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Cell-type specific molecular architecture for mu opioid receptor function in pain and addiction circuits

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Abstract

Opioids are potent analgesics broadly used for pain management; however, they can produce dangerous side effects including addiction and respiratory depression. These harmful effects have led to an epidemic of opioid abuse and overdose deaths, creating an urgent need for the development of both safer pain medications and treatments for opioid use disorders. Both the analgesic and addictive properties of opioids are mediated by the mu opioid receptor (MOR), making resolution of the cell types and neural circuits responsible for each of the effects of opioids a critical research goal. Single-cell RNA sequencing (scRNA-seq) technology is enabling the identification of MOR-expressing cell types throughout the nervous system, creating new opportunities for mapping distinct opioid effects onto newly discovered cell types. Here, we describe molecularly defined MOR-expressing neuronal cell types throughout the peripheral and central nervous systems and their potential contributions to opioid analgesia and addiction.

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Data availability
No data was used for the research described in the article.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2023.109597.

Declaration of competing interest
The authors have no competiting interests to disclose.

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Keywords

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1. Introduction

The mu opioid receptor (MOR) mediates both analgesic and harmful effects of opioids including addiction and respiratory depression (Bagley and Ingram, 2020; Charbogne et al., 2014; Contet et al., 2004; Darcq and Lina Kieffer, 2018; Fields and Margolis, 2015; Galaj and Xi, 2021; Kim et al., 2016, 2018; Massaly et al., 2021; Matthes et al., 1996; Paul et al., 2021). A variety of pharmacological, histological, and molecular approaches have been used to resolve the expression of MORs at the regional and cellular level and to identify the cell types mediating distinct properties of opioids. Initially, CNS sites with functional MOR expression were mapped by monitoring behavioral responses following intracranial injections of opioid receptor agonists and antagonists in animal models (Foster et al., 1967; Herz et al., 1970; Jacquet et al., 1977; Jacquet and Lajtha, 1973, 1974; Lotti et al., 1965, 1966; Mayer et al., 1971). Experiments using radiolabeled ligands revealed the distribution of MOR ligand binding sites in the brain, spinal cord and peripheral tissues (Fig. 1A and B) (Atweh and Kuhar, 1977; Kuhar et al., 1973; Pert and Snyder, 1973; Simon et al., 1977). Together these studies identified regions with MOR expression in the PNS and CNS in which MOR agonists can alter nociception and reward (Cohen and Melzack, 1985; Dickenson et al., 1979; Foster et al., 1967; Herz et al., 1970; Jacquet and Lajtha, 1973, 1974; Kuhar et al., 1973; Lotti et al., 1965, 1966; Mayer et al., 1971; Pert et al., 1976; Pert and Snyder, 1973; Phillips and LePiane, 1980; Simon et al., 1977; Yaksh et al., 1976; Yaksh and Rudy, 1976; Yeung et al., 1977).

In 1993, cloning of the *Oprm1* gene that encodes MOR provided novel means to investigate opioid receptor distribution (Thompson et al., 1993; Wang et al., 1993, 1994). With nucleotide and amino acid sequences known, RNA probes, antibodies, and transgenic mice expressing fusion proteins were developed to refine *Oprm1*/MOR localization in the nervous system (Delfs et al., 1994; Erbs et al., 2015; Fukuda et al., 1994; Hiller et al., 1994; Mansour et al., 1994, 1995b, 1994; Minami et al., 1994, 1995; Moriwaki et al., 1996; Peckys and Landwehrmeyer, 1999; Sternini et al., 1996; Taki et al., 2000) (Fig. 1A, B). *Oprm1* RNA and MOR protein expression patterns characterized in these studies largely overlapped with those found in ligand binding assays and provided enhanced spatial resolution (i.e., at the cellular and subcellular levels) of the opioid system organization in pain and addiction neural circuits.

Recent studies using single-cell RNA-sequencing (scRNA-seq) reveal which cell types express *Oprm1* and provide molecular marker genes for interrogating opioid-sensitive circuits with unprecedented specificity (Figs. 1B and 2B). In this review, we utilize single-cell data from dorsal root ganglia (DRG) (Renthal et al., 2020; Sharma et al., 2020), spinal cord (Häring et al., 2018; Russ et al., 2021; Sathyamurthy et al., 2018), and brain (Saunders et al., 2018; Zeisel et al., 2018) tissues to provide a catalog of *Oprm1*-expressing cell types

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and molecular markers identified in these cells (Fig. 1A and B; Sup. Table 1). We focus on a limited number of brain regions of particular importance for pain and addiction and for which scRNA-seq data exist. When the information is available, we also describe the function of molecularly defined *Oprm1*-expressing cell types and their contribution to opioid effects, with a focus on studies that employ modern viral and mouse genetic tools such as conditional knockout (cKO) mice. While experiments done in global knockout (KO) mice demonstrated that MOR mediates both the antinociceptive and rewarding properties of MOR agonists (Loh et al., 1998; Matthes et al., 1996; Sora et al., 1997, 2001), we describe here the results of cKO studies that employ knockin mice bearing a conditional allele (i.e., floxed mice) (Ehrich et al., 2015; Gaveriaux-Ruff et al., 2011; Weibel et al., 2013). The interpretation of the results obtained in these studies in light of the catalog of *Oprm1*-expressing cell types we provide reveals the molecular architecture principles by which opioid receptors expressed in specific cell types and circuits produce distinct opioid effects.

2. Brain cell types for opioid addiction

2.1. Ventral tegmental area

A prevailing hypothesis of opioid reward implicates MOR-mediated inhibition of ventral tegmental area (VTA) GABAergic interneurons and disinhibition of dopaminergic neurons. In rat and mouse VTA, *Oprm1* is detected by *in situ* hybridization and scRNA-seq in GABAergic (*Slc32a1*) but not dopaminergic (*Th*, *Slc6a3*) neurons (Galaj et al., 2020; Phillips et al., 2022). Additionally, VTA scRNA-seq data revealed the heterogeneity of MOR expression within GABAergic interneurons. All three inhibitory neuron subtypes express *Oprm1*, with one subtype, distinguished by the expression of *Chrna2*, *Lamp5*, and *Nox4*, expressing *Oprm1* at substantially higher levels (Phillips et al., 2022).

MOR-mediated disinhibition of dopaminergic neurons may involve other MOR+ inhibitory inputs, from the rostromedial tegmental nucleus (RMTg, the tail of the VTA) (Bourdy and Barrot, 2012; Jhou et al., 2009; Lecca et al., 2011, 2012; Matsui and Williams, 2011; Reeves et al., 2022), substantia nigra (SN) (Galaj et al., 2020; Galaj and Xi, 2021), nucleus accumbens (NAc), ventral pallidum (VP), or periaqueductal gray (PAG) (Reeves et al., 2022). Due to their close spatial proximity, scRNA-seq datasets do not distinguish between *Oprm1*+ VTA and RMTg cells, but convergent evidence suggests that RMTg neurons make important contributions to circuits mediating opioid reward. For example, the RMTg strongly expresses *Oprm1* mRNA and MOR protein (Galaj et al., 2020; Galaj and Xi, 2021; Jalabert et al., 2011), morphine induces greater inhibition of optically-evoked inhibitory postsynaptic currents (IPSCs) in the VTA from RMTg inputs compared to those from the NAc or from local GABAergic neurons (Galaj et al., 2020; Matsui et al., 2014), and inhibition or excitation of RMTg neurons bidirectionally modulates VTA dopaminergic neuronal activity (Galaj and Xi, 2021; Jalabert et al., 2011). However, scRNA-seq datasets with higher spatial resolution are needed to complement pharmacological and electrophysiological studies of RMTg neurons.

Although MOR/*Oprm1* expression across molecularly defined dopaminergic neuron types remains to be unequivocally established via RNA sequencing or histology experiments,

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some electrophysiological studies provide evidence for the direct action of MOR agonists onto dopaminergic neurons in VTA (Margolis et al., 2014, 2017). Additionally, dopaminergic neurons in nearby regions such as the SN express MOR and may also contribute to opioid reward (Baumeister et al., 1993; Galaj et al., 2020; Galaj and Xi, 2021; Hommer and Pert, 1983; Melis et al., 2000; Nazzaro et al., 1981; Walker et al., 1987). Finally, VTA dopamine release into the NAc likely represents only one facet of the neuronal signaling underlying opioid reward, and the extent to which dopamine signaling underlies opioid reward may be context-dependent (Galaj and Xi, 2021). For example, VTA glutamatergic signaling may also contribute to reward-seeking behaviors (Harris et al., 2004; Jalabert et al., 2011; Zell et al., 2020). Blockade of VTA NMDA or AMPA receptors interferes with morphine CPP, and intra-VTA injection of AMPA or NMDA receptor antagonists prevents morphine-induced dopamine neuron excitation *in vivo* (Jalabert et al., 2011), suggesting the importance of glutamate signaling to opioid reward. More recent evidence found that mice continue to seek optogenetic self-stimulation of either VTA VGLUT2+ cell bodies or VTA VGLUT2+ terminals in NAc when dopamine co-release is blocked using floxed *Th* mice or viral CRISPR/Cas9-based *Th* deletion (Zell et al., 2020). Similarly, dopamine co-release blockade does not impede real-time place preference for the optogenetically-paired chamber when VTA VGLUT2 + cell bodies or VTA VGLUT2 + terminals in NAc are excited (Zell et al., 2020). However, whether or not *Oprm1* is expressed in populations of VTA excitatory neurons is still an open question and the contribution of glutamatergic neurotransmission in VTA circuits to opioid reward warrants further investigation.

