserved pavlovian learning, attention and inhibitory control in a 5-CSRTt with highly palatable food- Mice were tested in a touch-screen based test, the 5-CSRTt, that classically measures attention and impulsivity but also provides measure of associative learning. The test consists of a training and a testing phase. **I-** Results from the training phase are presented in this figure. Given the complexity of this task, the training session comprises 4 steps respectively called "habituation", "initial touch", "must touch" and "must initiate". Number of sessions (**A to D**) and number of trials per session (**G to J**) are similar in both groups of mice. Consequently, the sum of sessions in training is similar in both groups (**E**), all groups reach criterion (30 trials per 30 minute session) and all groups have similar number of testing days (**F**). **II-** Results from the testing phase are presented in this figure. **A-** The average % of accuracy across different intervals of stimulus is similar for both genotypes. **B-** The average % of omission across different intervals of stimulus is similar for both genotypes. **C-** The average number of perseverative response across different intervals of stimulus is similar for both genotypes. n=7-8; Data are expressed as mean±s.e.m

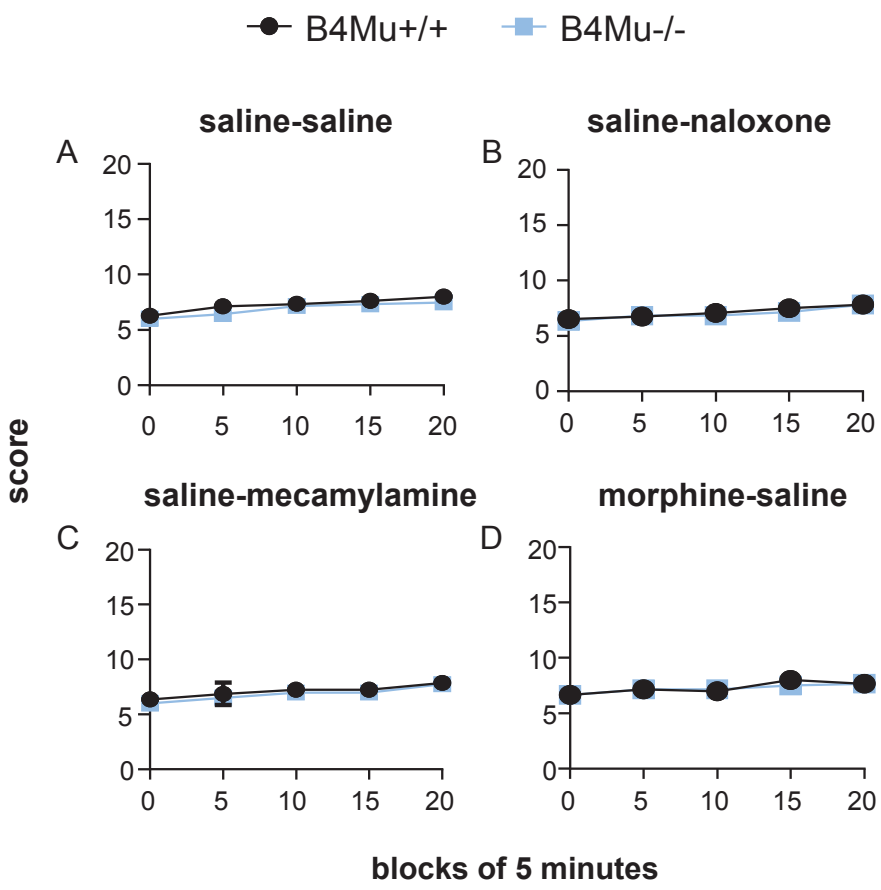
Supplementary figure 1

Deleted MORs are on both SP and ChAT positive habenular neurons



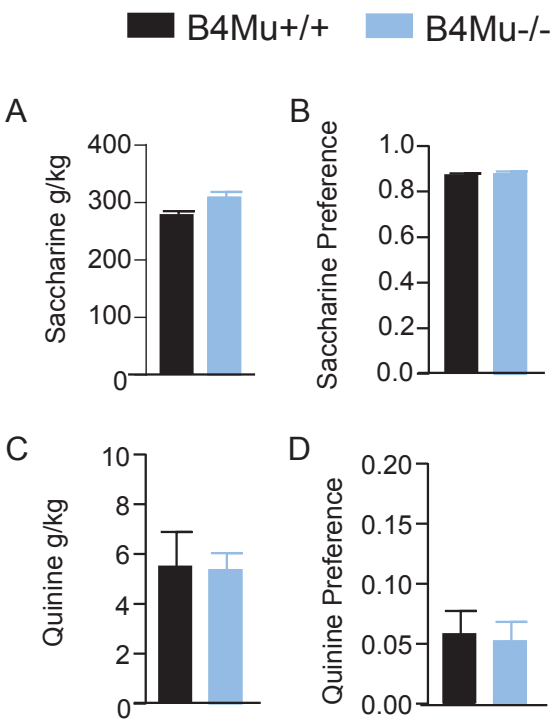
Supplementary figure 2

Somatic Withdrawal Controls



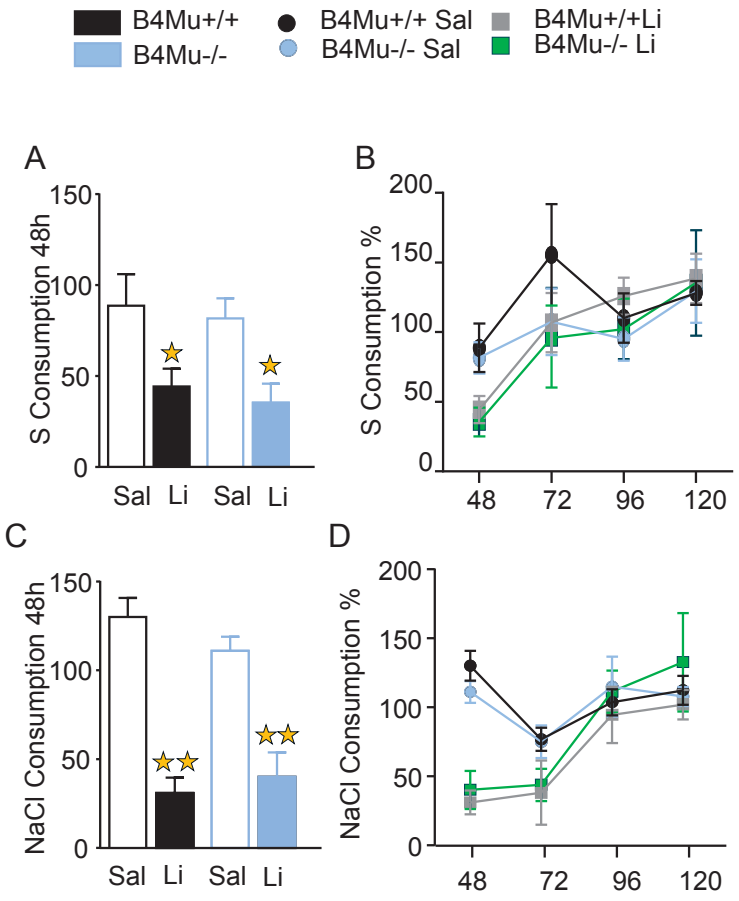
Supplementary figure 3

Preserved palatability



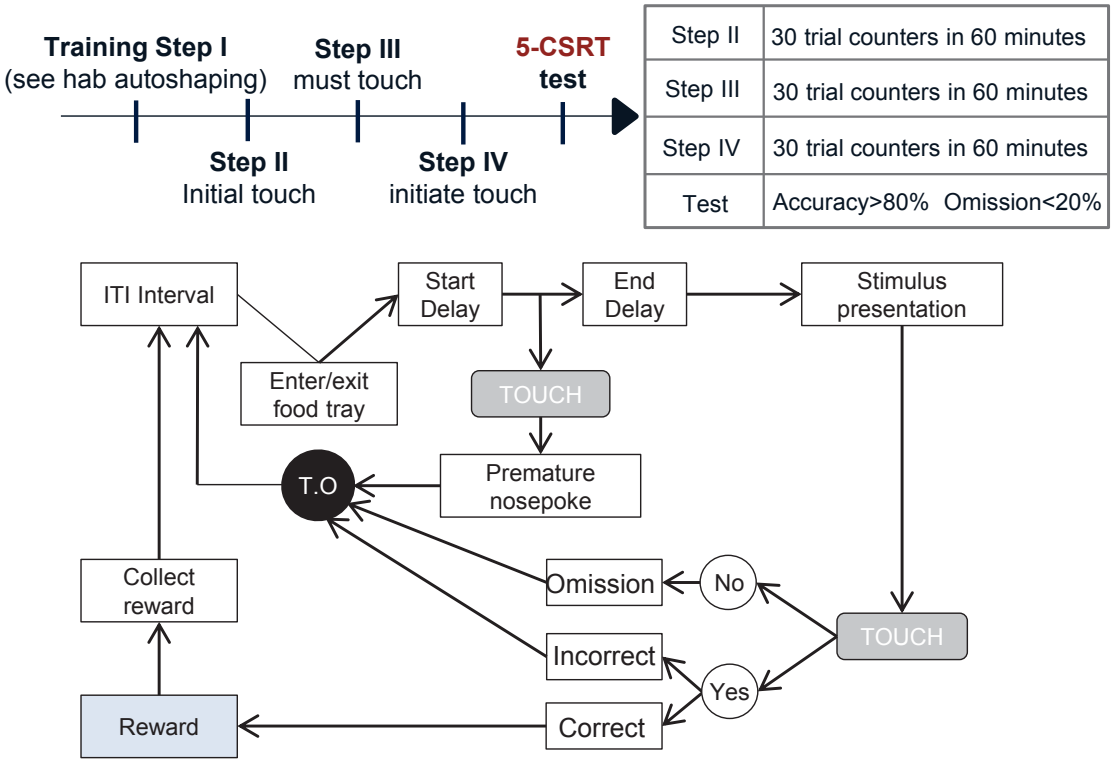
Supplementary figure 4

Preserved Conditioned Taste Aversion

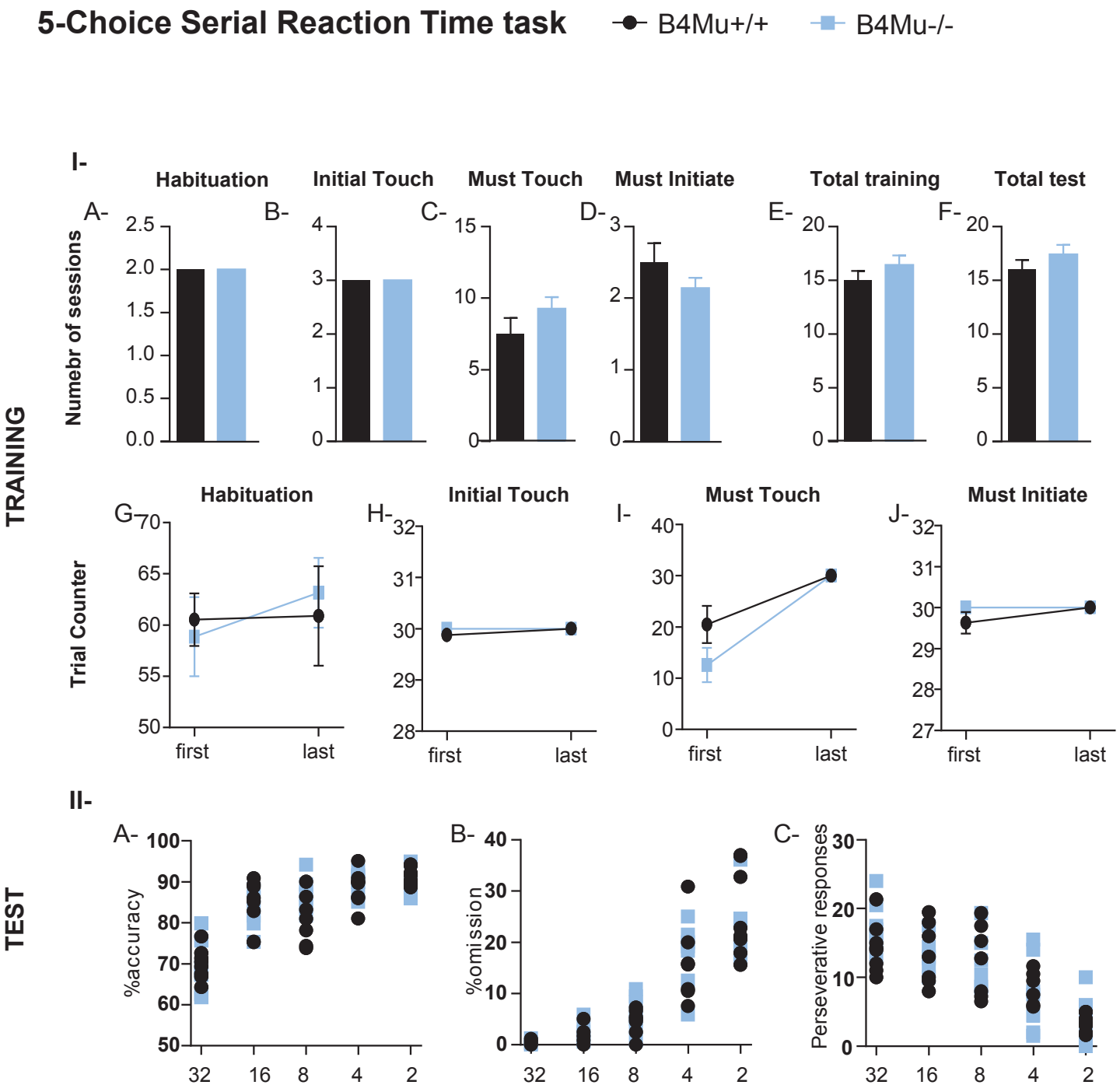


Supplementary figure 5

5-Choice Serial Reaction Time protocol



Supplementary figure 6



Study III- Mu opioid receptors in the striatum: alcohol reward

Aim of the chapter

MORs have been shown to contribute to alcohol addiction but the specific mechanisms through which the receptors participate to the disorder are still unclear. Here we explored the contribution of striatal MORs in alcohol behaviors with a focus on consumption and reward processing.

Why did we look at striatal mu opioid receptors and alcohol?

Alcohol, the most consumed addictive substance worldwide, can lead to alcohol use disorder (AUD) defined as continued drinking despite adverse consequences (Friedmann et al, 2013). In other words and consistent with general drug addiction patterns, alcoholism is a progressive brain disorder involving the transition from recreational or moderate use to loss of control over drug consumption. It is generally accepted that alcohol triggers opioid peptide release (Weerts et al, 2011) and that endogenous peptides and opioid receptors modulate both acute alcohol effects and alcohol dependence (Nutt, 2014). In fact, pharmacological modulation of the opioid system is considered an effective approach to treat alcoholism, and one of the few currently available treatments for alcohol use disorders uses mu opioid receptor (MOR) blockade as a strategy to reduce relapse (Drobes et al. 2003; Ripley et al. 2015; Soyka, Friede, & Schnitker 2016). A mechanism proposed to explain clinical efficacy of these compounds is that MOR blockade prevents MOR-mediated reward normally elicited by alcohol-induced endogenous opioid peptides, and this reduction of alcohol rewarding effect ultimately leads to diminished craving for alcohol and reduced risk of relapse (Nutt et al, 2015).

MORs have extensively been associated with reward (Charbogne et al, 2014; Fields and Margolis, 2015). Others and we have demonstrated that rewarding properties of both opiates (Matthes et al. 1996) and non-opioid drugs of abuse, including alcohol (Contet et al. 2004), are abolished in constitutive knockout mice lacking the Oprm1 gene (MOR KO mice). In particular, MOR KO mice drink less alcohol and show reduced alcohol place conditioning than their wild type counterparts (Roberts et al. 2000; Hall, Sora, & Uhl 2001), indicating that MORs are necessary for rewarding properties of

alcohol. Further, knockout mice lacking enkephalins and beta-endorphin show no elevation of stress-induced alcohol drinking (Racz et al. 2008), demonstrating altogether that endogenous opioid mechanisms involving MORs contribute to alcohol consumption, and thus substantiating the large pharmacological literature (Nutt et al, 2015). Circuit mechanisms underlying this particular MOR function, however, remain an open question and have not been investigated by genetic approaches. Mu opioid receptor is broadly expressed in the nervous system (Erbs et al. 2015) including reward (Le Merrer et al. 2009) and addiction-related circuitries (Koob & Volkow 2016). The dopamine (DA) mesolimbic circuitry is considered central to reward and motivational processes. Alcohol is self-administered in the ventral tegmental area (VTA) (Gatto et al. 1994) where DA neurons originate, and stimulates DA release in the ventral striatum (or nucleus accumbens, NAc) that receives DA inputs (Gonzales, Job, & Doyon 2004). Alcohol also directly regulates activity of the NAc, considered another hotspot for alcohol activity (Spanagel 2009), and MOR is present in both VTA and NAc. Hence, behavioral studies show that several sites of the mesolimbic DA system are key for the acquisition of alcohol self-administration and, interestingly, evidence also suggests that additional brain areas contribute to alcohol consummatory behaviors including the hypothalamus, amygdala and prefrontal cortex (Barson, Morganstern, & Leibowitz 2012). In sum, MOR-mediated alcohol reward and drinking may operate at the level of the VTA through disinhibition of DA neurons (Fields & Margolis 2015), or in the striatum that expresses high levels of endogenous opioid peptides, or even outside the DA mesolimbic system including for example the habenular circuitry where MORs are most densely expressed (Gardon et al. 2014) and modulate reward/aversion networks (Mechling et al. 2016).

How did we look at striatal mu opioid receptors and alcohol?

Within the complex meshwork that underlies alcohol reward and reinforcement, both MORs (Charbogne et al, 2014) and the striatum (Nutt et al, 2015) have been extensively implicated in alcohol-related brain functions that alter behavior. To test whether MORs expressed at the level of the striatum are necessary for the development

