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# EXPRESSION OF MU OPIOID RECEPTOR IN DORSAL DIENCEPHALIC CONDUCTION SYSTEM: NEW INSIGHTS FOR THE MEDIAL HABENULA

**O. GARDON,[†] L. FAGET,[†‡] P. CHU SIN CHUNG, A. MATIFAS, D. MASSOTTE[§] AND B. L. KIEFFER***

*Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/Université de Strasbourg, 1 rue Laurent Fries, F-67404 Illkirch, France*

**Abstract—The habenular complex, encompassing medial (MHb) and lateral (LHb) divisions, is a highly conserved epithalamic structure involved in the dorsal diencephalic conduction system (DDC). These brain nuclei regulate information flow between the limbic forebrain and the mid- and hindbrain, integrating cognitive with emotional and sensory processes. The MHb is also one of the strongest expression sites for mu opioid receptors (MORs), which mediate analgesic and rewarding properties of opiates. At present however, anatomical distribution and function of these receptors have been poorly studied in MHb pathways. Here we took advantage of a newly generated MOR-mcherry knock-in mouse line to characterize MOR expression sites in the DDC. MOR-mcherry fluorescent signal is weak in the LHb, but strong expression is visible in the MHb, fasciculus retroflexus (fr) and interpeduncular nucleus (IPN), indicating that MOR is mainly present in the MHb–IPN pathway. MOR-mcherry cell bodies are detected both in basolateral and apical parts of MHb, where the receptor co-localizes with cholinergic and substance P (SP) neurons, respectively, representing two main MHb neuronal populations. MOR-mcherry is expressed in most MHb-SP neurons, and is present in only a subpopulation of MHb-cholinergic neurons. Intense diffuse fluorescence detected in lateral and rostral parts of the IPN further suggests that MOR-mcherry is transported to terminals of these SP and cholinergic neurons. Finally, MOR-mcherry is present in septal regions projecting to the MHb, and in neurons of the central and intermediate IPN. Together, this study describes MOR expression in several compartments of the MHb–IPN circuitry. The remarkably high MOR density in the MHb–IPN pathway suggests that these receptors are in a unique position to mediate analgesic, autonomic and reward responses.**
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**Key words: medial habenula, mu opioid receptor, knock-in mice, interpeduncular nucleus, substance P, acetylcholine.**

## INTRODUCTION

The dorsal diencephalic conduction (DDC) is a highly conserved pathway present in all vertebrates, which interconnects limbic forebrain structures (septum, pallidum, striatum, lateral hypothalamus) to mid- and hindbrain regions including raphe and tegmental nuclei, locus coeruleus, ventral tegmental area (VTA) and the interpeduncular nucleus (IPN). The habenular complex (Hb) is central to DDC (Sutherland, 1982; Bianco and Wilson, 2009). The key role of this highly conserved epithalamic structure in integrating cognitive with emotional and sensory processing has raised increasing interest (Klemm, 2004; Lecourtier and Kelly, 2007; Hikosaka et al., 2008; Ikemoto, 2010; Darcq et al., 2011; Goutagny et al., 2013; Lee and Goto, 2013), and Hb contribution to motivational processes and value-based decision-making has been established (Hikosaka, 2010). The Hb comprises two nuclei, the medial habenula (MHb) and the lateral habenula (LHb), which show distinct anatomy and connectivity within brain networks, and there is some evidence of interconnections between the two nuclei (Sutherland, 1982; Kim and Chang, 2005). The LHb, which has pallidal and hypothalamic afferences and mainly projects to midbrain and hindbrain structures such as the raphe nuclei and VTA (Lecourtier and Kelly, 2007; Jhou et al., 2009; Kaufling et al., 2009; Kim, 2009), was shown involved in aversion and behavioral avoidance (Lammel et al., 2012; Stamatakis and Stuber, 2012; Ilango et al., 2013). The MHb receives septal inputs and projects primarily to the IPN, and has been less studied (Viswanath et al., 2013). This nucleus was proposed to regulate inhibitory controls, cognition-dependent

*Corresponding author. Present address: Douglas Research Centre, Department of Psychiatry, Faculty of Medicine, McGill University, Perry Pavilion Room E-3317.1, 6875, boulevard LaSalle, Montreal, Quebec H4H 1R3, Canada. Tel: +1-514-761-6131x3175; fax: +1-514-762-3033.