2.2. Striatum

The striatum contains patch and matrix compartments, both composed of two main medium spiny neuron (MSN) types and several interneuron types. MSNs are GABAergic and include direct MSNs (dMSNs), expressing the dopamine 1 receptor (D1R, encoded by *Drd1*), and indirect MSNs (iMSNs), expressing the dopamine 2 receptor (D2R, encoded by *Drd2*) (Allichon et al., 2021; Thompson et al., 2021; Yager et al., 2015). Primary interneuron types include *Pthlh (*includes *Pvalb cells), Sst/Npy, Npy/Mia, Th,* and *Chat* + populations, although recent studies suggest further complexity in these distinctions (Castro and Bruchas, 2019; Muñoz-Manchado et al., 2018). Both the dorsal and ventral striatum contain these compartments and cell types, with some variations in morphology, electrophysiological properties, and gene expression (Castro and Bruchas, 2019). Dopaminergic VTA inputs to the NAc are believed to underlie the motivational effects of opioids, and inhibition of this projection blocks heroin SA (Corre et al., 2018). Additionally, NAc dMSNs and iMSNs are differentially involved in cue-induced reinstatement of heroin SA and heroin CPP (O'Neal et al., 2022, 2020). Chemogenetic inhibition of dMSNs or activation of iMSNs can suppress reinstatement of heroin-seeking in rats classified as addiction-vulnerable after heroin SA experiments (O'Neal et al., 2020). Calcium indicator and dopamine sensor experiments using fiber photometry during heroin CPP additionally revealed increased calcium signaling and dopamine activity in dMSNs upon entry of the heroin-paired chamber and decreased calcium signaling and dopamine activity upon exit; the reverse was found in iMSNs (O'Neal et al., 2022). Together, these observations show an inverse and complex relationship for

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the role of dMSNs and iMSNs in neural activity and in heroin-seeking behavior itself, demonstrating the importance of NAc MSNs to opioid reward.

Although autoradiography and immunohistochemistry studies demonstrate MOR expression throughout the striatum (Arvidsson et al., 1995; Kaneko et al., 1995; Mansour et al., 1995a; Okunomiya et al., 2020; Pert et al., 1976) region-specific sequencing studies are necessary to compare relative *Oprm1* expression in striatal cell types. Transcriptomic studies reporting *Oprm1* expression have separately examined the dorsal and ventral striatum. In one study, single nucleus RNA-seq data was collected using FACS-sorted dorsal striatum tissue from *Oprm1*^{Cre} mice crossed with the nuclear-localized reporter mice H2B-GFP. RNAscope *in situ* hybridization experiments found 87% of *Oprm1* expression in dMSNs, which often coexpress *Pdyn* and *Tac1,* and only 9% in iMSNs, for which *Adora2a* is a marker gene (Gokce et al., 2016; Märtin et al., 2019). Another recent study found that *Sema5b* expression distinguishes *Oprm1* + patch neurons, while *Id4* marks *Oprm1*-matrix cells as well as *Oprm1* + exopatch cells, which are cells found in the matrix with physiological properties resembling those of MSNs found in the patch compartment (Märtin et al., 2019; Smith et al., 2016).

In the ventral striatum, we observe *Oprm1* expression in published scRNA-seq data at higher levels in an atypical MSN cell type, defined by *Drd1* and *Drd2* expression (Savell et al., 2020) This atypical class of MSNs is also distinguishable by the expression of *Grm8*, *Foxp2*, and *Bcl11b*. *Oprm1* expression was also detected in iMSNs, undefined GABAergic cells, and glutamatergic cells in the NAc (Savell et al., 2020). A study combining spatial transcriptomics and scRNA-seq in mice reported *Oprm1* enrichment in the ventromedial NAc and an increasing gradient of *Oprm1* expression along the anterior-posterior axis (Chen et al., 2021). Two multiplexed error-robust fluorescent *in situ* hybridization (MERFISH) clusters were enriched for *Oprm1*: D1_NA2, which was preferentially labeled by MERFISH probes against *Dlk1* and *Trhr*, and D2_NA2, which was distinguishable by probes against *Npy2r* and *Calr* (Chen et al., 2021). Three populations identified by sequencing, which express *Cidea, Arhgap36,* and *Ecm1* (D1_6), *Cidea*, *Calcr*, and *Gabrg1* (D2_1), and *Pls3* (D2_6), correspond to the MERFISH clusters. The two D2 cell populations identified by scRNA-seq express *Ngb*, whereas the cell clusters identified by MERFISH express *Pde1a, Peg10, Dlk1, Htr2c,* and *Arhgap36* and are located in the ventromedial core of the anterior segments of the NAc. An 'atypical' cell type (D1_AT1) in the posterior medial shell also expresses *Oprm1* at a high level and is enriched in *Cacna1g* and *Trh* (MERFISH) and *Nfix*, *Sphkap*, *Slc39a6*, and BC048546 (scRNA-seq). It should be noted that *Oprm1* expression is high in the NAc in general; this analysis indicates the cell types that are most enriched with *Oprm1* expression. Other cell types present in this dataset are also enriched for *Oprm1*; however, they are not considered to be part of the NAc based on MERFISH.

Before *Oprm1*$^{lox/lox}$ and *Oprm1*^{Cre} were generated, a study interrogated the function of MOR in a subpopulation of striatal neurons by crossing *Oprm1*$^{-/-}$ mice with transgenic mice in which a bacterial artificial chromosome vector containing the *Pdyn* promoter followed by MOR cDNA was inserted in the genome (Cui et al., 2014). This genetic strategy, which reinstated MOR expression in patch dMSNs in the dStr and NAc, restored morphine-induced CPP and increases in extracellular dopamine, and partially restored

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remifentanil SA, suggesting the importance of MOR expression in these dMSNs to opioid reward and opioid-seeking. Opioid-induced hyperlocomotion was also re-established. However, neither somatic signs of withdrawal nor antinociception were modified, suggesting that MOR expression in dMSNs is not sufficient to mediate these effects.

Several studies then took advantage of *Oprm1*$^{lox/lox}$ mice to reappraise and expand our understanding of MOR function in striatal neurons. A first study investigated MOR function in MSNs and other GABAergic forebrain neurons by crossing *Oprm1*$^{lox/lox}$ mice with *Dlx5/6*^{Cre} mice (*Dlx5/6* cKO) (Charbogne et al., 2017). *Dlx5* and *Dlx6* genes are involved in GABAergic cell fate and are expressed in all major classes of developing and adult GABAergic interneurons in the cortex, striatum, and hypothalamus (de Lombares et al., 2019). *Dlx5/6* cKO mice show reduced MOR binding by [^{3}H]DAMGO autoradiography primarily in the dorsolateral striatum, NAc, and VP (Charbogne et al., 2017), suggesting reduced MOR expression in these regions. Behavioral tests in *Dlx5/6* cKO mice revealed increased heroin SA and unchanged heroin CPP (Charbogne et al., 2017). Palatable food seeking was similarly increased. These results suggest that MORs in *Dlx5/6* + cells, and potentially in MSNs specifically, are implicated in motivation to seek opioids, and in addition to other rewarding stimuli and experiences. In the *Dlx5/6* cKO mice, morphine antinociception was unchanged, as was naloxone-precipitated withdrawal (Charbogne et al., 2017). However, note that a study in mice with partial sciatic nerve injury, a model of chronic neuropathic pain, suggests that MOR expression in *Dlx5/6* + cells may contribute to heat hyperalgesia in a sex-specific manner (Martínez-Navarro et al., 2020).

These studies were extended by the parallel phenotyping of multiple cKO mouse lines with *Oprm1* deletion in distinct populations of striatal neurons expressing D1R, D2R, A2a receptors, or choline acetyl-transferase (ChAT), generated by crossing of *Oprm1*$^{lox/lox}$ mice and *Drd1*^{Cre}, *Drd2*^{Cre}, *Adora2a*^{Cre}, or *Chat*^{Cre} mice, respectively (Severino et al., 2020). Notably, *Adora2a* is highly co-expressed with *Drd2* in the striatum, so differences in the observed phenotype of *Oprm1* cKO may be attributed to gene deletion in other regions (Gokce et al., 2016). Additionally, *Oprm1* is thought to be primarily expressed in the *Drd1* population (Banghart et al., 2015; Gokce et al., 2016; Märtin et al., 2019), therefore, conditional deletion from this population likely resulted in the broadest removal of the gene from striatal circuits. Striatum-specific scRNA-seq studies have not reported co-expression of *Oprm1* with *Chat*, possibly because the *Chat* + interneurons represent a small proportion of all neurons (Muñoz-Manchado et al., 2018). However, MOR agonists have been shown to cause inhibition of spontaneous activity in striatal cholinergic interneurons, which is blocked by the antagonist CTOP, suggesting co-expression of *Oprm1* and *Chat* (Ponterio et al., 2013). Finally, *Oprm1* is coexpressed with *Drd1, Drd2, Adora2a,* and *Chat* outside of the striatum, and this study did not address the extent to which these extrastriatal MORs may mediate behavioral phenotypes. For example, *Oprm1* is also enriched in *Drd1* + cells in layer 6 and 6b cells in cortex (*Sulf1, Prss12,* and *Cplx3, Nxph3* + respectively) (Zeisel et al., 2018) and in globus pallidus cells expressing *Ppp1r1b* and *Pde1c* (Saunders et al., 2018). *Oprm1*+/*Drd2*+ cells were also detected in the superior colliculus (*Fibcd1, Mgarp*) and both *Oprm1*+/*Drd2*+ and *Oprm1*+/*Adora2a*+ cells were detected in globus pallidus (*Ppp1r1b, Pde1c*) (Saunders et al., 2018). Finally, *Oprm1*+/*Chat*+ cells were found in cranial

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nerve VI-XII, medial pontine nuclei (*Gpx2*) (Zeisel et al., 2018) globus pallidus (*Nefm*), and habenula (*Tac2, Wif1*) (Saunders et al., 2018).

Oprm1 deletions from *Drd1, Drd2, Adora2a,* and *Chat* cells did not reveal differences in remifentanil SA acquisition or maintenance compared to control animals (Severino et al., 2020). However, *Adora2a* cKO and *Chat* cKO models each demonstrated increased lever pressing during remifentanil SA extinction. These four cKO models did show varying effects on opioid-induced locomotor sensitization, suggesting that these four populations may have a greater contribution to opioid-induced locomotor behavior and a smaller contribution to the extinction of opioid reward-seeking behavior.