of alcohol drinking behavior, we took advantage of a conditional MOR knockout mouse line (Dlx-MOR KO mice), which we recently developed in our lab using a Dlx5/6-Cre driver line targeting GABAergic neurons of the forebrain (Monory et al. 2006). Dlx-MOR KO mice show a predominant and almost complete MOR deletion in the striatum, whereas MORs expressed in the VTA are intact (Charbogne et al. 2017). We therefore compared Dlx-MOR KO mice and total MOR KO mice with their respective controls for voluntary alcohol consumption in continuous and intermittent two-bottle choice paradigms as well as alcohol rewarding effects in a conditioned place preference test.

MORs and the striatum in voluntary alcohol intake. Voluntary alcohol can be measured many ways, the most common way being in a two-botte choice test with access to one bottle of water and one bottle of alcohol (Crabbe et al, 2011). While continuous access to the two bottles typically induces moderate alcohol drinking, intermittent access stimulates excessive drinking (Crabbe et al, 2011).

MORs- Previous studies have demonstrated that MOR KO mice do not self-administer alcohol under different conditions including oral administration in the continuous two-bottle choice procedure (Roberts et al, 2000). Pharmacological studies have tried to locate this MOR effect neuroanatomically (Richard and Fields, 2017) but the exact neuronal substrates have never been investigated genetically and remain unknown.

The striatum- Extensive literature have accumulated to support a striatal importance in alcohol consumption (Ravan et al, 2014). This has been shown using different drinking protocols including chronic intermittent ethanol (DePoy et al, 2013; DePoy et al, 2015) and several two-bottle choice paradigms (Barker and Taylor, 2014). For a long time alcohol-striatum literature mainly rested on dopaminergic D2 receptors, found predominantly within the extended striatum and that are thought to be critical in neurobiological mechanisms leading to AUDs (Rominger et al, 2012). After having demonstrated the importance of D1 too in alcohol consumption (Bahi and Dreyer, 2012), more recent studies are focusing on both direct and indirect striatal pathways, revealing for instance that repeated cycles of excessive alcohol intake and withdrawal potential glutamatergic strength exclusively in D1-Medium Spiny Neurons and GABAergic strength specifically in D2-Medium Spiny Neurons of the dorsal striatum

(Cheng et al, 2017). Other work further identifies potential striatal therapeutic target for alcohol use disorders, among which the kinase IKKB in the nucleus accumbens (Truitt et al, 2016), the neurotrophic factor receptor p75 in the dorsal striatum (Darcq et al, 2016), specific microRNAs targeting the protective corticostriatal BDNF pathway (Logrip et al, 2015) and other striatal gene of which the expression has been profiled (Darlington et al, 2016). Given the pattern of striatal MOR expression and their presence on D1-Medium Spiny Neurons (Charbogne et al, 2017), this population is likely to contribute to alcohol excessive consumption.

MORs and the striatum in alcohol conditioned place preference. Increased/decreased alcohol consumption can be derived from multiple neuroadaptations but the key component argued here involves increased/decreased brain reward function (Koob et al, 2014).

MORs- One of the most widely studied manifestations of drugs of abuse addictive properties is the conditioned place preference (CPP) that indicates rewarding properties of the conditioning drug, i.e. alcohol (Urs et al, 2011; Sanchez-Segura and Spanagel, 2004). Alcohol CPP is abolished in constitutive MOR knockouts (Hall et al, 2001) but the exact anatomical structures and mechanisms underlying this effect haven't been elucidated (Charbogne et al, 2017).

The striatum- Different subdivisions of the striatum contribute to rewarding properties of alcohol as measured by CPP (Gremel and Cunningham, 2008; Bahi and Dreyer, 2012) and compulsive use of alcohol is associated with a shift in brain structures involved in the reward pathway, such as the ventral and the dorsal striatum (dos Santos Junior et al, 2017). Recent studies are revealing specific striatal molecular targets among which β-arrestin 2 (Arrb2), a crucial regulator of μ-opioid receptor function (Bjork et al, 2013). Arrb2 knockout mice express increased alcohol CPP that is associated with increased MOR signaling in both dorsal and ventral striatum (Bjork et al, 2013), strongly suggesting a contribution of striatal MORs in rewarding properties of alcohol as measured by CPP.

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Mu opioid receptors in GABAergic neurons of the forebrain promote alcohol reward and drinking

Sami Ben Hamida[1,2]*, Laura-Joy Boulos[1,2]*, Michael McNicholas[2], Pauline Charbogne[2,3] & Brigitte Lina Kieffer[1,2]

Département de Médecine Translationnelle et Neurogénétique, Institut de Génétique et de Biologie Moléculaire et Cellulaire, INSERM U-964, CNRS UMR-7104, Université de Strasbourg, France[1], Douglas Research Center, Department of Psychiatry, Faculty of Medicine, McGill University, Canada[2] and Current position: Department of Neuroscience, School of Medicine, Brain Science Institute, Johns Hopkins University, Baltimore, MD USA[3]

ABSTRACT

Mu opioid receptors (MORs) are widely distributed throughout brain reward circuits and their role in drug and social reward is well established. Substantial evidence has implicated MOR and the endogenous opioid system in alcohol reward, but circuit mechanisms of MOR-mediated alcohol reward and intake behavior remain elusive, and have not been investigated by genetic approaches. We recently created conditional knockout (KO) mice targeting the *Oprm1* gene in GABAergic forebrain neurons. These mice (Dlx-MOR KO) show a major MOR deletion in the striatum, whereas receptors in midbrain (including the Ventral Tegmental Area or VTA) and hindbrain are intact. Here, we compared alcohol-drinking behavior and rewarding effects in total (MOR KO) and conditional KO mice. Concordant with our previous work, MOR KO mice drank less alcohol in continuous and intermittent two-bottle choice protocols. Remarkably, Dlx-MOR KO mice showed reduced drinking similar to MOR KO mice, demonstrating that MOR in the forebrain is responsible for the observed phenotype. Further, alcohol-induced conditioned place preference was detected in control but not MOR KO mice, indicating that MOR is essential for alcohol reward and again, Dlx-MOR KO recapitulated the MOR KO phenotype. Taste preference and blood alcohol levels were otherwise unchanged in mutant lines. Together, our data demonstrate that MOR expressed in forebrain GABAergic neurons is essential for alcohol reward-driven behaviors, including drinking and place conditioning. Challenging the prevailing VTA-centric hypothesis, this study reveals another mechanism of MOR-mediated alcohol reward and consumption, which does not necessarily require local VTA MORs but rather engages striatal MOR-dependent mechanisms.