E-mail address: brigitte.kieffer@douglas.mcgill.ca (B. L. Kieffer).

[†] Co-first authors.

[‡] Present address: Department of Neurosciences, UCSD, 9500 Gilman Drive, La Jolla, CA 92093, USA.

[§] Present address: Institut des Neurosciences Cellulaires et Intégratives UPR3212, 5 rue Blaise Pascal, F-67084 Strasbourg cedex 03, France.

*Abbreviations:* BAC, bed nucleus of the anterior commissure; ChAT, choline acetyltransferase; DAPI, 4',6-diamidino-2-phenylindole; DB, diagonal band; DDC, dorsal diencephalic conduction; DTR, dorsal tegmental region; fr, fasciculus retroflexus; IPC, interpeduncular nucleus central subnucleus; IPI, interpeduncular nucleus intermediate subnucleus; IPL, interpeduncular nucleus lateral subnucleus; IPN, interpeduncular nucleus; IPR, interpeduncular nucleus rostral subnucleus; LDTg, laterodorsal tegmental nucleus; LHb, lateral habenula; Met-enk, Met-Enkephalin; MHb, medial habenula; MnR, median raphe nucleus; MORs, mu opioid receptors; PEnK, preproenkephalin; PPTg, pedunculopontine tegmental nucleus; SFi, septo-fimbrial septum; SP, substance P; TS, triangular septum; VGLUT2, type 2 vesicular glutamatergic transporter; VTA, ventral tegmental area.

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executive functions, place aversion learning (Darcq et al., 2011; Kobayashi et al., 2013) and the nicotine withdrawal syndrome (Dani and De Biasi, 2013, Kobayashi et al., 2013; Dao et al., 2014). A recent report showed that post-natal ablation of MHb cells in transgenic mice induces an abnormal phenotype, characterized by impulsive and compulsive behavior, environmental maladaptation and learning deficits (Kobayashi et al., 2013), underscoring functional implication of this structure in a broad range of behaviors.

MHb expresses remarkably high levels of mu opioid receptors (MORs). This G-protein-coupled receptor belongs to the opioid system, which plays a major role in pain control and autonomic functions, and modulates affective behavior and neuroendocrine physiology (Kieffer and Evans, 2009; Lutz and Kieffer, 2013). The MOR has unambiguously been established as the molecular target for opiate drugs, and mediates all the biological activities of morphine including both therapeutic and adverse effects (Matthes et al., 1996; Charbogne et al., 2014). Further, this receptor mediates reinforcing properties of non-opioid drugs of abuse including alcohol, cannabinoids, and nicotine (Kieffer and Gaveriaux-Ruff, 2002), as well as motivation for food (Papaleo et al., 2007) and social interactions (Moles et al., 2004), indicating a major role in reward processing. The broad MOR distribution throughout the brain supports all these roles (Erbs et al., 2014). Studies of this receptor have traditionally focused on the well described nociceptive and reward circuitries, but there is also some evidence for MOR-mediated morphine analgesia at the level of MHb (Terenzi and Prado, 1990; Terenzi et al., 1990; Darcq et al., 2012). Neuroanatomical distribution and function of MOR in the MHb, however, have been little explored despite strongest expression levels compared to all other brain areas (Kitchen et al., 1997) (reviews in Le Merrer et al., 2009; Lutz and Kieffer, 2013).