2.3. Dorsal raphe nucleus

The dorsal raphe nucleus (DRN) is one area modulating neural activity in the mesolimbic circuitry through bidirectional connections with the VTA (Li et al., 2019; Paquelet et al., 2022). The DRN contains primarily serotonergic neurons, defined by early *Pet1* expression, but also GABAergic, glutamatergic, peptidergic, and dopaminergic neurons (Huang et al., 2019; Okaty et al., 2020). The most transcriptionally distinct population in the DRN (*Met, P2ry1*, *Trpc3*+) is located in the posterior tail of the DRN (Huang et al., 2019) and is enriched in *Oprm1* (Okaty et al., 2020). Viral tracing experiments using *P2ry1*^{Cre}*; Pet1*^{Flp} mice revealed neuronal fibers primarily in the third, lateral, and fourth ventricles, with sparser fibers in regions such as the lateral hypothalamus, hippocampus, medial and lateral septum, olfactory bulb, amygdala, and lateral parabrachial nucleus (Okaty et al., 2020).

Few studies address the contributions of *Oprm1*+ DRN neurons themselves to opioid reward and dependence, however chronic chemogenetic elevation of the *Oprm1*+ rostral VTA→DRN projection activity disrupts formation of morphine CPP (Li et al., 2019), and Crispr/Cas9 genetic ablation or optogenetic inhibition of *Th*+ DRN neurons disrupts expression of morphine CPP (Lin et al., 2020), suggesting that VTA inputs and DRN neurons themselves are important to opioid reward-context association. Interestingly, genetic ablation or optogenetic inhibition of *Th*+ DRN neurons can also reduce formation and expression of opioid withdrawal-induced conditioned place aversion (CPA), suggesting a broader role for dopaminergic DRN neurons in expression of opioid-related memories (Lin et al., 2020).

A recent study examining the contribution of DRN MORs to reward found that MORs expressed on DRN neurons mediate sucrose reward consumption through a *Penk*+ lateral DRN→medial NAc shell (mNAcSh) circuit (Castro et al., 2021). Unlike wild-type control animals, global *Oprm1* KO mice demonstrated no increase in sucrose consumption after food deprivation (FD). *Penk* cKO mice, but not *Pdyn* cKO mice, similarly decrease FD sucrose consumption. However, conditional deletion of *Oprm1* in the mNAcSh did not reduce FD intake, suggesting that MORs expressed presynaptically to the mNAcSh may mediate this effect. Selective viral rescue of MOR on *Penk*+ lateral DRN → mNAcSh projections using *Oprm1* KO; *Penk*^{Cre} mice restored FD sucrose intake and partially restored morphine CPP without restoring morphine analgesia, demonstrating that MORs expressed on this projection are sufficient for components of exogenous and endogenous opioid reward but separable from those mediating opioid analgesia (Castro et al., 2021).

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2.4. Habenula

The habenula mediates aversive states, highly expresses MOR, and is strongly connected to regions implicated in opioid reward (Bailly et al., 2022; Gardon et al., 2014). It contains primarily glutamatergic neurons, but ventrally located *Chat*+ MHb neurons corelease acetylcholine and more dorsal *Tac1*+ MHb neurons corelease substance P and glycine (Ables et al., 2023). MOR is highly expressed in the medial habenula (MHb), the interpeduncular nucleus (IPN), the projections from the MHb to the IPN (fasciculus retroflexus), and to a lesser extent, in the lateral habenula (LHb) (Gardon et al., 2014; McLaughlin et al., 2017; Xu et al., 2018). LHb neurons are also bidirectionally connected to the VTA (Stamatakis et al., 2013), where they synapse onto GABAergic neurons (Lammel et al., 2012) and onto prefrontal cortex (PFC)-projecting dopaminergic neurons (Galaj and Xi, 2021).

Although the current resolution of whole-brain scRNA-seq studies makes identification of *Oprm1*-expressing neurons in the habenula challenging, a few studies have examined MOR function in distinct habenular neuron-types. For example, the contribution of MOR to opioid withdrawal was examined using cKO of *Oprm1* in *Chrnb4*+ neurons, a population of neurons expressing nicotinic receptors that are highly enriched in the MHb (*Chrnb4* cKO mice) (Boulos et al., 2020). Our analysis of published scRNA-seq studies confirm *Oprm1+ Chrnb4*+ neurons are enriched in the habenula (*Tac2, Wif1, Syt15*+ habenular neuronal populations), but also reveals *Oprm1+ Chrnb4*+ neurons in other structures such as the nucleus of the solitary tract (*Npff, Tfap2b*+ neurons) (Saunders et al., 2018). *Chrnb4* cKO mice showed decreased naloxone-precipitated withdrawal and reduced naloxone-conditioned place aversion, but normal morphine-induced CPP and response to palatable food reward (Boulos et al., 2020). Acute morphine antinociception in the tail immersion test was minimally reduced in *Chrnb4* cKO mice, while chronic morphine studies revealed intact antinociceptive tolerance (Boulos et al., 2020). Other habenular neurons and circuits may contribute more significantly to opioid analgesia (Waung et al., 2022). Thus, microinjection of the MOR agonist DAMGO into LHb decreased mechanical sensitivity in mice with partial sciatic nerve injury and Complete Freund's Adjuvant (CFA) models of neuropathic and inflammatory pain, respectively, without changing heat sensitivity (Waung et al., 2022). Specifically, excitatory LHb inputs from the lateral preoptic area (LPO) may be one opioid-sensitive projection underlying pain aversion that does not contribute to reward in pain-free animals. This projection expresses *Oprm1* and its optogenetic inhibition in animals with chronic pain specifically causes CPP. Additionally, while optogenetic activation of this projection is sufficient to cause CPA, LHb DAMGO microinjection produces CPP in optogenetically-stimulated animals only (Waung et al., 2022).

As expected, given that habenular neurons express VGLUT2, *Slc17a6* cKO mice similarly show decreased opioid withdrawal signs (Reeves et al., 2021; Zhang et al., 2020c). Two studies found *Slc17a6* cKO mice demonstrate altered withdrawal symptoms; while Zhang et al. reported naloxone-precipitated withdrawal signs were absent, Reeves et al. reported that withdrawal symptoms were observed at baseline and not increased by naloxone administration. Opioid-induced locomotor stimulation and opioid-induced CPP were unchanged in *Chrnb4* cKO mice; this suggests that the VGLUT2 populations

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mediating these effects are not included in the *Chrnb4* population. In *Slc17a6* cKO mice, CPP to oxycodone is abolished and CPA to oxycodone forms at higher doses. (Reeves et al., 2021). Furthermore, SA assays revealed decreased oxycodone consumption and decreased preference in *Slc17a6* cKO mice (Reeves et al., 2021). Morphine and oxycodone-induced hyperlocomotion were also abolished in *Slc17a6* cKO mice (Reeves et al., 2021; Zhang et al., 2020c). Interestingly, ethanol CPP and sucrose preference tests did not reveal differences between *Slc17a6* cKO mice and controls, suggesting that motivation to seek non-opioid rewards was intact (Reeves et al., 2021). Importantly, numerous neuronal populations throughout the nervous system, and specifically along pain and addiction circuits, express VGLUT2. Cells co-expressing MOR and VGLUT2 are expressed in the thalamus, habenula, PAG (Li et al., 2016), and parabrachial nucleus (Pauli et al., 2022), among other regions; glutamatergic neurons in the spinal cord and primary afferent neurons also express VGLUT2 (Russ et al., 2021; Zeisel et al., 2018; Zhang et al., 2020c), making the identification of the neurons responsible for opioid effects based on the *Slc17a6* cKO mouse phenotype challenging.

2.5. Prefrontal cortex

Although whole-brain scRNA-seq datasets lack the resolution to identify all cortical cell types enriched in *Oprm1*, the higher sequencing depth of cortex-specific studies allow detection of *Oprm1*+ cell types in the mouse cerebral cortex, including prefrontal cortex (PFC). One study sampled over 1.3 million cells across all 26 regions of the isocortex and 2 main regions of the hippocampus, identifying 388 distinct neuronal clusters using both 10x and SMART-seq technology (Yao et al., 2021). Here we consider data generated using SMART-seq technology, which provides considerably higher sensitivity for sparse and lowly expressed transcripts, such as those of opioid receptor genes. This study revealed that inhibitory neurons (defined by *Gad1*, *Gad2*, and *Slc32a1* expression) are generally transcriptionally similar across cortical regions, whereas for excitatory neurons (defined by *Slc17a7* expression), cell clusters differ across cortical regions. Within inhibitory neuron populations, at least half of the inhibitory clusters in the *Lamp5*, *Vip*, and *Sst* categories express *Oprm1* (trimmed mean normalized expression >1; Fig. 1B, Sup. Table 1). Within excitatory neuron populations, we observe that over half of the cell clusters designated as L6 corticothalamic, L5/6 near-projecting, or L5 pyramidal tract, and all L6b neuron clusters, also meet these criteria (Fig. 1B, Sup. Table 1), suggesting *Oprm1*$^{+}$ neurons are enriched within deeper laminae throughout the cortex. These findings suggest that *Oprm1* expression is differentially enriched across cell types, cortical layers, and cortical regions. Among cortical regions, many rodent and human studies of opioid analgesia and/or addiction mechanisms have focused on the PFC. Despite this, knowledge of which of the neuron-types express *Oprm1* specifically in PFC is lacking.

Recent efforts to investigate how exogenous opioids act on PFC circuits found that morphine decreases inhibitory synaptic transmission onto pyramidal neurons via MOR signaling on parvalbumin (PV) interneurons in the prelimbic cortex (PL). (Jiang et al., 2021). *Oprm1* knockdown in PL PV neurons using small-hairpin RNA did not affect morphine CPP but decreased opioid-induced hyperlocomotion and behavioral sensitization to morphine (Jiang et al., 2021). We do not observe *Oprm1* in *Pvalb*+ cells to meet our enrichment

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criteria in cortical scRNAseq datasets, however several inhibitory PV + neuronal subtypes still express high levels of *Oprm1.* Markers for these clusters include *Pde3a* (123) and *Etv1* (85, 100, 101, 114) (Yao et al., 2021). However, it should be noted that some of these neuronal populations (85, 100, 101) also express *Sst,* and further validation will be needed to demonstrate that these clusters are present in PL. Finally, the lack of definitive characterization of *Oprm1*+ cell types in PL leaves open the possibility that other *Oprm1*-expressing neurons could make important contributions to opioid context-reward association.