Keywords alcohol intake, mu opioid receptor, forebrain GABAergic neurons.

Correspondence to: Brigitte L. Kieffer, Douglas Research Center, Department of Psychiatry, Faculty of Medicine, McGill University, Perry Pavilion Room E-3317.1, 6875 boulevard LaSalle, Montreal (Quebec) H4H 1R3, Canada. E-mail: brigitte.kieffer@douglas.mcgill.ca
*contributed equally to this work

INTRODUCTION

Alcohol is the most consumed addictive substance worldwide and can lead to alcohol addiction. As for every substance use disorder, alcoholism is a progressive brain disorder involving the transition from recreational or moderate use to loss of control over drug consumption. It is well recognized that alcohol triggers opioid peptide release, and that endogenous opioid peptides and opioid receptors are involved in both acute alcohol effects and alcohol dependence (Nutt 2014). In fact, pharmacological modulation of the opioid system is considered an effective approach to treat alcoholism, and one of the few currently available treatments for alcohol use disorders uses mu opioid receptor (MOR) blockade as a strategy. Naltrexone and more recently nalmefene, two long-acting antagonists that are shown to occupy MORs in human [11C] carfentanyl Positron-emission tomography studies, significantly reduce drinking, craving and relapse in heavy alcohol drinkers (Drobes *et al.* 2003; Soyka, Friede, & Schnitker 2016), and compounds with higher MOR selectivity are currently under development (Ripley *et al.* 2015). A mechanism proposed to explain clinical efficacy of these compounds is that MOR blockade prevents MOR-mediated reward normally elicited by alcohol-induced endogenous opioid

peptides, and this reduction of alcohol rewarding effect ultimately leads to diminished craving for alcohol and reduced risk of relapse (Nutt 2014).

In pre-clinical research, gene targeting has definitely established the essential role of MORs in mediating drug (Charbogne, Kieffer, & Befort 2014) and natural (Moles, Kieffer, & D'Amato 2004; Becker *et al.* 2014) rewards. Others and we have demonstrated that rewarding properties of both opiates (Matthes *et al.* 1996; Roberts *et al.* 2000; Contet, Kieffer, & Befort 2004) and non-opioid drugs of abuse (Contet *et al.* 2004), including alcohol, are abolished in constitutive knockout mice lacking the *Oprm1* gene (MOR KO mice). In particular, MOR KO mice do not self-administer alcohol, drink less alcohol, show lower alcohol preference and reduced alcohol place conditioning than their wild type counterparts (Roberts *et al.* 2000; Hall, Sora, & Uhl 2001), indicating that MORs are necessary for rewarding properties of alcohol. Further, knockout mice lacking enkephalins and beta-endorphin show no elevation of stress-induced alcohol drinking (Racz *et al.* 2008), demonstrating altogether that endogenous opioid mechanisms involving MORs contribute to alcohol consumption, and thus substantiating the large pharmacological literature (Nutt 2014). Circuit mechanisms underlying this particular MOR function, however, remain an open question and have not been investigated by genetic approaches.

Mu opioid receptor is broadly expressed in the nervous system (Erbs *et al.* 2015) including reward (Le Merrer *et al.* 2009) and addiction-related circuitries (Koob & Volkow 2016). The dopamine (DA) mesolimbic circuitry is considered central to reward and motivational processes. Alcohol is self-administered in the ventral tegmental area (VTA) (Gatto *et al.* 1994) where DA neurons originate, and stimulates DA release in the ventral striatum (or nucleus accumbens, NAc) that receives DA inputs (Gonzales, Job, & Doyon 2004). Alcohol also directly regulates activity of the NAc, considered another hotspot for alcohol activity (Spanagel 2009), and MOR is present in both VTA and NAc. Hence, behavioral studies show that several sites of the mesolimbic DA system are key for the acquisition of alcohol self-administration and, interestingly, evidence also suggests that additional brain areas contribute to alcohol consummatory behaviors including the hypothalamus, amygdala and prefrontal cortex (Barson, Morganstern, & Leibowitz 2012). In sum, MOR-mediated alcohol reward and drinking may operate at the level of the VTA through disinhibition of DA neurons (Fields & Margolis 2015), or in the striatum that expresses high levels of endogenous opioid peptides, or even outside the DA mesolimbic system including for example the habenular circuitry where MORs are most densely expressed (Gardon *et al.* 2014) and modulate reward/aversion networks (Mechling *et al.* 2016).

Here, we tested whether MORs expressed at the level of the striatum are necessary for the development of alcohol drinking behavior and alcohol reward. We took advantage of a conditional MOR knockout mouse line (Dlx-MOR KO mice), which we recently developed using a Dlx5/6-Cre driver line targeting GABAergic neurons of the forebrain (Monory *et al.* 2006). Dlx-MOR KO mice show a predominant and almost complete MOR deletion in the striatum, whereas MORs expressed in the VTA are intact (Charbogne *et al.* 2017) and (Fig. 1). We therefore compared Dlx-MOR KO mice and total MOR KO mice with their respective controls for voluntary alcohol consumption in continuous and intermittent two-bottle choice paradigms, and for alcohol rewarding effect using conditioned place preference test. Our data show that Dlx-MOR KO mice fully recapitulate the behavioral phenotype of total MOR KO mice in these tests, demonstrating unambiguously that MORs expressed in GABAergic forebrain neurons are essential for alcohol reward and drinking behavior.

MATERIAL AND METHODS

Animals

MOR KO line. Male MOR KO mice and their wild-type controls (MOR Ctl) were bred in-house on a hybrid 50 percent 129SvPas—50 percent C57Bl/6 J background (Matthes *et al.* 1996).

Dlx-MOR KO line. Briefly, the floxed Oprm1 mouse line (Oprm1fl/fl) harboring exons two and three of the MOR gene flanked by loxP sites, previously reported by our group (Weibel *et al.* 2013), was crossed with the Dlx5/6-Cre driver transgenic Cre mouse line (obtained from Beat Lutz laboratory, Institute of Physiological Chemistry, Johannes Gutenberg University, Germany). Cre-positive conditional mutant animals (Dlx5/6-Cre-Oprm1fl/fl) were obtained (hereafter named Dlx-MOR KO) and Cre-negative animals (Oprm1fl/fl) were used as controls (hereafter named Dlx-MOR Ctl). Genetic background of Dlx-MOR KO and their corresponding controls is 63 percent C57BL/6 J-37 percent 129SvPas.

Animals studied in two-bottle choice paradigm were housed individually under a 12 hours reversed light/dark cycle, whereas animals used for the other experiments were group-housed (3–5 animals per cage) under a 12 hours light/dark cycle. In both cases, the mice were 3–5 months old and weighed 25–35 g at the time of the experiments. Temperature and humidity were controlled, and food and water were available *ad libitum*.

All animals procedures in this report were conducted in accordance with (1) the European Communities Council Directive of November 24, 1986 (86/609/EEC) and approved by both the Comité Régional d'Ethique en

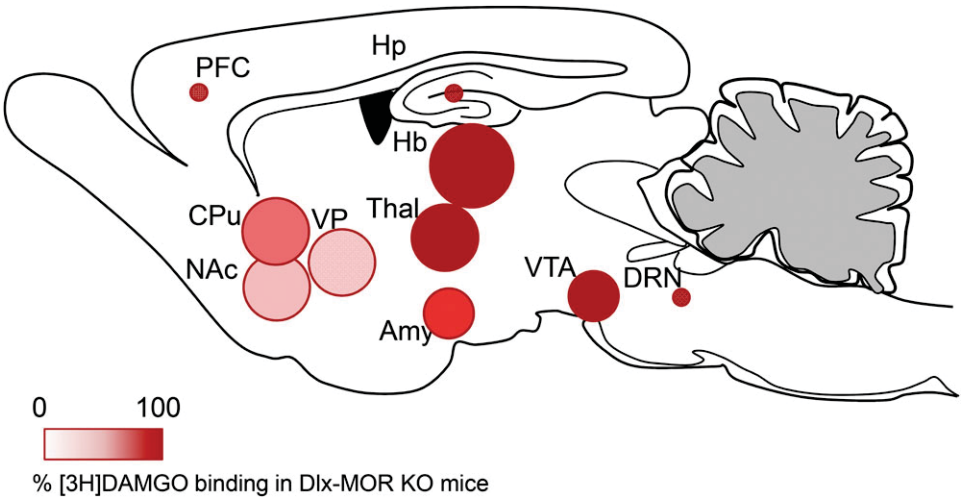

Figure 1 Anatomical distribution of the MOR deletion in Dlx-MOR KO mice. The scheme shows the quantification of MOR binding levels throughout the brain using [3H]DAMGO binding autoradiography in areas with sufficiently high signal (≥60 fmoles/mg tissue) adapted from Charbogne *et al.* 2017. Circle size represents MOR density in control mice, red color intensity represents decrease of MOR agonist binding in Dlx-MOR KO mice (from dark red to white: 0 to 100 percent decrease). In Dlx-MOR KO mice, autoradiograms show strong receptor deletion in the NAc, VP and CPu, three structures that show abundant MOR expression in control mice. Binding is slightly decreased (Amy) or remains intact in other high-MOR (Hb, Thal, VTA) or low-MOR expressing structures (PFC, Hp, DRN). Abbreviations: Amy, amygdala; CPu, caudate putamen; DRN, dorsal raphe nucleus; Hb, habenula; Hp, hippocampus; PFC, prefrontal cortex; NAc, nucleus accumbens; Thal, thalamus; VP, ventral pallidum; VTA, ventral tegmental area; MOR, mu opioid receptor [Colour figure can be viewed at wileyonlinelibrary.com]

Matière d'Expérimentation Animale de Strasbourg [2003–2010-08-(1)-58] and the local ethical comity (Comité d'Ethique en Experimentation Animale IGBMC-ICS, Com'Eth) or (2) the guidelines set forth by the Canadian Council of Animal Care and by the Animal Care Committees of McGill University/Douglas Mental Health University Institute.

Drugs and treatments

Alcohol solution for the drinking experiments was prepared from absolute anhydrous alcohol diluted to 10 or 20 percent alcohol (*v*/v) in tap water, whereas alcohol solution for systemic administration was diluted to 20 percent alcohol (v/v) in saline.

Behavioral procedures

First set of experiments

Two-bottle choice—continuous access. Oral alcohol intake was determined using continuous access to alcohol in a two-bottle choice drinking paradigm. Drinking sessions were conducted 24 hours a day during 14 consecutive days, with one bottle containing tap water, while the other contained alcohol diluted to 10 percent alcohol (*v*/v) in tap water. The bottles were weighed every day, and the mice were weighed at the beginning of the experiment. The position (left or right) of each solution was alternated between sessions, as a control for side preference. Possible loss of solution due to bottle handling was controlled by weighing bottles in empty cages.

Second set of experiments

Two-bottle choice—intermittent access. New groups of MOR KO and Dlx-MOR KO mice and their corresponding controls were first given continuous access to a solution of 10 percent alcohol for 4 days as described above (Fig. S1). Next, animals were tested for intermittent access to 20 percent alcohol in two-bottle choice procedure. Animals were given 24 hours of concurrent access to one bottle of 20 percent alcohol (v/v) in tap water and another bottle of water starting at 12 p.m. on Monday, Wednesday and Friday with 24 or 48 hours alcohol-deprivation periods (only water available) between the alcohol-drinking sessions. Water and alcohol bottles were weighed after 24 hours of access.

Quinine and saccharin consumption. One week after the alcohol-drinking study, mice were tested for saccharin (0.066 percent) (sweet) or quinine (0.06 mM) (bitter) intake. Each solution was offered for 3 days, and the amount of fluid intake and preference were recorded every day.