MOR distribution throughout the nervous system has been reported at RNA and protein levels (for a review Le Merrer et al., 2009). Low MOR mRNA abundance has rendered *in situ* hybridization experiments on mouse brain truly challenging, and data have been mainly reported from rat (George et al., 1994; Mansour et al., 1994). Also, protein detection at neuron level has been difficult due to poor availability of specific antibodies in tissue sections. MOR protein levels have otherwise been mapped and quantified using ligand autoradiography (Kitchen et al., 1997; Slowe et al., 1999; Goody et al., 2002), however this approach does not allow cellular resolution and the precise MOR distribution within MHb and associated circuitry is unknown. Our laboratory has recently generated knock-in mice expressing MOR in fusion with the red fluorescent protein mcherry (MOR-mcherry) in place of the native receptor. This mouse has enabled characterizing MOR distribution throughout the entire brain, as well as MOR colocalization with the delta opioid receptor with cellular resolution (Erbs et al., 2014). In the present study, we used this unique tool to investigate MOR distribution in neurons of the two Hb nuclei, and characterized MOR-expressing neuronal populations in the MHb–IPN pathway. Our data demonstrate prominent MOR expression in different subregions of MHb and IPN, and provides a basis for MOR-mediated mechanisms operating at the level of both substance P (SP)-ergic and cholinergic systems, in interaction with glutamatergic transmission in the MHb.

## EXPERIMENTAL PROCEDURES

### Animals

MOR-mcherry knock-in mice were generated by homologous recombination, as done earlier by our laboratory to generate DOR-eGFP knock-in mice, expressing delta opioid receptor coupled to a green fluorescent protein (Scherrer et al., 2006). In these mice, the mcherry cDNA was introduced into exon 4 of the MOR gene, in frame and 5′ of the stop codon, as described in Erbs et al., 2014. This C-terminal construct was designed to allow correct native-like MOR expression at sub-cellular level (see (Erbs et al., 2014) and leads to visualize the best described MOR protein population. Proteins that may arise from alternative splicing lacking exon 4 would not be visualized in these mice (Pasternak and Pan, 2013). The genetic background of all mice was C57/BL6J;129svPas (50:50%). Functional properties of MOR are maintained in MOR-mcherry mice both *in vitro* and *in vivo* (Erbs et al., 2014), as previously observed for DOR-eGFP mice (Scherrer et al., 2006; Pradhan et al., 2009).

Mice were housed in a temperature- and humidity-controlled animal facility (21 ± 2 °C, 45 ± 5% humidity) on a 12-h dark-light cycle with food and water *ad libitum*. Male ($n$ = 2) and female ($n$ = 3) mice aged 8–12 weeks were used. All experiments were performed in accordance with the European Communities Council Directive of 26 May 2010 and approved by the local ethics committee (Com'Eth 2010-003).

### Tissue preparation and immunohistochemistry

Mice were anaesthetized with ketamine (Virbac, Carros, France)/xylazine (Rompun, Kiel, Germany) (100/10 mg/kg, i.p.) and perfused intracardially with 100 ml of 4% paraformaldehyde (at 2–4 °C) in phosphate buffer (PB) 0.1 M (Sigma, St. Louis, MO, USA) pH 7.4. Brains were post-fixed for 24 h at 4 °C in the 4% PFA solution, cryoprotected at 4 °C in a 30% sucrose (Sigma, St. Louis, MO, USA), PB 0.1 M pH 7.4 solution and finally embedded in OCT (Optimal Cutting Temperature medium, Thermo Scientific, Waltham, MA, USA) frozen and kept at −80 °C. Frozen brains were sliced coronally into 30-μm-thick serial sections by using a cryostat (CM3050, Leica, Wetzlar, Germany) and kept floating in PB 0.1 M pH 7.4.

Immunohistochemistry was performed according to standard protocols. Briefly, 30-μm-thick sections were incubated in blocking solution (PB 0.1 M pH 7.4, 0.5% Triton X100 (Sigma, St Louis, MO, USA), 5% normal goat or donkey serum (Invitrogen, Paisley, UK) (depending on the secondary antibody) for 1 h at room temperature (RT). Sections were incubated overnight at 4 °C in the blocking solution with appropriate primary antibodies (see the section below for characterization of the primary antibodies used). After three washes in PB

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