Similarly to infralimbic cortex (IL) and PL, no specific sequencing resources have outlined the populations expressing *Oprm1* in the anterior cingulate cortex (ACC), but recent immunohistochemical evidence using *Oprm1*$^{\mathrm{mCherry}}$ mice suggests that MOR is expressed in a variety of excitatory and inhibitory populations across cortical layers, with the highest level of expression found in excitatory neurons in layer 6 and in inhibitory neurons expressing somatostatin (Wang et al., 2018; Zamfir et al., 2022). This is in agreement with scRNA-seq data (Yao et al., 2021) with which we observe *Oprm1* expression both in *Sst* and in *Lamp5* inhibitory neuron-types, as well as in *Cplx3* excitatory layer 6b neurons. We also note moderate *Oprm1* expression in other cell types, including inhibitory *Vip* and *Pvalb*+ neurons, as well as excitatory intratelencephalic, pyramidal tract, and near-projecting neurons across cortical layers (Yao et al., 2021). While a recent study using slice electrophysiology found that measures of ACC L5 thick-tufted pyramidal neuron excitability increased during spontaneous opioid withdrawal and that chemogenetic inhibition of ACC blocks opioid-induced withdrawal behaviors (McDevitt et al., 2021), functional studies to date have not manipulated ACC *Oprm1*+ cell types to determine their function in opioid addiction. Given the diversity of *Oprm1*+ neurons in cortex, such studies will likely require modern intersectional genetic strategies that enable the specific investigation of molecularly or projection-defined *Oprm1*+ cortical neurons.

3. Cell types for opioid analgesia in ascending and descending nociceptive circuits

3.1. Dorsal root ganglia

Evidence for MOR expression in dorsal root ganglia (DRG) was first provided by radioligand binding studies that revealed MOR presence in small-diameter fibers projecting to the spinal cord dorsal horn (Fields et al., 1980). Later, i*n situ* hybridization experiments confirmed the presence of *Oprm1* in DRG and trigeminal ganglia (TG) neurons (Mansour et al., 1994). Anti-MOR antibodies then documented MOR expression in small-diameter, TRPV1-immunoreactive and peptidergic (i.e., expressing the neuropeptides substance P and calcitonin gene-related peptide (CGRP)) C-nociceptors, as well as in large-diameter, neurofilament-positive, peptidergic DRG neurons (Arvidsson et al., 1995; Bardoni et al., 2014; Scherrer et al., 2009).

Recent scRNA-seq studies have clarified the molecular identity of *Oprm1*+ DRG cells; the first scRNA-seq study (Usoskin et al., 2015) classified DRG neurons into 11 sub-types. These cell types consist of five neurofilament (NF1–5), three non-peptidergic (NP1–3),

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two peptidergic (PEP1–2) and one TH population. *Oprm1* expression was confirmed in peptidergic neurons and, to a lesser degree, in some non-peptidergic neurons. *Oprm1* was enriched in *Calca*+ (encoding CGRP) neurons, including *Nefh*- (neurofilament heavy chain-negative, unmyelinated C nociceptors) and *Nefh*+ populations (neurofilament heavy chain-positive, myelinated nociceptors) (Fig. 1A, B). A more recent scRNA-seq study revealed that *Calca*+ DRG neurons include six transcriptionally discrete subtypes, of which *Oprm1* is found in CGRP-Alpha, CGRP-Gamma, and CGRP-Epsilon neurons (Sharma et al., 2020). *Oprm1* is also found in *Sst*+ neurons, which also express both *Calca* and *Trpv1*, but are considered pruriceptors rather than nociceptors (Renthal et al., 2020), and in *Calca*-negative neurons, including *Trpm8*+ cold sensing neurons. In addition, scRNA-seq has been used to resolve the changes in gene expression that occur in DRG following nerve injury, revealing 9 neuronal clusters and 6 non-neuronal cell clusters in DRG. Of these cell clusters, *Oprm1* was only detected in peptidergic neuronal clusters, specifically the PEP1, PEP2 and SST clusters (Renthal et al., 2020), confirming previous literature. Interestingly, *Oprm1* expression was downregulated in various models of nerve injury, consistent with early radioligand binding studies, and revealing the DRG cell types in which this reduction in *Oprm1* expression occurs. Finally, these scRNA-seq studies of mouse DRG neurons also unequivocally validate the model of segregated expression of the MOR and the delta opioid receptor (DOR, encoded by the *Oprd1* gene), which, rather than being co-expressed with MOR in substance P- and TRPV1-expressing peptidergic C nociceptors as originally postulated (Dado et al., 1993; Guan et al., 2005), is predominantly expressed by mechanosensory neurons and non-peptidergic C nociceptors (Bardoni et al., 2014; Corder et al., 2018; François and Scherrer, 2018; Scherrer et al., 2009). Recently, a study of human DRG combining electrophysiology and pharmacology found that 50% of cultured DRG neurons express functional MOR, of which 80% were responsive to capsaicin and presumably express TRPV1 (Moy et al., 2020). Although experiments in intact tissue are necessary to confirm these findings, another study also reports a significant proportion of peptidergic nociceptor fibers express MOR in human epidermal tissue (Ständer et al., 2002).

Functionally, conditional deletion of MOR in nociceptors by crossing *Oprm1*$^{lox/lox}$ mice with *Trpv1*^{Cre} mice (*Trpv1* cKO mice), in which *Trpv1* developmental expression also drives Cre-mediated recombination in non-peptidergic and large-diameter DRG nociceptors (Cavanaugh et al., 2011) revealed intact antinociception following systemic morphine, suggesting that activation of MOR in CNS nociceptive circuits is sufficient to produce pain relief (Corder et al., 2017). By contrast, morphine antinociceptive tolerance and OIH are profoundly reduced in *Trpv1* cKO mice, a phenotype that can be mimicked by pharmacological blockade of peripheral MORs with methylnaltrexone bromide (Corder et al., 2017). Mechanistically, optogenetic electrophysiology studies established that whereas in littermate control animals, DAMGO acutely decreases neurotransmission between C nociceptors and spinal cord projection neurons and then potentiates neurotransmission (i.e., producing a form of long-term potentiation [LTP] that facilitates nociception), upon washout and opioid withdrawal both effects were abolished in *Trpv1* cKO mice. These findings suggest that, paradoxically, pro-nociceptive MOR-mediated signaling in C nociceptors at the first synapse of the pain circuitry represents an essential neural substrate for the development of morphine antinociceptive tolerance and OIH (Corder et al., 2017). Note, however, that

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other cKO studies that used Cre mouse lines with broader Cre activity patterns in PNS and/or CNS (Shields et al., 2012; Tenza-Ferrer et al., 2022) such as *Avil*^{Cre} (Sun et al., 2019, 2020) and *Nav1.8*^{Cre} (Weibel et al., 2013) reached different conclusions.

3.2. Spinal cord

MOR is expressed in both the dorsal and ventral horns of the spinal cord (Aicher et al., 2000; Gouardères et al., 1991; Kar and Quirion, 1995; Kemp et al., 1996; Spike et al., 2002; Wang et al., 2018; Zhang et al., 2020b). Recently, the *Oprm1*^{mCherry} mouse line has enabled the detection and study of MOR signaling in various types of spinal neurons (Wang et al., 2018). In the dorsal horn, *Oprm1*+ neurons are predominantly found in the superficial laminae I-IIinner, and include both projection neurons of the anterolateral tract and glutamatergic and GABAergic interneurons. In sharp contrast with the functional organization of opioid receptors in DRG, MOR and DOR are often co-expressed by these projections neurons, in which the two receptor types may coordinately, or differentially, regulate nociception (Wang et al., 2018). The mechanistic consequences of this co-expression in spinal neurons *in vivo* have yet to be fully elucidated, as MOR-DOR co-internalization and co-degradation, which were originally proposed based on studies using heterologous expression systems or cultured neurons, have not been observed in tissues (Wang et al., 2018). *Oprm1*+ interneurons include *Penk*+ dorsal horn interneurons, which can be either excitatory or inhibitory, and may participate in enkephalinergic-MOR auto-signaling (François et al., 2017; Häring et al., 2018). In the ventral horn, MOR expression is observed in pre-motor neurons, including inhibitory *Slc32a1+ En1*+ neurons, and sparsely in discrete *Chat*+ motoneurons (Wang et al., 2018).

A recent publication integrating six independent scRNA-seq studies on the spinal cord confirmed high levels of *Oprm1* expression in excitatory and inhibitory neurons of lamina I/IIinner, consistent with previous histological analysis from Wang et al. Cell types expressing the highest levels of *Oprm1* consist of a *Trhr*+ dorsal excitatory cluster and three inhibitory interneuron clusters distinguished by *Nppc, Rxfp2*, and *Pdyn*/*Gal* (Russ et al., 2021). In the ventral horn, *Oprm1* was detected in an excitatory population enriched in lamina 7 and defined by the expression of *Chx10*, which marks V2a premotor neurons, and in a population of inhibitory neurons defined by *Lmx1b/Zfhx3*. *Oprm1* expression was not detected in motoneurons in this study. Although the existence of MOR in ventral horn neurons has been observed in several studies (Gouardères et al., 1991; Kao et al., 2012; Kar and Quirion, 1995; Mansour et al., 1994; Wang et al., 2018), the functional relevance of these findings remains to be fully established. Further investigations are necessary to determine whether MOR function in ventral horn neurons contributes to clinically relevant opioid motor side effects, such as muscle rigidity (Baliga and Sanford, 2006 n.d.; Haouzi and Tubbs, 2022; Kinshella et al., 2018; Klausner et al., 1988).

To investigate the function of MOR in specific subpopulations of spinal neurons, one study employed a "knockout first" model (*Oprm1*$^{KI/KI}$) to rescue *Oprm1* expression in genetically defined neuron types (Zhang et al., 2020b). A stop cassette flanked by loxP sites was inserted into the *Oprm1* locus to disrupt MOR expression. In the presence of Cre activity, the stop cassette is excised and *Oprm1* expression is restored. Crossing MOR$^{KI/KI}$ with

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Lbx1^{Cre}, which restored MOR expression in *Lbx1*+ dorsal horn neurons, rescued morphine antinociception. In agreement with this, crossing *Lbx1*^{Cre} with *Oprm1*^{fl} revealed a decreased antinociceptive effect of morphine, indicating that part of the analgesic effect of exogenous opioids is mediated by these neurons (Zhang et al., 2020b). Note, however, that *Lbx1* is broadly expressed by projection neurons and interneurons in the dorsal horn, preventing the study of subtypes of *Oprm1*+ dorsal horn neurons, as well as in the cerebellum and in brainstem nuclei, notably in the medulla (Lein et al., 2007; Pagliardini et al., 2008; Sjöstedt et al., 2020).