Third set of experiments

Conditioned place preference. Conditioned place preference (CPP) was assessed in eight place preference boxes

(Panlab, Harvard Apparatus, Spain). Each box consists of two chambers (20 × 18 × 25 cm) with distinct visual and tactile cues separated by a clear acrylic rectangular corridor. On day 1, mice were given access to the entire apparatus for 20 minutes (preconditioning). For the conditioning phase, we used a biased CPP design in which alcohol treatment was associated with the less preferred compartment during the preconditioning test. On day 2, conditioning training started with one conditioning trial per day for 6 days as follows: mice were administered (i.p.) saline solution and confined immediately to one of the compartments for 5 minutes (saline-paired compartment). The next day, mice were administered saline solution (saline group) or alcohol (1.8 g/kg, 20 percent *v*/v, alcohol group) and were confined to the other compartment (drug-paired compartment). This schedule was repeated three times until day 7. On day 8, animals were allowed to explore the entire apparatus for 20 minutes (post-conditioning test).

Blood alcohol concentrations. An independent animal cohort was used for this experiment. Animals were given a single dose of alcohol (3.2 g/kg, 20 percent v/v, i.p.), and blood samples were taken from the tail at 10, 60 and 120 minutes after injection. Serum was extracted with 3.4 percent trichloroacetic acid followed by a 5-minute centrifugation at 420×*g* and assayed for alcohol content using the NAD + −NADH enzyme spectrophotometric method. Blood alcohol concentrations were determined using a standard calibration curve.

Statistical analysis

Data were analyzed using unpaired *t*-test or two or three-way ANOVA with or without repeated measures (RM-ANOVA). Significant main effects and interactions of the ANOVAs were further investigated with the pairwise comparisons (Bonferroni *t*-test). Statistical significance was set at $P < 0.05$.

RESULTS

Moderate alcohol drinking is similarly reduced in total MOR KO and Dlx-MOR KO mice.

Our previous study showed that MOR KO mice consume less alcohol and more water than control animals using continuous access to 10 percent alcohol in a two bottle-choice procedure, leading to significantly lower alcohol preference (Roberts *et al.* 2000). Here, we used the same continuous alcohol access protocol to compare drinking behaviors of total MOR KO and Dlx-MOR KO mice with their respective controls.

As shown in Fig. 2, both MOR KO and Dlx-MOR displayed reduced levels of alcohol intake compared with their corresponding controls. Two way ANOVA with RM showed significant main effects of Genotype for [MOR KO, Fig. 2a left panel: $F_{(1,\ 17)} = 4.5$, $P = 0.049$; Dlx-MOR KO, Fig. 2b left panel: $F_{(1,19)} = 4.6$, $P = 0.048$]. A significant main effect of Sessions was observed for MOR KO [$F_{(13,221)} = 4.5$, $P < 0.001$] and Dlx-MOR [$F_{(13,247)} = 6.99$, $P < 0.001$]. No significant interaction between Genotype x Session was seen for both genotypes [MOR KO: $F_{(13,221)} = 0.26$, $P = 0.99$; Dlx-MOR KO: $F_{(13,247)} = 1.09$, $P = 0.37$]. For each Fig. 2a and b, the right panel depicts mean daily alcohol intake during the experiment and showed a significant decrease of alcohol intake by 37 percent in MOR KO ($t_{17} = 2.1$, $P < 0.05$) and 36 percent in Dlx-MOR KO mice ($t_{19} = 2.1$, $P < 0.05$) compared with control animals. Water intake was unchanged for both genotypes (Fig. 2c & d). Consequently, as shown in Fig. 2e and f right panels, preference for alcohol was also reduced by 33 percent in MOR KO (Fig. 2e: $t_{17} = 2.37$, $P = 0.03$) and 37 percent in Dlx-MOR KO mice (Fig. 2f: $t_{19} = 2.3$, $P = 0.03$) compared with their control counterparts. Two-way ANOVA RM showed a significant Genotype effect [MOR KO Fig. 2e left panel: $F_{(1,17)} = 5.6$, $P = 0.03$; Dlx-MOR KO Fig. 2f left panel: $F_{(1,19)} = 5.5$, $P = 0.03$] and Session effect [MOR KO: $F_{(13,221)} = 3.6$, $P < 0.001$; Dlx-MOR KO: $F_{(13,247)} = 7.1$, $P < 0.001$] but no significant Genotype x Session interaction effect [MOR KO: $F_{(13,221)} = 0.69$, $P = 0.77$; Dlx-MOR KO: $F_{(13,247)} = 1.67$, $P = 0.07$].

Taken together, data from MOR KO mice first confirm our previous results (Roberts *et al.* 2000) demonstrating that MORs contribute to moderate alcohol consumption. Further, the strongly reduced alcohol drinking phenotype was similarly observed in both MOR KO and Dlx-MOR KO mice, and we therefore conclude that MORs expressed in forebrain GABAergic neurons are responsible for this phenotype.

Intermittent high alcohol drinking is similarly reduced in total MOR KO and Dlx-MOR KO mice.

It is acknowledged that continuous 24-hour free choice access to alcohol does not lead to voluntary alcohol intake at levels sufficient to induce intoxication, or to engender dependence. A number of procedures have been designed to trigger 'excessive' levels of voluntary alcohol drinking in rodents that more closely mimic heavy drinking in humans, including the modification of temporal availability of the drug from continuous to intermittent (Becker 2013). We therefore further tested a 20 percent alcohol intermittent two-bottle choice drinking procedure, to determine whether the total and/or conditional MOR deletion would also affect excessive alcohol intake.

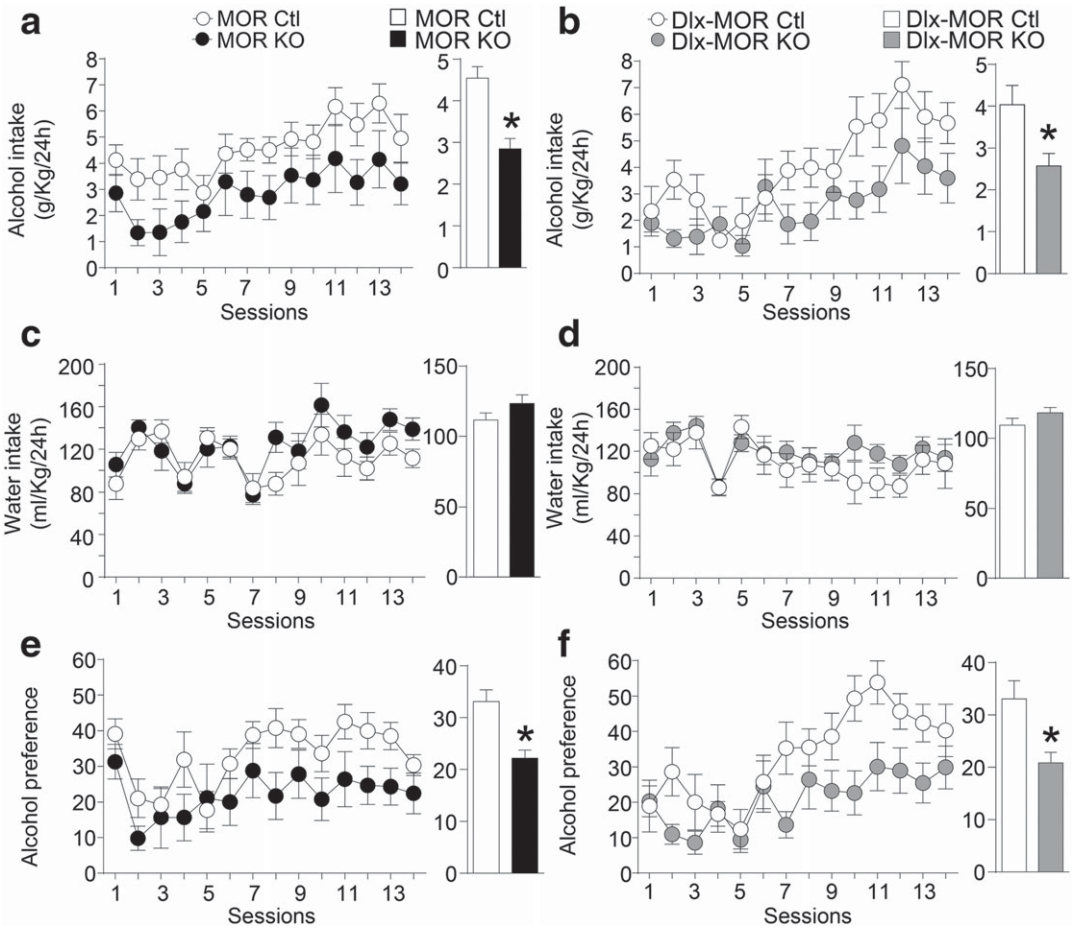

Figure 2 MOR KO and Dlx-MOR KO mice show reduced moderate alcohol consumption. (a) MOR KO and (b) Dlx-MOR KO mice consume less alcohol than their corresponding controls in an alcohol continuous-access 2-bottle-choice drinking paradigm. Animals were offered access to alcohol (10 percent *v*/v) (a–b) and water (c–d) in their home cages for 14 consecutive days (14 sessions). Values are presented as the daily mean g/kg of alcohol intake (±SEM) and ml/kg of water intake (±SEM), respectively. Alcohol preference (e–f) was calculated by dividing the total alcohol solution consumed by total fluid (alcohol plus water) consumption. Left panels represent the mean (±SEM) of alcohol and water consumption or alcohol preference per session; right panels represent mean (±SEM) of daily alcohol and water consumption or alcohol preference during the entire experiment. (a, c, e) n = 9–10, (b, d, f) n = 9–12 for each group. *$P < 0.05$

To do so, new mice (second set of experiments) were first tested in continuous access to 10 percent alcohol in a two bottle-choice procedure for 4 days to confirm our above described phenotype (Fig. S1) and then switched to intermittent 20 percent alcohol access over 4 weeks. As shown in Fig. 3a and b (right panels), this procedure indeed led to an escalation of mean daily alcohol intake for all the four groups, compared with continuous access procedure (MOR KO +65 percent and their corresponding controls +69 percent; Dlx-MOR KO mice +56 percent and their controls +54 percent). Importantly, and as for continuous access drinking, alcohol intake was remarkably lower in both total KO and those lacking MORs in GABAergic forebrain neurons compared with their respective controls (Fig. 3a & b left panels). Two way ANOVA with RM showed significant main effects of Genotype [MOR KO Fig. 3a left panel: $F_{(1,24)} = 30.3$, $P < 0.001$; Dlx-MOR KO Fig. 3b left panel: $F_{(1,45)} = 17.7$, $P < 0.001$] and Sessions [MOR KO: $F_{(11,264)} = 3.6$, $P < 0.001$; Dlx-MOR KO: $F_{(10,450)} = 3.4$, $P < 0.001$] but no significant Genotype x Session interaction effect [MOR KO: $F_{(11,264)} = 0.8$, $P = 0.61$; Dlx-MOR KO: $F_{(10,450)} = 0.9$, $P = 0.58$]. Analyses using the method of contrasts revealed a significant difference for all of the sessions (p's < 0.05) except for session 2 for MOR KO mice, as well as significant difference for sessions 3, 4, 5, 6, 7, 8, 9, 10 and 11 (p's < 0.05) for Dlx-MOR KO animals. The right panels of Fig. 3a and b represent the average of daily alcohol intake and show a significant decrease of 55 percent in MOR KO ($t_{24} = 5.5$, $P < 0.001$) and 39 percent in Dlx-MOR KO ($t_{45} = 4.2$, $P < 0.001$) mice compared with their corresponding controls. As observed above in the moderate access paradigm, there was no difference in water consumption for both mouse genotypes (Fig. 3c & d). Consequently, preference for alcohol was reduced by