3.3. Ascending pain pathways in the brain

Dorsal horn projection neurons convey nociceptive information to third order nociceptive neurons in several brain regions including the parabrachial nucleus (PB), PAG, and thalamus, which then project to the amygdala, striatum, and cortical regions including the ACC, PFC, insula, primary somatosensory, and secondary somatosensory cortex, as well as to other areas for further processing of nociceptive information (Bushnell et al., 2013; Todd, 2010). Neural activity in these networks generates the sensory-discriminative, affective-motivational, and cognitive-evaluative dimensions of the pain experience (Mercer Lindsay et al., 2021). We focus here on the PB (Chiang et al., 2019; Palmiter, 2018; Pauli et al., 2022), thalamus, amygdala, and ACC, structures in which cell-type specific interrogation of MOR function has begun (Mercer Lindsay et al., 2021; Okunomiya et al., 2020).

The great majority of PB and thalamic neurons are excitatory neurons that express *Slc17a6* and utilize VGLUT2 to load synaptic vesicles with glutamate (Eacret et al., 2023; Phillips et al., 2019). In the PB nucleus, *Oprm1* is found in the lateral region (PBl). *Oprm1*+ PBl neurons receive nociceptive inputs and project to limbic areas such as the central nucleus of the amygdala, parvocellular subparafascicular nucleus, and the bed nucleus of the stria terminalis (BNST) as well as areas relaying sensory information, such as the nucleus tractus solitarius, superior colliculus, and the spinal cord (Liu et al., 2022; Pauli et al., 2022). Although single PBl *Oprm1*+ neurons show increased calcium activity in response to both breathing and painful pinch, as a population these neurons anatomically separate into a “core” and a “shell” region (Liu et al., 2022). *Oprm1*+ neurons in the core, containing the external lateral PB, primarily project to forebrain areas including the CeA, BNST, and intralaminar nucleus, while *Oprm1*+ neurons in the shell, containing the central lateral, lateral crescent, and ventral external lateral PB, project primarily to hindbrain areas including the pre-Bötzinger nucleus (Liu et al., 2022). scRNA-seq of PB neurons found *Oprm1* to be primarily expressed in *Calca* + cell clusters (Pauli et al., 2022). However, ISH data suggest *Oprm1*+ neurons are a diverse population, as *Oprm1* colocalizes with a variety of markers, including *Calca, Tac1, Nts, Ntr2a, FoxP2, Pdyn,* and *Tacr1* to varying extents ranging from 10 to 40% of the *Oprm1*+ population (Liu et al., 2022).

Chemogenetic inhibition of PBl *Oprm1* + neurons does not impact thermal or mechanical pain thresholds in the hot plate and von Frey test (Liu et al., 2022). However, chemogenetic inhibition of PBl *Oprm1* +neurons results in increased jump latency on the hot pate and decreased paw-licking behaviors in the second phase of the formalin test, suggesting reduced affective-motivational pain behaviors (Liu et al., 2022). *Oprm1*+ neurons in the

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PBl that project to neurons in the pre-Bötzinger nucleus are believed to contribute to opioid-induced respiratory depression (OIRD) (Liu et al., 2021) and coordination of breathing with pain and emotional states (Liu et al., 2022). Specifically, *Oprm1* deletion from PBl neurons weakens OIRD phenotypes, while reintroduction of human MOR into the PBl of *Oprm1* KO mice restores OIRD (Liu et al., 2021). Chemogenetic or prolonged optogenetic inhibition of PBl *Oprm1* neurons mimics the OIRD phenotype or causes apnea, respectively, while chemogenetic or pharmacologic activation of these neurons improves breathing in mice experiencing OIRD (Liu et al., 2021). These experiments demonstrate that while the *Oprm1* neuronal population may appear heterogeneous, anatomical and functional structure exists to allow these circuits to mediate both OIRD and affective pain behaviors.

In the thalamus, *Oprm1* is highly expressed in the intralaminar and midline thalamic nuclei, including the paracentral, paraventricular (PVT), central medial, centrolateral, xiphoid, and rhomboid nuclei (Brodsky et al., 1995; Eacret et al., 2023; Erbs et al., 2015; Okunomiya et al., 2020). Neurophysiological studies indicate that MOR activation in PVT and parataenial thalamus reduces the activity of thalamic neurons projecting onto both central amygdala and basal amygdala neurons (Goedecke et al., 2019), whereas MOR activation in mediodorsal thalamus and centrolateral thalamus inhibits thalamic inputs to striatal MSNs and ACC pyramidal neurons (Birdsong et al., 2019). MOR expression in the PVT is particularly of interest given that recent studies suggest that PVT projections to the NAc and to the CeA contribute to opioid dependence and withdrawal memory (Keyes et al., 2020; Zhu et al., 2016). Consistent with this idea, *Slc17a6* cKO mice show reduced or abolished opioid withdrawal symptoms (Reeves et al., 2021; Zhang et al., 2020c). Additionally, inhibition of *Oprm1*+ PVT neurons during morphine SA in the dark cycle decreased morphine-induced wakefulness, suggesting their involvement in morphine-induced sleep disturbance (Eacret et al., 2023). Our analysis of *Slc17a6*+/*Oprm1*+ PVT neuron identity using whole-brain scRNA-seq datasets indicate that these neurons can be distinguished by *Foxp2*, *Shox2*, and *Gbx2* expression (Zeisel et al., 2018). Overall, we observe *Rora* and *Nptxr* expression to distinguish the thalamic neurons with the greatest enrichment for *Oprm1* (Zeisel et al., 2018).

The amygdala receives nociceptive information from the PB and thalamus and produces, along with other cortical and subcortical structures, the affective-motivational dimension of pain, i.e., pain unpleasantness (Corder et al., 2019; Kimmey et al., 2022; Mercer Lindsay et al., 2021; Neugebauer, 2020; Neugebauer et al., 2020). Histological and physiological studies showed that MOR is highly expressed in the amygdala, particularly in the capsular division of the CeA and in the intercalated cells (ITC) (Cooper et al., 2022; Corder et al., 2019; Mansour et al., 1995b; Mercer Lindsay et al., 2021; Neugebauer, 2020; Okunomiya et al., 2020; Pert et al., 1976; Wang et al., 2018; Winters et al., 2017). The CeA contains primarily GABAergic neurons and is the output nucleus of the amygdala, containing spatially-segregated cell types projecting to the substantia nigra, PAG, PB, parafasciular thalamus, BNST, and lateral hypothalamus (O'Leary et al., 2022). About 40% *Oprm1*+ neurons in CeA are PKCδ+ neurons (Cooper et al., 2022), a cell type found in the lateral CeA that is known to inhibit medial CeA output neurons (McCullough et al., 2018; O'Leary et al., 2022). Conversely, the basolateral amygdala contains primarily glutamatergic neurons and transmits information to the CeA, NAc, and cortical regions including the ACC, IL,

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PL, and insular cortices (Thompson and Neugebauer, 2017, 2019); this region contains high levels of DOR, but low levels of MOR compared to the CeA and ITC (Wang et al., 2018). MOR blockade in the CeA with naloxone reinstates mechanical hypersensitivity in a hindpaw inflammation model in male mice only, while intra-CeA CTAP reinstated hypersensitivity in both sexes (Cooper et al., 2022), suggesting the importance of CeA MORs in preventing the transition from acute to chronic pain. Further study is needed to address whether these effects are mediated pre- or postsynaptically in the CeA, and to uncover the molecular and functional roles of *Oprm1*+ CeA neurons. Amygdala cell clusters reported in whole-brain scRNAseq datasets do not meet our criteria for *Oprm1* enrichment, and recent CeA scRNAseq studies do not report which clusters express *Oprm1* (O'Leary et al., 2022; Saunders et al., 2018; Zeisel et al., 2018).

The mediodorsal, intralaminar and midline thalamic nuclei, and the BLA are reciprocally connected with the ACC, and neurons in these regions can together process nociceptive information to shape the affective-motivational dimension of pain. Studies in both humans and rodents suggest that MOR signaling in the ACC can reduce the affective-motivational dimension of pain, often without consequences on on sensory thresholds and nocifensive reflexes (Barthas et al., 2015; Eippert et al., 2009; Gomtsian et al., 2018; Kuo and Yen, 2005; LaGraize et al., 2004, 2006; Navratilova et al., 2015a, 2015b, 2020; Petrovic et al., 2002; Sprenger et al., 2006; Wang et al., 2020; Zubieta et al., 2001). Furthermore, ACC MORs also contribute to rewarding aspects of pain relief, particularly in rostral areas of the structure (Navratilova et al., 2013, 2015a, 2015b, 2020). The precise identification of the ACC neurons that express MOR and mediate these effects is currently being resolved (Zamfir et al., 2022), which will enable cell type specific investigation of MOR function in the ACC.

Several studies have used broad cKO of *Oprm1* in GABAergic or glutamatergic neurons to identify the *Oprm1*+ cell types responsible for the antinociceptive effects of MOR. However, cells co-expressing *Slc32a1* (the gene encoding VGAT*)* and *Oprm1* are present in nearly all the central nervous system regions where MOR is expressed. Like-wise, in addition to the DRG, spinal cord, and thalamus, analysis of whole-brain scRNA-seq data indicates that *Oprm1* is co-expressed with *Slc17a6* (the gene encoding VGLUT2) in medullary adrenergic neurons (*Npff, Tfap2b, Chodl*), cholinergic neurons of the hindbrain (*Gpx2, Cabp7*) (Zeisel et al., 2018), habenular neurons (*Tac2, Wif1, Syt15*), and thalamic neurons (*Rora, Nptxr*) (Saunders et al., 2018).

Use of the *Slc32a1*^{Cre}/*Oprm1*$^{KI/KI}$ "knockout first" mice demonstrated that MORs on GABAergic neurons contribute to endogenous opioid functions. Only some measures of exogenous antinociception were impacted, such as increased latency for tail withdrawal after morphine administration in the tail immersion test. However, in a chronic CFA-induced inflammatory pain model, *Slc32a1*^{Cre}/*Oprm1*$^{KI/KI}$ mice recovered from hyperalgesia significantly more rapidly than controls, an effect that was confirmed by slower recovery in *Slc32a1*^{Cre} cKO mice (Zhang et al., 2020b). By contrast, broad manipulations of MOR on VGLUT2+ glutamatergic cells using both "knockout-first" and cKO models suggest MORs on VGLUT2+ cells contribute to exogenous morphine antinociception (Zhang et al., 2020b,c). MOR on VGLUT2+ cells contribute to systemic morphine's analgesic effect in

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the hot plate, tail immersion test, and the von Frey test, both for acute nociceptive pain and after intraplantar CFA persistent inflammatory pain (Zhang et al., 2020b). However, MORs expressed on VGLUT2 cells on a *Oprm1*$^{-/-}$ background did not affect recovery time from CFA-induced inflammatory pain, thought to be governed by endogenous opioids (Zhang et al., 2020b). This conditional knockout was described to remove MOR from subcortical regions such as the habenula, thalamus, and PBN (Zhang et al., 2020c). In another study utilizing VGLUT2 cKO mice, no changes in oxycodone-mediated antinociception were observed in the shock-flinch test, but other methods of evaluating opioid analgesia were not measured (Reeves et al., 2021). Ultimately, both GABAergic and glutamatergic MOR + cell types are likely to contribute to analgesia, but the localization of these effects to more specific structures and cell types is still to come.

3.4. Descending pain modulation circuits

MOR is also expressed by neurons in brain regions that are part of descending pain modulation systems, including forebrain ACC and amygdalar neurons projecting to the periaqueductal gray (PAG), PAG neurons projecting to the rostral ventromedial medulla (RVM) (Harasawa et al., 2016; Kim et al., 2018; McPherson and Ingram, 2022; Zhang et al., 2015), and RVM neurons projecting to the spinal cord dorsal horn (Marinelli et al., 2002; McPherson and Ingram, 2022; Mercer Lindsay et al., 2021). The PAG integrates activity from forebrain inputs and can modulate nociception through projections to specific RVM neuronal populations that either facilitate or inhibit nociception at the spinal level (Heinricher et al., 2009; Mercer Lindsay et al., 2021). MOR has been reported to be expressed by both excitatory and inhibitory PAG neurons, and to inhibit both GABA and glutamate release (Bagley and Ingram, 2020; Li et al., 2016; McPherson and Ingram, 2022; Park et al., 2010), a finding consistent with recent scRNA-seq data (Vaughn et al., 2022). Interestingly, one slice electrophysiology study demonstrated that application of DAMGO onto *Th*+, *Slc17a6* + DRN/PAG neurons that release dopamine and glutamate reduced evoked IPSCs and mIPSC frequency and amplitude (Li et al., 2016). Of note, these cells did not project to the RVM, but instead to the BNST and CeA (Li et al., 2016). Other slice electrophysiology experiments examining GABAergic inputs to PAG cells retrogradely labeled from the RVM indicated that DAMGO decreases evoked IPSCs in RVM-projecting neurons more potently than in unlabeled PAG neurons (Lau et al., 2020). These studies suggest that MOR primarily decreases inhibitory input onto *Slc17a6*+ excitatory PAG cells, disinhibiting these cells and increasing PAG excitatory output to the RVM. Further study should explore the extent to which pain modulation mechanisms in the PAG may be sex-specific, as sex differences have been reported both in MOR expression (Loyd et al., 2008) and in the function of dopaminergic PAG/DRN to BNST nociceptive circuits (Yu et al., 2021).

In the RVM, electrophysiological characteristics determine ON-cells, OFF-cells, and neutral cells, which have facilitating, inhibiting, or no effect, on nociception, respectively (Fields et al., 1983). Evidence suggests that the opioid system modulates the activity of these cell types (Fields, 2000); for example, systemic chronic morphine shifts RVM ON and OFF cell responses to a more pronociceptive state (Viisanen et al., 2020). Importantly, technical challenges associated with sequencing neurons in brainstem regions with abundant

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myelination, such as the RVM, have hampered the comprehensive identification of cell types expressing *Oprm1* and MOR (Zeisel et al., 2018). Despite these challenges, work from our lab and others has shed light on the possible mechanisms underlying these effects. For example, tetanus toxin-mediated silencing of inhibitory, enkephalinergic RVM neurons that project to the spinal cord causes increased nocifensive behaviors in the hot plate and tail flick tests (Zhang et al., 2015). Ablation of these neurons using a viral caspase approach resulted in increased mechanical sensitivity in the von Frey assay (Zhang et al., 2015). We demonstrated that spinally projecting, MOR-immunoreactive, inhibitory RVM neurons facilitate mechanical pain via inhibition of *Pen*k+ GABAergic, spinal interneurons that presynaptically inhibit somatosensory neurons (François et al., 2017). Finally, KOR+ RVM neurons may also contribute to descending inhibition of nociception, as chemogenetic activation of these neurons causes antinociception in the cold plantar, Hargreaves, and tail flick assays (Nguyen et al., 2022). Notably, these neurons are primarily GABAergic and have a 30% overlap with *Penk*+ neurons and a 30% overlap with *Oprm1*+ neurons in the RVM (Nguyen et al., 2022). Further study will be needed to thoroughly elucidate the relationships between the electrophysiological profiles, functional importance to pain facilitation or suppression, and genetic identity of the remaining cell types in this pathway.

4. MOR expression in non-neuronal cells

The extent to which opioid receptors are expressed by non-neuronal cells, in particular by CNS glial cells such as microglia, has long been a matter of debate. Several immunolabeling studies using anti-MOR antibodies have reported MOR expression in microglia, astrocytes, and oligodendrocytes (Ferrini et al., 2013; Horvath et al., 2010; Maduna et al., 2018; Nam et al., 2018; Stiene-Martin et al., 2001; Vacca et al., 2012). However, studies relying predominantly on MOR-immunolabeling rarely include definitive negative controls, such as absence of labeling in glial cells from global *Oprm1* KO mice, that would establish the specificity of the signal reported. Here, we limit our assessment of *Oprm1* and MOR expression patterns to *in vivo* studies as culture conditions can alter gene expression (Bohlen et al., 2017; Wangzhou et al., 2020).

Cell sorting offers an alternative approach to investigate *Oprm1*/MOR expression in distinct cell populations by measuring gene and protein expression in purified cell types. Studies measuring *Oprm1* expression in sorted microglia and astrocyte populations by quantitative PCR have produced conflicting results (Leduc-Pessah et al., 2017; Schwarz et al., 2013). High-throughput molecular profiling techniques like RNA-seq and proteomics do not rely on target-specific antibodies or probes and therefore provide a less biased approach to RNA and protein quantification (Zhang et al., 2014). A meta-analysis of microarray and bulk RNA-seq datasets on sorted microglia from various brain and spinal cord samples reported *Oprm1* transcripts in 17/18 rodent studies and 16/17 human studies (Maduna et al., 2018). However, these studies all exhibit a degree of neuronal contamination based on gene cluster analysis and therefore cannot conclusively rule out *Oprm1* expression from synaptic debris. Additional bulk RNA-seq studies on sorted microglia either did not detect *Oprm1* (Corder et al., 2017), consistent with the authors' histological data using *Oprm1* ISH, MOR immunolabeling, and *Oprm1*^{mCherry} mice, or detected *Oprm1* in only a subset of samples at low levels (Bennett et al., 2016), which the authors attribute to

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synaptic contamination. Thus, whether *Oprm1* detection in glia sorted from naive animals is biological or arises from neuronally-derived RNA contamination is unclear. Interestingly, a recent study detected *Oprm1* from sorted CD11c + spinal cord microglia that appear after nerve injury and resolve hypersensitivity, suggesting that glial cell *Oprm1*/MOR expression may change under pathological conditions (Kohno et al., 2022). Contrary to RNA detection, MOR protein was not detected in proteomic studies using mass-spectrometry on sorted microglia (Rangaraju et al., 2018; Rayaprolu et al., 2020).

scRNA-seq provides a greater degree of sample specificity to bulk studies wherein doublets and cellular debris contamination can be detected and removed. Numerous studies that performed high-throughput scRNA-seq on unsorted mouse brain tissues and sorted microglia failed to detect *Oprm1* in non-neuronal cells at considerable levels (Hammond et al., 2019; Mathys et al., 2019; Saunders et al., 2018; Zeisel et al., 2018). While *Oprm1* transcripts are detected in a small number of non-neuronal cells in these datasets, expression is remarkably low and inconsistent across samples which is suggestive of debris contamination. One shortcoming of high-throughput approaches is that high cell abundance comes at the expense of lower sequencing depth per cell, which may yield false negatives in gene detection. In addition to high-throughput datasets, recent studies using SMART-seq technology failed to detect *Oprm1* in non-neuronal cells (trimmed normalized expression >0.0) despite having sequencing depths well over 1 million reads per cell (Tasic et al., 2016; Yao et al., 2021). Collectively, scRNA-seq experiments indicate that *Oprm1* detection in non-neuronal glial cells is either extremely rare or the result of technical artifacts. Future studies using spatial transcriptomics and single-cell proteomics will provide further insight into *Oprm1*/MOR expression patterns in non-neuronal cells.

5. Conclusion: the complexity of *Oprm1* functional organization beyond transcriptional mechanisms

The remarkable advancements in scRNA-seq technology have opened new horizons in our understanding of the complexity of biological systems, including the molecular architecture of MOR expression and function in neural circuits. However, as for all techniques, scRNA-seq has limitations. One limitation is the difficulty in capturing rare cells and detecting low-abundance transcripts, such as *Oprm1*. Another limitation of scRNA-seq is that mRNA expression levels do not always directly correlate with protein expression levels. Furthermore, post-transcriptional modifications, including phosphorylation, can significantly alter the expression and function of MOR.