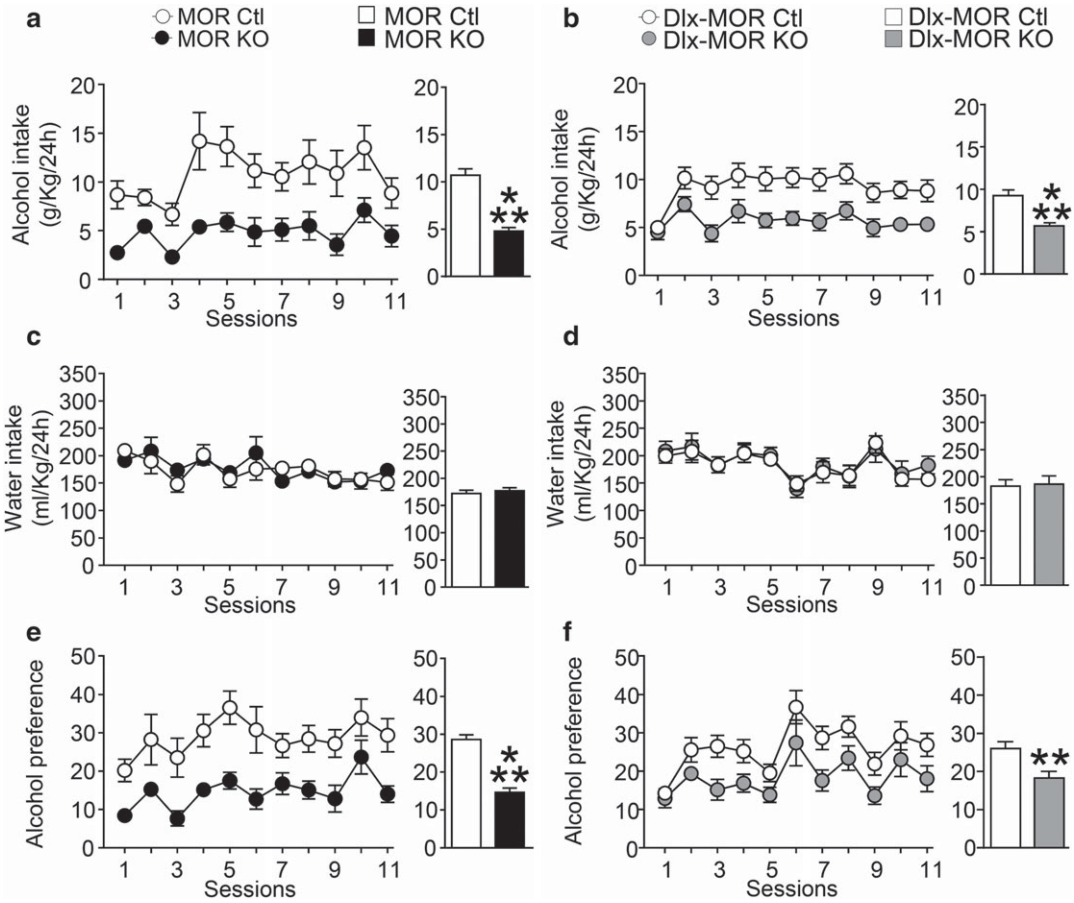

Figure 3 MOR KO and Dlx-MOR KO mice show reduced excessive alcohol consumption. (a) MOR KO and (b) Dlx-MOR KO mice consume less alcohol than their corresponding controls in a 20 percent alcohol intermittent-access 2-bottle-choice drinking paradigm. Animals were offered access to alcohol (20 percent v/v) (a–b) and water (c–d) in their home cages for 4 weeks (11 sessions). Values are presented as the daily mean g/kg of alcohol intake (±SEM) and ml/kg of water intake (±SEM), respectively. Alcohol preference (e–f) was calculated by dividing the total alcohol solution consumed by total fluid (alcohol plus water) consumption. Left panels represent the mean (±SEM) of alcohol and water consumption or alcohol preference per session; right panels represent mean (±SEM) of daily alcohol and water consumption or alcohol preference during the entire experiment. (a, c, e) n = 12–14, (b, d, f) n = 19–28 for each group. $^{**}P < 0.01$; $^{***}P < 0.001$

49 percent in MOR KO (Fig. 3e: $t_{24} = 6.3$, $P < 0.001$) and by 30 percent in Dlx-MOR KO (Fig. 3f: $t_{45} = 2.9$, $P = 0.0052$) compared with their control groups. Two way ANOVA with RM showed significant main effects of Genotype [MOR KO Fig. 3e left panel: $F_{(11,264)} = 39.8$, $P < 0.001$; Dlx-MOR Fig. 3f left panel: $F_{(1,45)} = 8.6$, $P = 0.005$] and Sessions [MOR KO: $F_{(11,264)} = 3.01$, $P < 0.001$; Dlx-MOR KO: $F_{(10,450)} = 6.99$, $P < 0.001$] but no significant Genotype x Session interaction effect [MOR KO: $F_{(11,264)} = 0.4$, $P = 0.9$; Dlx-MOR KO: $F_{(10,450)} = 0.5$, $P = 0.88$]. Analyses using the method of contrasts showed a significant difference across all sessions for MOR KO, as well as a significant difference in session 3, 7, 9 (p's < 0.05) plus a trend in sessions 6 and 11 (p's = 0.06) for Dlx-MOR KO animals.

Together, these results demonstrate that MORs, which contribute to moderate drinking, also significantly contribute to excessive alcohol drinking. Further, as for moderate drinking, the comparable phenotype of MOR KO and Dlx-MOR KO mice in this procedure indicates that MORs expressed in forebrain GABAergic neurons are essential for this behavior.

Taste palatability and alcohol metabolism are intact in MOR KO and Dlx-MOR mice.

Diminished alcohol drinking in the two groups of mutant mice could be a consequence of altered perception of alcohol flavor (Bachmanov *et al.* 2003). One week after moderate and excessive alcohol-drinking experiments were terminated, a group of animals from each genotype were tested for saccharin (0.066 percent) (sweet) or quinine (0.06 mM) (bitter) intake. Both MOR KO and Dlx-MOR KO mice, as well as their corresponding control animals, drank similar amounts of saccharin (MOR KO: Fig. 4a: $t_{24} = 1.5$, $P = 0.15$; Dlx-MOR: Fig. 4c: $t_{23} = 0.5$, $P = 0.6$) and quinine (MOR KO: Fig. 4e: $t_{24} = 0.42$, $P = 0.67$; Dlx-MOR: Fig. 4g: $t_{23} = 0.43$, $P = 0.7$) and

expressed an equivalent high preference for saccharin (MOR KO: Fig. 4b: $t_{24} = 0.9$, $P = 0.36$; Dlx-MOR: Fig. 4d: $t_{23} = 0.51$, $P = 0.62$) and low preference for quinine (MOR KO: Fig. 4f: $t_{24} = 0.38$, $P = 0.7$; Dl x-MOR: Fig. 4h: $t_{23} = 0.74$, $P = 0.46$). This result indicates that neither the total nor the conditional MOR deletion in forebrain GABAergic neurons affects quinine and saccharin consumption, suggesting that taste palatability is unchanged.

Another factor influencing alcohol-drinking levels is alcohol metabolism. We thus used a separate animal cohort to measure blood alcohol levels in mutant mice and their controls after acute alcohol administration. As shown in Fig. 4i and j, there was no difference in blood alcohol levels between MOR KO mice and their controls, as well as Dlx-MOR KO and their corresponding controls. Two way ANOVA with RM showed no effect of Genotype [MOR KO: $F_{(1,11)} = 0.86$, $P = 0.37$; Dlx-MOR KO: $F_{(1,11)} = 0.01$, $P = 0.89$], a significant effect of time [MOR KO: $F_{(2,22)} = 7.9$, $P = 0.002$; Dlx-MOR KO: $F_{(2,22)} = 56.62$, $P < 0.001$] and no significant interactions [MOR KO: $F_{(2,22)} = 0.96$, $P = 0.39$; Dlx-MOR KO: $F_{(2,22)} = 0.49$, $P = 0.61$]. This result shows that alcohol clearance is not affected by the MOR deletion, either total or conditional, indicating that the lesser alcohol consumption in both MOR KO and Dlx-MOR KO mice is not due to differences in alcohol metabolism.

Conditioned place preference to alcohol is undetectable in MOR KO and Dlx-MOR mice.

A most likely explanation for diminished voluntary alcohol intake in mutant mice is that rewarding effects of alcohol are reduced in these animals, as proposed in our (Roberts *et al.* 2000) and other (Hall *et al.* 2001) earlier studies. To substantiate this hypothesis, we re-tested the expression of alcohol-induced CPP in MOR KO mice, and then compared MOR KO mice with Dlx-MOR KO mice in this test.

As shown in Fig. 5, both the total and the conditional MOR deletion resulted in a complete loss of alcohol-induced CPP. Data for MOR KO mice are shown in Fig. 5a. Three way ANOVA comparing place preference between preconditioning and post-conditioning sessions revealed significant Treatment effect [$F_{(1,66)} = 8.1$, $P = 0.006$], Session effect [$F_{(1,66)} = 8.2$, $P = 0.005$], Session x Treatment interaction [$F_{(1,66)} = 5.5$, $P = 0.02$] and Genotype x Session x Treatment interaction [$F_{(1,663)} = 4.2$,

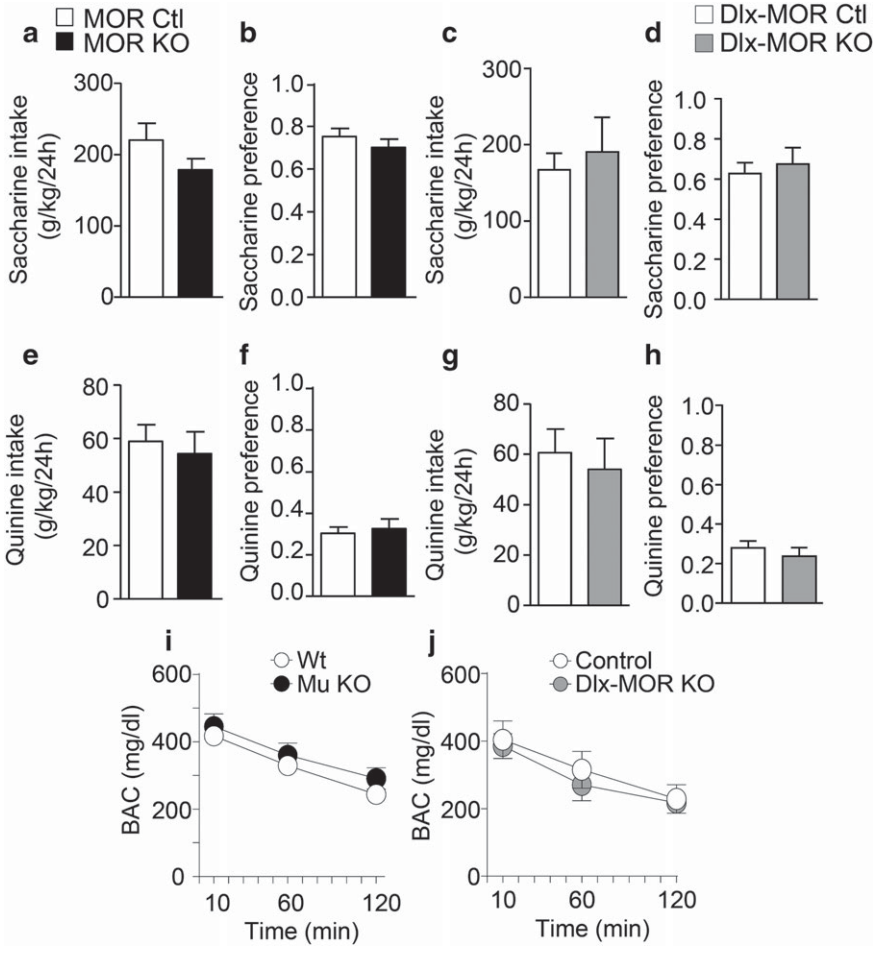

Figure 4 MOR KO and Dlx-MOR KO mice show intact taste palatability and alcohol metabolism. (a–h) No difference in consumption (a, c, e, g) or preference (b, d, f, h) for sweet (saccharin; a–d) or bitter (quinine, e–h) solutions between MOR KO and Dlx-MOR KO compared with their corresponding controls. Data are mean ± SEM of daily fluid intake in g/kg. (i–j) MORs KO and Dlx-MOR KO mice showed similar blood alcohol concentrations after injection of alcohol compared with WT controls. Data represent level of blood alcohol in mg/dl. (a–h) MORs KO, n = 12–14; Dlx-MOR KO, n = 9–16 for each group. (i–j), n = 6–7 for each group

$P = 0.04$]. Subsequent pairwise comparisons (Bonferroni *t*-test) indicated that control mice spent significantly more time in the alcohol-associated environment during post-conditioning compared with preconditioning phase ($P < 0.001$). This effect was not observed in MOR KO. Data for Dlx-MOR KO mice are shown in Fig. 5b. Three way ANOVA comparing place preference between preconditioning and post-conditioning sessions revealed significant Treatment effect [$F_{(1,160)} = 9.93$, $P = 0.002$], Session effect [$F_{(1,160)} = 9.96$, $P = 0.002$], Genotype x Treatment interaction [$F_{(1,160)} = 4.47$, $P = 0.036$], Session x Treatment interaction [$F_{(1,160)} = 5.8$, $P = 0.017$], Genotype x Session interaction [$F_{(1,160)} = 9.46$, $P = 0.002$] and no significant Genotype x Session x Treatment interaction [$F_{(1,160)} = 1.97$, $P = 0.16$]. Analyses using the method of contrasts indicated that control mice, but not Dlx-MOR KO mice, spent more time in the alcohol-associated compartment during the post-conditioning phase versus preconditioning ($P < 0.001$).