Thus, resolving the mechanisms of function of MOR presents unique and complex challenges at multiple levels. At the gene level, the extensive splicing of the *Oprm1* gene can generate multiple receptor isoforms with distinct receptor structural, signaling and trafficking properties (Pasternak, 2018; Pasternak and Pan, 2011), which could contribute to the heterogeneity of analgesic effects produced by MOR agonists (Andoh et al., 2008; Pasternak et al., 2004; Ravindranathan et al., 2009). At the signaling level, MOR activation by different agonists can lead to biased signaling through preferential association with G proteins (including multiple G protein classes) or β-arrestin, in various subcellular

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localizations and organelles, including plasma membrane, endosomes, and Golgi (Stoeber et al., 2018). MOR can also form heterodimers with a variety of other receptors (Valentino and Volkow, 2018; Zhang et al., 2020a), expanding the potential for diverse signaling mechanisms. At the cellular level, MOR can be located pre-, post-, and extra-synaptically (Haberstock-Debic et al., 2003; Svingos et al., 1996, 1997) to differentially modulate cell function and neurotransmission. As addressed in this review, at the tissue level, MOR is broadly expressed across the peripheral and central nervous systems, where receptor interactions between endogenous opioid peptides and MOR modulate a broad variety of physiological functions beyond reward and pain perception. Future studies combining scRNA-seq findings with innovative techniques that characterize receptor properties at these different biological levels will be crucial to fully unravel the long-standing mysteries of MOR function in specific cell types and pathways, and to identify new therapeutic targets for diseases such as pain and addiction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

MOR	Mu opioid receptor
scRNA-seq	single cell RNA sequencing

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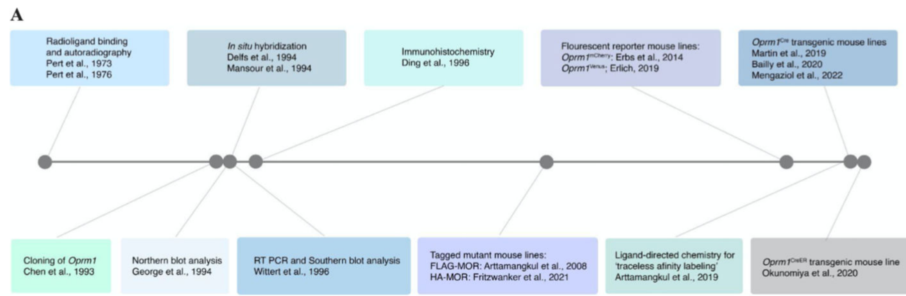
A



B

Technique	Nervous system tissues with highest MOR/*Oprm1* levels in brain-wide studies	Ref. (metric for Oprm1/MOR enrichment)
Histological studies		
Radioligand binding	Cortical nucleus of the amygdala, caudate-putamen, periaqueductal gray, locus coeruleus, midbrain reticular formation, interpeduncular nucleus, superficial spinal cord, dorsal root ganglion	(Atweh and Kuhar, 1977; Kuhar et al., 1973; Pert et al., 1976; Pert and Snyder, 1973; Simon et al., 1977) (fmol radiolabeled morphine / mg protein > 90th percentile
Oprm1 ISH	Olfactory bulb, caudate-putamen, nucleus accumbens, lateral and medial septum, diagonal band of Broca, bed nucleus of the stria terminalis, most thalamic nuclei, hippocampus, amygdala, medial preoptic area, superior and inferior colliculi, central gray, dorsal and median raphe, raphe magnus, locus coeruleus, parabrachial nucleus, pontine and medullary reticular nuclei, nucleus ambiguus, nucleus of the solitary tract, nucleus gracilis and cuneatus, dorsal motor nucleus of vagus, spinal cord, and dorsal root ganglia.	(Delfs et al., 1994) (neuron or probe density >= +++ on 4-plus scale
anti-MOR antibodies	Anterior olfactory nucleus, piriform cortex, ventrolateral orbital cortex, lateral orbital cortex, cingulate cortex, agranular insular cortex, frontal cortex, parietal cortex, occipital cortex, entorhinal cortex, pre-parasubiculum, medial habenula, intercalated amygdalar nucleus, lateral geniculate nucleus, paraventricular hypothalamic nucleus, dorsomedial hypothalamic nucleus, perifornical hypothalamic nucleus, ventromedial hypothalamus, nucleus accumbens, superior and inferior colliculi, nucleus of the optic tract, median raphe nucleus, parabrachial nucleus, locus coeruleus, interpeduncular nucleus, paratrigeminal nucleus, nucleus of the solitary tract, rostral ambiguus nucleus, spinal lamina I & II	(Arvidsson et al., 1995; Ding et al., 1996; Hiller et al., 1994) (immunoreactivity intensity >= ++ on 3-plus scale)
Oprm1 mutant mice (*Oprm1*^{mCherry}; *Oprm1*^{CreER})	Intermediate central amygdala, anterior dorsal medial amygdala, fasciculus retroflexus, anterior paraventricular thalamic nucleus, paratenial thalamic nucleus, rhomboid thalamic nucleus, xiphoid thalamic nucleus, rostral central medial thalamic nucleus, centrolateral thalamic nucleus, medial mammillary nucleus, medial supramammillary nucleus, lateral hypothalamus, parathrochlear nucleus, external lateral parabrachial nucleus, caudal dorsal raphe, dorsal endopiriform nucleus, somatosensory cortex, orbitofrontal cortex, retrosplenial cortex, dorsolateral entorhinal cortex, bed nucleus of the stria terminalis, medial preoptic hypothalamus, dorsal cochlear nucleus	(Erbs et al., 2015; Okunomiya et al., 2020) (immunoreactivity intensity >= +++ on 4-plus scale)

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RNA-seq cell atlases: Brain		
Bulk RNA-seq on sorted neuron populations (neuroseq.janelia.org) Region (Neuron type, *sorting gene*)	Jugular ganglia (Glut; *Trpv1*), central lateral thalamus (Glut; *Grp*), paracentral nucleus of the thalamus (Glut; *Grp*), lateral parabrachial nucleus (Glut; *Chat*), paraventricular nucleus of the thalamus (Glut; *Gpr26*), DRG (Glut; *Trpv1*), medial mammillary nucleus of the hypothalamus (Glut; *Hsd11b2*), spinal cord (GABA; *Sla*), spinal cord (Glut; *Grp*), nucleus raphe pallidus (5HT; *Tph2*), hippocampus CA1 (GABA; *Pvalb*), locus coeruleus (NE; *Th*), area postrema (GABA), nucleus raphe pallidus (GABA; 5HT), medial central amygdala (GABA; *Crh*), periaqueductal gray (GABA; *Slc6a5*), arcuate hypothalamic nucleus (Glut; *Pomc*), accessory olfactory bulb (Glut; *Rbp4*), spinal cord (GABA)	(Sugino et al., 2019) (mean expr > 90th percentile;
10x Genomics (mousebrain.org) Cluster ID (region; *marker*)	TEGLU2 (cortex L6b; *Cplx3*), TEGLU20 (cortex L6; *Sulf1*), TEINH20 (hippocampus sr/slm; *Cyp26b1*), HBCHO3 (cranial nerves VI-XII; *Slc5a7*), HBADR (NTS; *Npff*), SCINH5 (spinal cord laminae 2-6; *Serpinb1b*), SCGLU7 (spinal cord; laminae 1-2; *Grp*), HBGLU10 (NTS; *Ebf2*), HBGLU2 (NTS; *Prlr*), DECHO2 (habenula; *Gng8*), DEGLU3 (PVT; *Gbx2*), MEINH6 (superior colliculus; *Fibcd1*), HBINH5 (gigantocellular reticular nucleus; *Slc6a5*), HBINH3 (paragigantocellular reticular nucleus; *Hoxb5*), HBCHO2 (medial pontine nuclei; *Gpx2*), ENT2 (myenteric plexus; *Cartpt*) , ENT3 (myenteric plexus; *Cox8b*), ENT6 (myenteric plexus; *Htr2b*), ENT7 (myenteric plexus; *Cckar*), ENT8 (myenteric plexus; *Ucn3*), PSPEP8 (peptidergic TrpM8 DRG; *Rarres1*), PSPEP7 (peptidergic TrpM8 DRG; *Gpsm3*), PSPEP6 (peptidergic TrpM8 DRG; *Gfra3*), PSPEP5 (peptidergic DRG; *Trpv1*), PSNF3 (neurofilament DRG; *Trappc3l*), PSNP5 (non-peptidergic DRG; *Mrgpra3*), PSNP6 (neurofilament DRG; *Nppb*)	(Zeisel et al., 2018) (>90TH PERC; 0.03114)
Drop-seq (dropviz.org) ClusterID (region, subcluster; *marker*)	Neuron_Chat#1 (globus pallidus; *Slc17a8-Nefm*), Neuron_Habenula_Tac2#1 (thalamus; *Tac2-Wif1)*, Neuron_Ppp1r1b#3 (globus pallidus; *Adora2a-Pde1c*), Neuron_Ppp1r1b#3 (globus pallidus; *Sphkap-Tmem255a*), Neuron_Habenula_Tac2#1 (thalamus; *Tac2-Syt15*), Neuron_Rora#2 (thalamus; *Rora-Nptxr*), Interneuron_MGE_Sst-Pvalb#2 (frontal cortex; *Sst-Pdyn*), Interneuron_MGE_Sst-Pvalb#2 (frontal cortex; *Sst-Crh*), Interneuron_MGE_Sst-Pvalb#2 (frontal cortex; *Pvalb-Unc5b*), Neuron_Layer5b_Fezf2#4 (frontal cortex; *Slc17a8*), Neuron_Claustrum_Nr4a2#5 (frontal cortex; *Nr4a2*), Neuron_Layer6Subplate_Syt6#1 (posterior cortex; *Cplx3*), Neuron_Layer6Subplate_Syt6#1 (posterior cortex; *Lancl3*), Fibroblast-like_Dcn#14 (posterior cortex; *Coch*), Interneuron_MGE_Sst-Pvalb#5 (posterior cortex; *Sst-Pdyn*), Interneuron_MGE_Sst-Pvalb#5 (posterior cortex; *Sst-Nr2f2*), Layer5b_Fezf2#7 (posterior cortex; *Slc17a8*), Neuron_Syt1#4 (entopeduncular, *Cbln1*), Neuron_Syt1#4 (entopeduncular; *Tcf7l2*), Neuron_Gabra1#2 (globus pallidus; *Tac2*), Neuron_Gabra1#2 (globus pallidus; *Satb1*), Neuron_Gabra1#2 (globus pallidus; *Cbln1*), Neuron_Gabra1#2 (globus pallidus; *Rspo3*), Neuron_Gabra1#2 (globus pallidus; *Neurod2-C1ql3*), Neuron_Gabra1#2 (globus pallidus; *Trdn*), Neuron_Gabra1#2 (globus pallidus; *Elfn1-Grik3*), Neuron_Gabra1#2 (globus pallidus; *Trh*), Neuron_Gabra1#2 (globus pallidus; *Igfbp4-Adora1*), Neuron_Ppp1r1b#3 (globus pallidus; *Drd1-Pdyn*), Neuron_Ppp1r1b#3 (globus pallidus; *Drd1-Pde1c*), Neuron_Ppp1r1b#3 (globus pallidus; *Sphkap-Cpne4*), Neuron_Ppp1r1b#3 (globus pallidus; *Th-Adora2a*), Interneuron_Gad2#1 (hippocampus; *Ncam2*), Interneuron_Gad2#1 (hippocampus; *Sst-Spon1*), Interneuron_Gad2#1 (hippocampus; *Sst-Grm1*), Interneuron_Gad2#1 (hippocampus; *Sst-Pcdh11x*), Interneuron_Gad2#1 (hippocampus; *Id1-Prlr*), Interneuron_Gad2#1 (hippocampus; *Htr3a-Nnat*), Interneuron_Gad2#1 (hippocampus; *Htr3a-Chrm2*), Neuron_Subiculum_Entorhinal_Nxph3#3 (hippocampus; *Nts*), Neuron_Subiculum_Entorhinal_Nxph3#3 (hippocampus; *Cplx3-Hs6st2*), Neuron_Subiculum_Entorhinal_Nxph3#3 (hippocampus; *Cplx3-Pappa2*), Interneuron_Chat#12 (striatum; *Slc17a8-Chat*), Neuron_Gad2#3 (substantia nigra; *Nfib-Satb1*), Neuron_Gad2#3 (substantia nigra; *Nts*), Neuron_Gad2#3 (substantia nigra; *Shox2*), Neuron_Gad2#3 (substantia nigra; *Pax5*), Neuron_Habenula_Tac2 (thalamus; *Htr2c*), Neuron_Rora#2 (thalamus; *Atp2b4*), Neuron_Rora#2 (thalamus, *Calca*), Neuron_Gad2-Ahi1 (thalamus; *Col25a1*), Neuron_Gad2-Ahi1 (thalamus; *Nrgn*), Fibroblast-like_DCN (thalamus; *Nenf*).	(Saunders et al., 2018) (subclusters; normalized avg. log2 > 0).