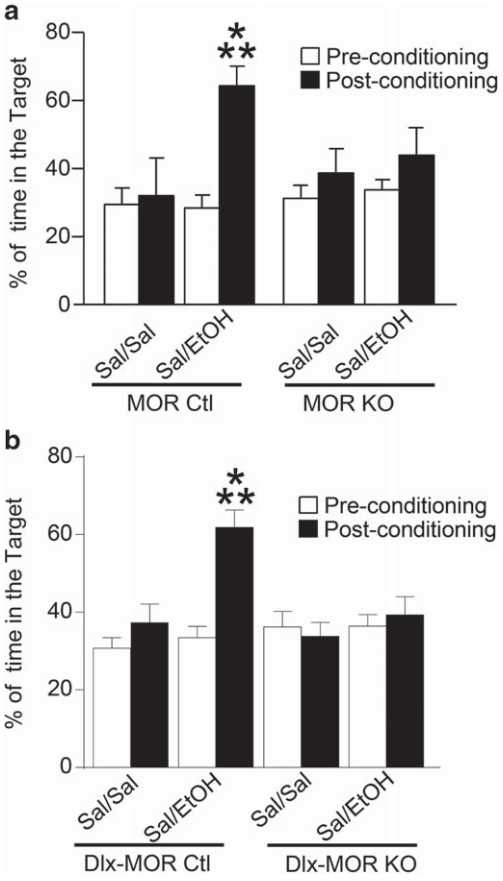

Figure 5 MOR KO and Dlx-MOR mice lack alcohol-induced conditioned place preference. (a) MOR KO and (b) Dlx-MOR KO mice do not develop alcohol-induced conditioned place preference. The habituation day (preconditioning; day 1) was designed to evaluate drug-free baseline preference for the compartments. Animals were placed in the neutral compartment and had free access to the entire apparatus for 20 minutes. Next, during the conditioning phase (6 days), animals were daily administered (i.p.) alcohol (1.8 g/kg) or saline solution and were then confined in the drug-paired or non-drug-paired compartment for 5 minutes. 24 hours after the last conditioning day, mice went through the post-conditioning test that was similar to the habituation phase. Data represent percentage of total test time spent in the alcohol-paired or saline-paired compartment during the preconditioning and the post-conditioning tests. Data represent mean (± SEM). (a) $n = 7$–12, (b) $n = 19$–23 for each group. ***$P < 0.001$

These data first confirm that MOR is essential for the expression of place preference to alcohol, and the lack of detectable alcohol CPP under our experimental conditions suggests that reduced moderate and excessive alcohol drinking in these mice both result from lower alcohol reward MOR (Discussion Section). Second, the lack of detectable alcohol CPP in both MOR KO and Dlx-MOR KO mice demonstrates that MORs expressed in GABAergic forebrain neurons are responsible for this behavioral alcohol phenotype.

DISCUSSION

In our study, MOR KO mice show reduced moderate and excessive alcohol drinking, and CPP to alcohol was undetectable under our experimental conditions, strengthening previous evidence that the MOR is essential for alcohol reward-driven behavioral responses. Further, our data show that Dlx-MOR KO and MOR KO mice show a comparable phenotype in all the behavioral experiments, demonstrating for the first time that MORs expressed in GABAergic forebrain neurons, i.e. predominantly in the striatum (Fig. 1), are responsible for MOR-mediated alcohol reward. This study therefore identifies a circuit mechanism underlying alcohol reward and consumption, which engages endogenous MOR-mediated neurotransmission at the level of striatal networks.

The reduction of moderate alcohol drinking in MOR KO mice, in this study, confirms previous evidence showing that MOR KO mice do not self-administer alcohol under several conditions, including oral self-administration and the continuous two-bottle choice procedure (Roberts *et al.* 2000). Our further observation that MOR KO mice also show reduced alcohol drinking in the intermittent alcohol access procedure is novel, and indicates that MOR-driven alcohol intake remains a prominent mechanism after animals have shifted from moderate to excessive alcohol consumption. While neurobiological mechanisms driving increases in free-choice alcohol consumption in this procedure are not completely understood, evidence for neuroadaptations at the level of several signaling pathways and at distinct sites of the reward system have been described (Carnicella, Ron, & Barak 2014). It is interesting to note that MOR remains a key player for alcohol intake even when neurochemical and circuit modifications have developed in response to higher levels of alcohol

exposure. MOR therefore plays a major role in several aspects of alcohol consumption and dependence, including the transition from the controlled/moderate drinking to excessive/compulsive use. This is concordant with human data reporting that naltrexone, a fairly low selective MOR antagonist, reduces heavy drinking (Guardia *et al.* 2002; Balldin *et al.* 2003; Pettinati *et al.* 2006; Gueorguieva *et al.* 2010) and shows significant efficacy to treat alcohol use disorders (Nutt 2014), and supports ongoing strategies to develop specific MOR antagonists (Ripley *et al.* 2015).

Our study reveals that MORs expressed in GABAergic forebrain neurons are responsible for MOR-mediated alcohol reward-driven behavior. Brain sites associated with alcohol reward have been identified throughout the corticomesolimbic dopaminergic pathway originating in the VTA and projecting to the NAc, prefrontal cortex and amygdala. The VTA, in return, is regulated by GABAergic and enkephalinergic projections from the ventral pallidum and NAc, as well as glutamatergic afferents from the prefrontal cortex (Sesack & Grace 2010). MORs and opioid peptides are weakly present in cortical areas, but are strongly expressed at striatal and VTA levels (Le Merrer *et al.* 2009; Erbs *et al.* 2015), and can therefore regulate alcohol reward and drinking at both sites. Our previous molecular characterization of Dlx-MOR KO mice indicate that the conditional mutants lack receptors predominantly in striatal GABAergic neurons, both at the level of medium spiny neuron cell bodies and at their terminals within the ventral pallidum and VTA, while MORs expressed locally in the VTA are preserved (Charbogne *et al.* 2017). Thus, our finding that this particular MOR population is responsible for MOR-driven alcohol reward is remarkable for two reasons, first, because we identify a main circuit mechanism for alcohol reward, and second, because the data suggest that MORs in the VTA do not substantially contribute to this MOR function.

The latter conclusion is at odds with the classical disinhibition hypothesis of alcohol reward (see Rev Spanagel 2009). In this model, alcohol exposure would trigger endogenous opioid release in the VTA, which in turn would activate MORs expressed in VTA local GABAergic interneurons, remove an inhibitory tone on DA neurons and promote enhanced DA release in the NAc, contributing to rewarding properties of alcohol (Spanagel & Weiss 1999). In support of this model, acute alcohol administration increased beta-endorphin release in the VTA (Hall *et al.* 2001; Jarjour, Bai, & Gianoulakis 2009). Further, VTA electrophysiology in acute midbrain slices from rats indicated that alcohol enhances MOR agonist-induced facilitation of DA neuron firing and interacts with MOR agonists/antagonists to regulate GABAergic transmission and plasticity (see (Guan & Ye 2010) and references therein). Finally, MOR signaling and levels of the GRK2 protein were increased in the lower midbrain upon alcohol treatment (Shibasaki *et al.* 2013). Our study suggests that, despite evidence for reciprocal MOR/alcohol effects locally in the VTA, the mechanism that would involve MOR-mediated disinhibition of DA neurons in the VTA is not the only mechanism contributing to rewarding effects of alcohol and the development of alcohol drinking behavior.

Our demonstration that striatal MORs are required for alcohol reward and drinking is otherwise consistent with previous pharmacological evidence that the NAc is critical site for the reinforcing and rewarding property of alcohol (reviewed in Spanagel 2009). Notably, and with regards to involvement of the opioid system, a moderate alcohol dose elevates levels of the MOR-preferring opioid peptide beta-endorphin (Lam *et al.* 2010) and Met-enkephalin (Marinelli *et al.* 2006) targeting the kappa opioid receptor and aversive responses. Further, intra-accumbal infusions of MOR agonists and antagonists enhance or decrease alcohol consumption intake and preference, respectively, in rat studies (Heyser *et al.* 1999; Hyytia & Kiianmaa 2001; Barson *et al.* 2009; Nealey *et al.* 2011). In humans, several functional magnetic resonance imaging studies indicate that brain responses to alcohol cues are associated to the OPRM1 A118G variant in ventral and dorsal striatum, the ventromedial prefrontal and the orbitofrontal cortex (Filbey *et al.* 2008; Ray *et al.* 2014; Bach *et al.* 2015). Further, a recent report using [11C]carfentanyl Positron-emission tomography imaging showed correlated MOR occupancy/opioid release in both NAc and orbitofrontal cortex upon alcohol drinking, and changes in [11C] carfentanyl binding in the cortex correlated with heavy drinking (Mitchell *et al.* 2012). These studies reveal an intriguing striato-cortical MOR implication in alcohol use disorder, consistent with the importance of MORs in the forebrain in our study. Together therefore, and beyond prior evidence, our data establish the causality between MOR activation in GABAergic forebrain neurons of the striatum and alcohol consummatory behaviors. Whether this primary event further recruits dopaminergic or non-dopaminergic mesocorticostriatal pathways to drive alcohol intake will require additional studies.

In our previous study, Dlx-MOR mice showed intact morphine and heroin CPP (Charbogne *et al.* 2017), suggesting that striatal MORs are not required for opioid reward. This finding is consistent with the notion that MOR-mediated disinhibition of DA neurons in the VTA is essential for opioid reward (Fields & Margolis 2015), and the fact that MORs are intact in the VTA of Dlx-MOR KO mice. In contrast, the present study shows that alcohol place preference is not expressed in these mice, indicating that the VTA-centric disinhibition model is not the only mechanism underlying MOR-dependent

alcohol reward. Circuit mechanisms subserving opioid and alcohol reward therefore differ. First, this is in accordance with a recent study showing that MOR blockade in the VTA prevents morphine—but not alcohol-stimulated DA release in the NAc (Valenta *et al.* 2013). Second, this conclusion substantiates the notion of divergent reward mechanisms across distinct drugs of abuse, which is notably well documented when comparing opiates and psychostimulants (Badiani *et al.* 2011; Becker, Kieffer, & Le Merrer 2017). Although the exact explanation for the alcohol/opioid difference is yet to be determined, it is well accepted that alcohol, in contrast to opiates and most other drugs of abuse, hits numerous molecular targets distributed throughout brain circuits, including ionotropic receptors and ion channels, and therefore acts via multiple and much more complex mechanisms than other abused substances (Spanagel 2009). To this respect, our genetic approach enabled the identification of a mechanism essential for alcohol reward-driven behaviors, which is mediated by a striatal MOR population and does not operate for opioid reward.

In conclusion, endogenous opioids have been implicated in pharmacological effects of alcohol and the development of alcohol use disorders (Drews & Zimmer 2010). Here, we show for the first time that rewarding effects of alcohol and alcohol consummatory behaviors are driven by MORs expressed in forebrain GABAergic neurons. Future experiments will identify the exact nature of striatal MOR-positive neurons responsible for these behaviors, and also the origin of endogenous opioid peptides responsible for this critical MOR function within neural circuits of alcohol use disorders.

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AUTHORS CONTRIBUTION

SBH and BLK designed the experiments. SBH and LJB performed and analyzed behavioral experiments. SBH, LJB and BLK interpreted the results and wrote the article. All authors have critically reviewed content and approved final version submitted for publication.

DISCLOSURE/CONFLICT OF INTEREST

The authors have no conflict of interest to disclose.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Figure S1. 4-days continuous alcohol drinking in 2nd set of experiments. MOR KO (A) and Dlx-MOR KO (B) mice consume less alcohol than their corresponding controls in the alcohol continuous-access 2-bottle-choice drinking exposure preceding intermittent access drinking. Animals were offered access to alcohol (10% *v*/v) (A-B) and water (C-D) in their home cages for 4 consecutive days (4 sessions). Values are presented as the daily mean g/kg of alcohol intake (±SEM) and ml/kg of water intake (±SEM), respectively. Alcohol preference (E-F) was calculated by dividing the total alcohol solution consumed by total fluid (alcohol plus water) consumption. Left panels represent the mean (±SEM) of alcohol and water consumption or alcohol preference per session; Right Panels represent mean (±SEM) of daily alcohol and water consumption or alcohol preference during the entire experiment. (A, C, E) $n = 12–14$, (B, D, F) $n = 19–28$ for each group. $^{**}p < 0.01$; $^{***}p < 0.001$.

General discussion

General aim of the thesis

Given their analgesic and addictive properties, MORs are the target of both widely used molecules in the clinic and widely abused drugs outside of the clinic. As part of a global health problem, MORs have thus been extensively studied and efforts are still ongoing to uncover the precise role of these receptors at the level of neural pathways with the ultimate goal to find the perfect treatment to addiction. My thesis combined genetic approaches and behavioral testing to investigate the role of MORs in reward, aversion and cognition with a focus on habenular MORs. My main finding is the importance of MORs beyond reward processing, mainly in compulsive-like behavior and aversive states, two crucial components of addiction cycles.

Habenular MORs and aversion

While the importance of MORs in reward processing is well known and generally attributed to their presence in the mesocorticolimbic circuitry, their role in the medial habenula (MHb), the structure in which MORs are most densely expressed, is unclear. This is quite surprising given the increasing literature on the habenula's role in addiction. Here we generated a conditional knockout mouse model that lacks MORs solely in the MHb and we investigated the contribution of habenular MORs in brain functions and behavioural outcomes with emphasis on reward, aversion and cognition. While the performance of our mutant model did not differ in locomotor, analgesic and reward responses to morphine nor in cognitive tasks compared to control mice, we uncovered a novel role for MORs in aversive states (see **Figure 8A**). This is the first report demonstrating that MORs control both somatic and affective aversion specifically at the level of the MHb.

Our results more specifically demonstrate that MORs in the MHb limit aversive states when naloxone is injected acutely in naïve mice or in morphine dependent mice, which strongly suggests a role of habenular MORs as a break to aversion in a healthy brain. However, whether endogenous opioids are released in aversive situations has not been investigated yet. Therefore more research is needed to understand the link between MOR and aversion in healthy brains.

As for the the role of MORs in aversion related to addiction, we have not entirely modeled prolonged abuse that is known to induce molecular adaptations in the brain. Previous reports have shown that, depending on the dose and the duration of the treatment, chronic morphine exposure either increases(Muldoon, Jackson et al. 2014) or decreases (Neugebauer, Einstein et al. 2013) c-fos activity in the MHb whereas morphine withdrawal increases glucose metabolism in the same brain structure, suggestive of homeostatic imbalance at the level of MHb in morphine-dependent animals(Kimes and London 1989). It is therefore likely that, in the context of addiction, MORs in the MHb contribute to the alteration of MHb function, as receptors are chronically activated along opioid exposure and downstream signaling adaptations take place. Interesting to note, similar patterns of cfos activation were reported in animals seeking sucrose following abstinence (Madsen, Brown et al. 2012), suggesting that neuronal populations coding for withdrawal to morphine in the MHb, including those expressing MOR, also encode drug-seeking. Further studies are needed to understand whether the dysregulation of the MHb-IPN system induces long term adaptability that confers in fine a role to MORs in the transition from reward to misery.

MORs and approach/avoidance behavior

Notably, in a distinct study using another conditional MOR knockout mouse, we identified a striatal MOR population that contributes to alcohol consumption and reward. Taken together, our results strengthen the widely-accepted idea that MORs in the mesocorticolimbic networks facilitate reward processes and further reveal that MORs within the MHb-IPN circuitry limit aversion, the two mechanisms contributing together to increase approach and decrease avoidance.

This echoes the increasingly documented ability of MORs in humans to contribute to a wide array of positive and negative affects through their modulation of approach vs. avoidance behavior (Bryant, Roberts et al. 2009, Holbrook, Galarneau et al. 2010, Comer, Zacny et al. 2012). Together these human PET and fMRI data as well as the preclinical findings that we and others have demonstrated converge to the

hypothesis that MORs constitute an important factor to optimism and resilience (Nummenmaa and Tuominen 2017).

Short term perspectives to sharpen this postulate in mice include opto- and chemogenetic targeting of neurons on which MORs are located in both the mesocorticolimbic and the MHb-IPN networks in order to validate and localize the observed results at a systemic level and further understand the contribution of MORs in the reward/aversion balance. Another interesting perspective would be to further investigate both pre- and clinically whether specific MORs networks contribute to the evaluation (attributing a value) of a given state/stimulus or to the execution of the behavior. Finally, it would be considerably salient to investigate the interactions that occur between appetitive and aversive conditioning at a behavioral level –by assessing the learning that occurs when a Pavlovian appetitive conditioned stimulus serves as a conditioned stimulus for shock in Pavlovian fear conditioning- and to study how value coding neurons, including in the habenula, contribute to both.

MORs and Addiction Models

In addition to their role in reward and aversion processing, we also demonstrated that MORs facilitate compulsive-like behavior, a crucial component in addiction. In the context of addiction, MORs thus appear to play a role not only in the initial recreational phase but also in the shift from impulsive to compulsive loss of control, as well as in the aversive withdrawal states that are thought to increase craving and prevent success in quitting (Changeux 2010). In other words, MORs contribute to every stage of an addiction cycle as conceived by Koob, Volkow, Everitt and Robbins.

At the beginning of this work we had mentioned two divergent models of addiction: the three-staged addiction cycle (Koob 2017) and the incentive salience model (Robinson and Berridge 2008). Although they fall at opposite ends of a spectrum ranging from incentive sensitization to aversion, one does not invalidate the other. Here we propose a model of addiction in which both co-exist, only underpinned by different mechanisms (see **Figure 8B**). We place dopamine at the center of brain mechanisms underlying an incentive-driven addiction cycle, whereas the opioid system preferentially

contributes to an addiction cycle driven by a surfeit of the negative. This is of course a very schematic theory, the main idea behind it being that addiction and related disorders, whether they involve self-control on mood dysregulations, ought to be thought of in the plural, with the possibility of different underlying mechanisms to different “types of addiction”.

Eventually, exploratory efforts aimed at dissecting different components of addiction may lead to the discovery of novel molecular events responsible of symptoms rather than the disorder as currently classified and thus result in the development of tailored, more effective therapeutics for substance use cessation and related psychiatric disorders treatment.

Neuroimaging and clinical perspectives

In parallel, the development of neuroimaging methods is increasing the understanding of underlying mechanisms and holds the potential to lead to biomarkers of addiction(s) as well as novel anti-addiction therapies. Interestingly, a growing number of fMRI studies performed at 3T are reporting neural activation of the habenula (Ely et al, 2016; Hétu et al, 2016; Lawson et al, 2017), opening the possibility to explore the function of this structure in human addiction. I have personally designed, together with Dr. Brigitte Kieffer and Dr. Marco Leyton, a protocol that aims at studying habenular resting state functional connectivity with related brain structures in heroin users. We want to compare ex-heroin/abstinent users stabilized under methadone to control subjects who never used heroin or other opiates. Our aim is to identify phenotypes of opioid use that focus on the aversive aspects of withdrawal. We could further add a third group of current heroin users and compare connectivity correlated with aversive withdrawal to connectivity correlated with craving.

In order to address the opioid/nicotinic cross-talk that we have observed in our work on B4MOR mice and that is well documented in both rodents (Glick et al, 2005; Neugebauer et al, 2013) and humans (Erlich et al, 2010), we have also added to the human protocol a Fagerström Test for Cigarette Dependence, a standard instrument for

assessing the intensity of physical addiction to nicotine. If this pilot study works, a perspective would be to further explore genetic variations of opioid and nicotinic receptors. Another perspective would be to explore cognitive functions during fMRI tasks. The list of possibilities is long.

“Tout future est fabuleux.”

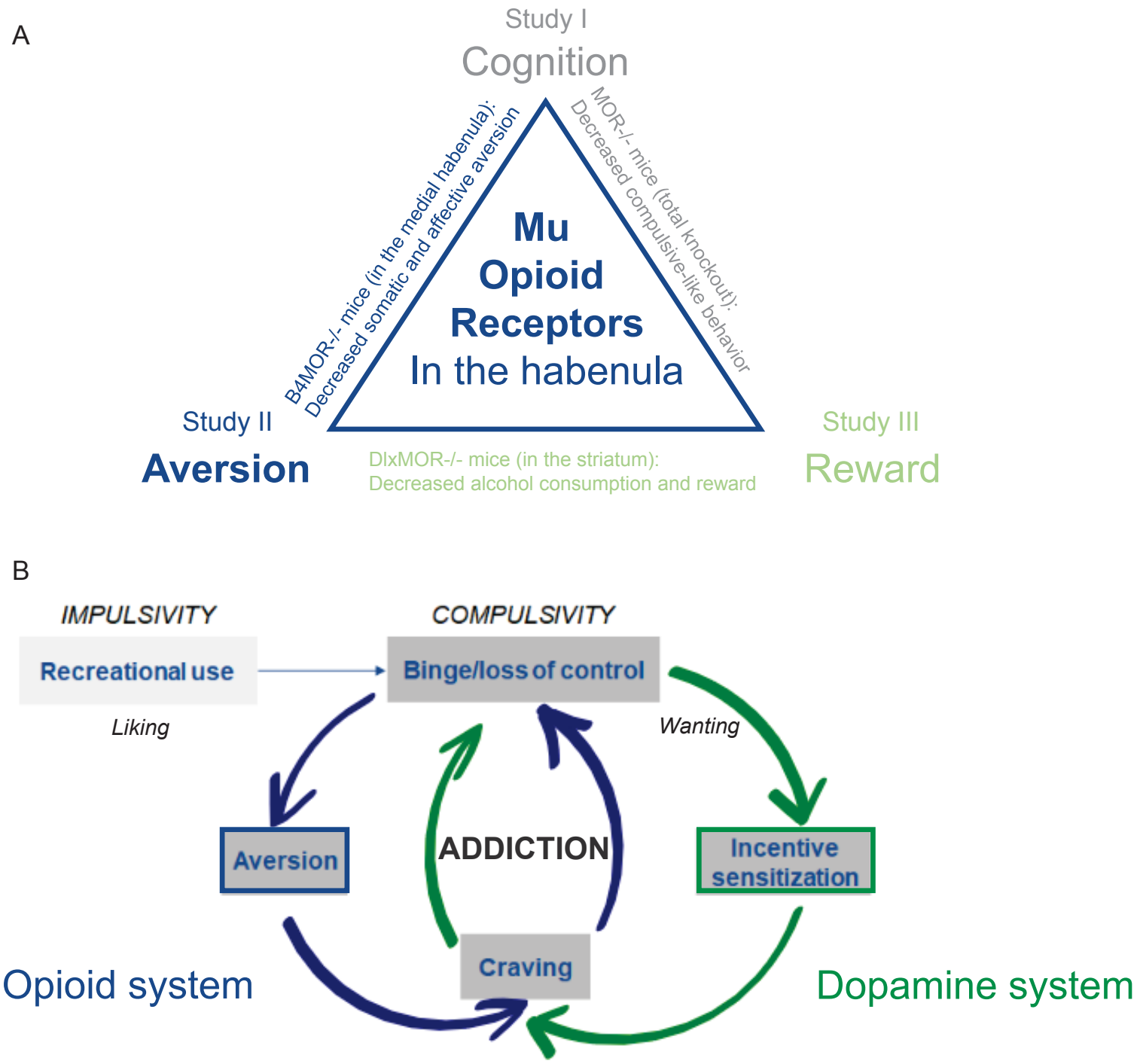

Figure 8. Discussing mu opioid receptors in addiction

A- Conclusion on our findings. Habenular mu opioid receptors are crucial for aversive states but not for cognitive or reward processing. Striatal mu opioid receptors contribute to alcohol consumption and reward. Mu opioid receptors in general are not only important for impulsivity but also for compulsive-like behavior.

B- Another model of addiction(s). Two distinct cycles of addiction: In both cycles, Stage I or binge/intoxicationis mediated by both opiates and dopamine. In Stage II for the blue cycle, negative emotional states of withdrawal are thought to increase craving and precipitate the shift from impulsive to compulsive-like loss of control. This stage may include deficits in MHb-IPN MORs (and recruitment of brain stress systems from the amygdala). In Stage II for the green cycle, the transition from casual drug use to compulsive addiction is posited to be owing to drug-induced sensitization of mesocorticolimbic mechanisms of incentive salience, namely dopamine.

Résumé en français

J’ai effectué ma thèse de neurosciences en co-tutelle entre l’université de Strasbourg (à l’Institut de Génétique et de Biologie Moléculaire et Cellulaire) en France et l’université de McGill (à l’Institut Douglas en Santé Mentale) au Canada. J’ai mené un projet principal (étude II dans la thèse) et initié deux autres projets (études I et III). Chacun des trois projets a abouti à une publication en premier auteur (respectivement acceptée, en révision et en préparation, voir dernière partie de ce résumé). J’ai aussi rédigé une revue de la littérature publiée en 2017 dans Biological Psychiatry. Les trois projets ont en commun les récepteurs mu aux opioïdes qui font partie du système opioïde, domaine d’expertise de mes deux directrices de thèse : Dr. Brigitte Kieffer et Dr. Claire Gavériaux-Ruff.

Projet I : Rôle des récepteurs mu aux opioïdes de l’habénula dans les fonctions cognitives

Les récepteurs mu aux opioïdes sont très fortement impliqués dans l’addiction. L’addiction est à son tour associée à des troubles des fonctions cognitives. Le lien entre récepteurs mu et fonctions cognitives est cependant très peu exploré. Dans cette première étude, nous utilisons des tests comportementaux qui permettent d’évaluer les fonctions cognitives et exécutives en utilisant un appareil comportant un écran tactile (TouchScreen®, Cambridge). Cet apparatus permet de tester plusieurs protocoles dans un même contexte, en utilisant les même stimuli et les mêmes récompenses. Il réduit donc le biais « anxiété » chez les rongeurs, augmente la reproductibilité et favorise l’interprétation translationnelle des résultats obtenus, puisqu’un équivalent existe chez l’humain (Cantab®, Cambridge). Nous avons principalement utilisé deux tests comportementaux (autoshaping et 5-CSRT) permettant de mesurer l’apprentissage par association, l’attention, l’impulsivité et la compulsivité. Nos résultats montrent que les souris knockouts constitutives aux récepteurs mu ont un délai dans l’acquisition de l’association stimulus-récompense et une réduction drastique de l’impulsivité/compulsivité, mais que les souris apprennent malgré le délai et que leur capacités attentionnelles semblent préservées. Ces résultats suggèrent que les récepteurs mu aux opioides contribuent au contrôle cognitif avec un rôle particulièrement important dans le contrôle inhibiteur. En montrant que les récepteurs mu facilitent un comportement dit compulsif, notre travail suggère que les récepteurs mu jouent un rôle non seulement dans le contrôle inhibiteur en présence de récompense, mais aussi dans le contrôle

inhibiteur malgré l'absence de récompense. Ces résultats sont intéressants dans la clinique de l'addiction et dans le cadre plus général des troubles du contrôle.

Projet II : Récepteurs mu aux opioides dans l'habénula : balance récompense/aversion dans l'addiction

L'habénula est une petite structure cérébrale épithalamique qui lie le cerveau antérieur au mésencéphale. Malgré sa petite taille, cette structure cérébrale peut-être divisée en deux sous-parties: l'habénula latérale et l'habénula médiale (Aizawa et al, 2011). La partie latérale fait l'objet depuis dix ans (Matsumoto et Hikosaka, 2007) de très nombreuses recherches neuroscientifiques alors que la partie médiale demeure beaucoup moins étudiée (Viswanath et al, 2013). Selon des études précliniques récentes, l'habénula médiale semble toutefois jouer un rôle majeur dans les processus aversifs (Frahm et al, 2013 ; Fowler et al, 2011). Plus particulièrement, les articles datant de 2015 à 2017 convergent pour conférer à l'habénula médiale un rôle dans les aspects aversifs de l'addiction avec mise en exergue plus spécifique encore des processus aversifs dans l'addiction à la nicotine (Zhao-Shea et al, 2015 ; Soria-Gomez et al, 2015). La sous-unité B4 des récepteurs nicotiniques, qui possède un pattern d'expression spécifique à l'habénula médiale, est placée aux centres de ce rôle dans les processus aversifs de l'addiction à la nicotine (Slimak et al, 2014). L'habénula médiale est d'autant plus intéressante qu'elle contient aussi la plus grande densité de récepteurs mu aux opioides (mu). Les récepteurs mu font partie du système opioide et contribuent à diverses fonctions avec un rôle majeur dans les effets récompensants (/renforcement positif) des opiacés (Matthes et al, 1996), d'autres drogues (Fields and Margolis, 2015) et des récompenses naturelles (Charbogne et al, 2014). Des travaux plus récents démontrent la contribution de ces récepteurs dans les changements thymiques associés au sevrage spontané (Goeldner et al, 2011) mais le rôle potentiel des récepteurs mu dans l'aversion liée ou non au sevrage n'a jamais été exploré.

Notre projet formule l'hypothèse suivante : les récepteurs mu aux opioides de l'habénula contribuent aux effets aversifs associés au sevrage aux opiacés. Pour tester cette hypothèse, nous avons construit une souris transgénique dans laquelle les récepteurs mu sont supprimés spécifiquement dans l'habénula. Pour ce faire, nous avons croisé une souris Mu-Flox et une souris B4-Cre. Etant donné la colocalization des récepteurs mu sur des neurones B4 positifs, nous avons dressé

une deuxième hypothèse, celle d'une communication (« cross-talk ») entre le système opioïdergique et le système nicotinique dans l'habénula médiale. Nos résultats démontrent d'abord que 50% des neurones exprimant des récepteurs mu n'en expriment plus dans le modèle de knockout conditionnel que nous avons construit. Nous démontrons ensuite que la population ciblée de récepteurs mu dans l'habénula contribue au sevrage physique (scoring de symptômes de sevrage), au sevrage émotionnel (conditionnement d'aversion de place) et au conditionnement d'aversion de place chez des animaux naïfs, en l'absence de traitement aux opiacés. En revanche, cette population de récepteurs mu aux opioïdes de l'habénula ne semblent pas contribuer aux effets récompensant des drogues tels que mesurés dans un conditionnement de préférence de place ou dans une batterie de tests cognitifs avec écran tactile. Nous montrons donc pour la première fois que les récepteurs mu, habituellement associés aux effets récompensant des drogues, peuvent aussi contribuer aux effets aversifs de ces derniers, ce qui confère auxdits récepteurs une complexité fonctionnelle supplémentaire. Nous démontrons aussi qu'un antagoniste nicotinique peut précipiter des symptômes de sevrage chez des animaux ayant reçu un traitement chronique à la morphine mais que ces signes sont diminués chez nos souris mutantes, indiquant un rôle croisé des récepteurs mu de l'habénula et de la sous-unité B4 des récepteurs nicotiniques de cette même structure dans la communication système opioïde / système nicotinique. Ces résultats sont intéressants dans la clinique des addictions aux opiacés et à la nicotine et particulièrement dans l'aspect aversif du sevrage.

Projet III : Implication des récepteur opioïdes mu dans l'addiction à l'alcool

C'est un projet que j'avais commencé en Master aux côtés du Dr. Ben Hamida auprès de qui je me suis engagée à continuer mes travaux pendant la première année de ma thèse. Le projet avait pour but d'explorer l'implication des récepteurs mu dans la consommation d'alcool. Nous avons d'abord montrer que des souris knockout constitutives n'exprimant pas le récepteur mu aux opioïdes consommaient significativement moins d'alcool dans un protocole d'accès continu à deux bouteilles (alcool ou eau) ainsi que dans un protocole d'accès intermittent à l'alcool. Nous avons ensuite voulu identifier la population spécifique des récepteurs mu responsables du phénotype observé. Nous avons donc utilisé un modèle de souris knockout conditionnelles chez lesquelles les récepteurs mu sont supprimés dans les neurones GABAergiques du cerveau antérieur uniquement. Nous avons démontré

que, comme les knockouts constitutifs, ces souris réduisent leur consommation d'alcool. Nous avons aussi soumis ces deux modèles de souris knockout – conditionnelles et constitutives- à une batterie de tests comportementaux dans le but de comprendre les causes de la diminution de consommation d'alcool chez ces souris. Les résultats indiquent que les récepteurs mu striataux sont essentiels à la consommation d'alcool via leur action sur les effets récompensant de cette drogue. Ces résultats sont intéressants dans la clinique de l'addiction à l'alcool et la comorbidité alcool-opiacés.

Publications

Boulos LJ, Darcq E, Kieffer BL, Translating the habenula: from rodents to humans, Biol Psychiatry. 2016 Jun 7. pii: S0006-3223(16)32430-1. doi: 10.1016/j.biopsych.2016.06.003.

Boulos LJ*, Ben Hamida S*, McNicholas M, Charbogne P, Kieffer BL, Mu opioid receptors in GABAergic neurons of the forebrain promote alcohol reward and drinking, Addiction Biology, *in press*

Boulos LJ, Kieffer BL, Ben Hamida S, Genetic knockout of mu opioid receptors alters reward-related learning and associated higher-order cognitive functions, *in revision*

Ben Hamida ., Netto S, Arefin TM, Boulos LJ, McNicholas M, Ehrlich AT, Moquin L, Gratton A, Darcq E, Harsan LA, Maldonado R and Kieffer BL, Increased alcohol seeking in mice lacking Gpr88 involves dysfunctional mesocorticolimbic networks, Biol Psychiatry, *in revision*

Boulos LJ, Ben Hamida S, Darcq E, Kieffer BL, Conditional knockout of mu opioid receptors in the habenula alters the reward/aversion balance in mice, *in preparation*

Invited Talks

March 2017 - Talk at the DGKJP Psychiatry Congress in Ulm, Germany
How to link Neuroscience and art?

September 2016 - Talk at the University of Toronto, Donnelly Research Center
Targeting mu opioid receptors in the habenula

March 2014 - Talk at the department retreat of the IGBMC
Striatal mu opioid receptors and addiction to alcohol

Pitch Talks

June 2017 Douglas Research Day, Douglas Mental Health Institute, Verdun, Canada
Mu opioid receptors in substance use disorders

March 2017 3 Minute Thesis, McGill Graduate School, Montreal, Canada
From small molecules to existential questions: heroin addiction

Posters (selection)

September 2015 IPN Retreat, McGill, Montreal, Canada
Mu opioid receptors in the habenula
Boulos LJ, Darcq E, Ben Hamida S, Kieffer BL

September 2016 IPN Retreat, McGill, Montreal, Canada
Mu opioid receptors in the habenula: dissecting reward and aversion in addiction
Boulos LJ, Ben Hamida S, Darcq E, Kieffer BL

November 2016 Society of Neuroscience (SfN), San Diego, USA
Mu opioid receptors in the habenula: dissecting reward and aversion in addiction
Boulos LJ, Darcq E, Gavériaux-Ruff C, Kieffer BL

June 2017 Douglas Research Day, Douglas Research Institute, Montreal, Canada
Mu opioid receptors in the habenula: dissecting reward and aversion in addiction
Boulos LJ, Ben Hamida S, Kieffer BL

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