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RNA-seq cell atlases: Spinal cord		
Multiple scRNA-seq methods; integrated datasets (seqseek.ninds.nih.gov)	Excit-8 (*Bnc2*), Inhib-3 (*Nwd2*) , Inhib4 (*Rxfp2*), Excit35 (*Tmsb10*)	(Russ et al., 2021) (expressed in >= 25% cells)
C1 Fluidigm (linnarssonlab.org/dorsalhorn): ClusterID (*Marker genes*)	GABA-7 (*Npy*, *Crabp1*), GABA-9 (*Calb2*, *Tac1*)	(Häring et al., 2018) (Bayesian posterior probability distribution > 99.9%)
RNA-seq cell atlases: DRG		
10x Genomics (painseq.org): ClusterID (cell type; *marker gene*)	PEP1 (peptidergic nociceptor; *Gpx3*), PEP2 (peptidergic nociceptor; *Hpca*), SST (Sst+ pruriceptor; *Il31ra*)	(Renthal et al., 2020) (Detected in > 5% of cells per cluster in naïve samples)
10x Genomics: ClusterID - Cluster number (*Marker gene*)	TRPM8-18 (*Foxp2*), SST-0 (*Mustn1*)	(Sharma et al., 2020)(Cluster-specific differential gene expression; Wilcoxon rank-sum Bonferroni corrected p-value < 0.01)
MOR Transgenic Mice		
MOR transgenic mice (strategy)		**Refs**
FLAG-MOR (TH-FLAG-MOR-Tg; *Th* promoter)		(Arttamangkul et al., 2008)
MOR-mCherry (mCherry cDNA inserted in frame instead of exon 4 stop codon)		(Erbs et al., 2015)
MOR-Venus (Venus cDNA inserted in frame instead of exon 4 stop codon)		(Ehrlich et al., 2019)
MOR-eGFP/Cre (T2A-eGFP-Cre construct replaced exon 4 stop codon)		(Bailly et al., 2020)
MOR-Cre (T2A-Cre construct replaced exon 5 stop codon).		(Märtin et al., 2019)
MOR-Cre (T2A-Cre construct replaced exon 4 stop codon).		(Mengaziol et al., 2022)
MOR-CreER (T2A-CreERT2 construct replaced exon 4 stop codon)		(Okunomiya et al., 2020)
HA-MOR (HA-tag inserted downstream of exon 1 start codon)		(Fritzwanker et al., 2021)

Fig. 1. Historical perspective: elucidation of the expression pattern of *Oprm1* and MOR.
Although radioligand binding and autoradiography studies provided the first window into the tissues and regions in which MOR was expressed, the cloning of the *Oprm1* gene in 1993 allowed for a myriad of new genetics-based approaches to reappraise and refine *Oprm1* and MOR distribution in tissues. Today, this rapidly expanding set of techniques, paired with high-throughput RNA-seq, enables the comprehensive elucidation of MOR expression patterns at the single cell level, in the DRG, spinal cord, and brain. (A) Timeline showing the evolution of techniques used for detection of *Oprm1*/MOR. (B) Table indicating *Oprm1/*

MOR+ regions in DRG, spinal cord, and brain via these techniques. (Arttamangkul et al., 2008; Bailly et al., 2020; Ding et al., 1996; Ehrlich et al., 2019; Fritzwanker et al., 2021; Mengaziol et al., 2022; Sugino et al., 2019).

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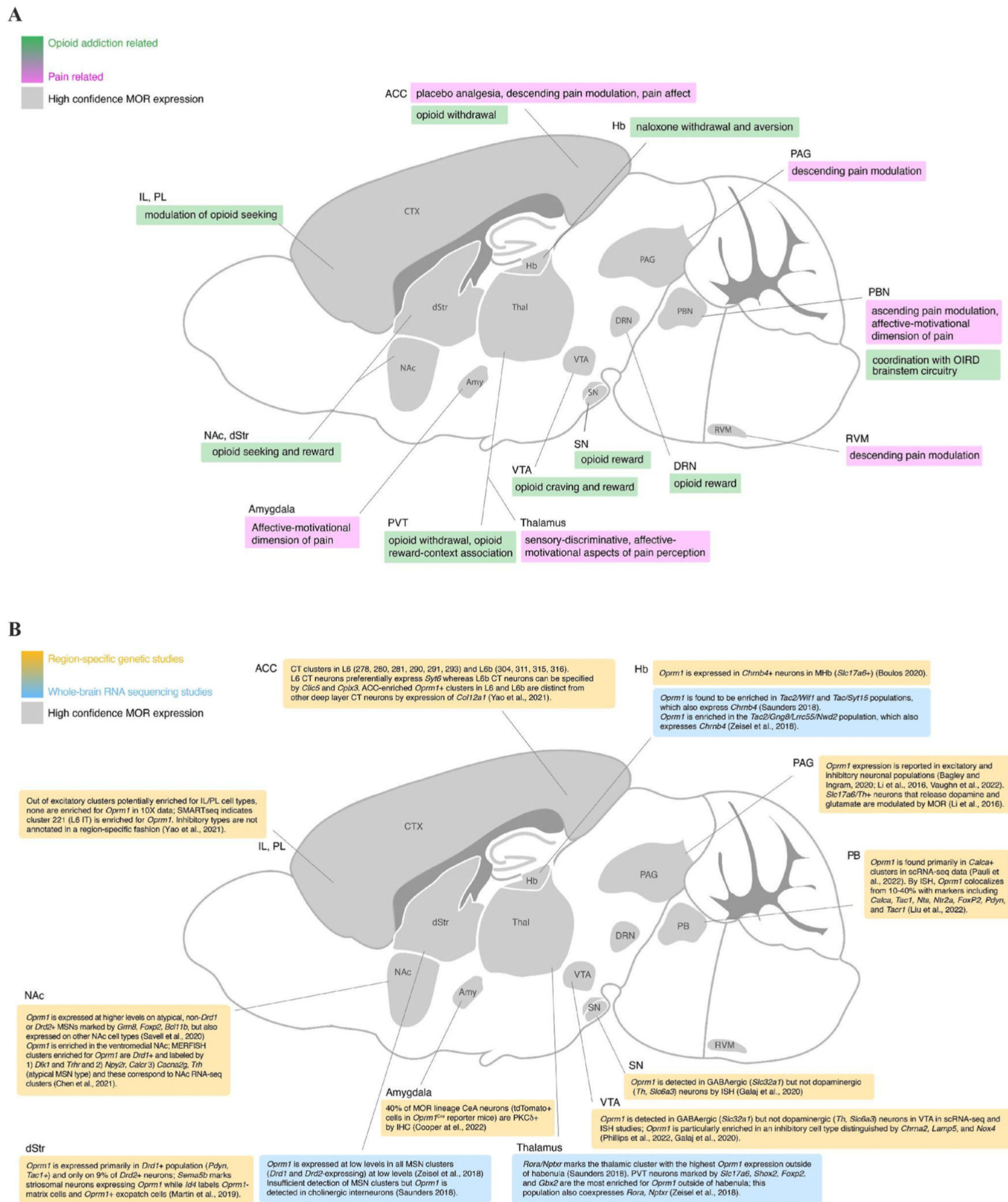

Fig. 2. High-confidence *Oprm1*+/MOR + brain regions and their associated functions in opioid addiction and antinociception.

(A) Regions validated as being enriched in *Oprm1* and MOR using multiple techniques are designated in gray. Functional contributions to opioid reward and dependence (green) and opioid antinociception (magenta) are indicated. (B) For regions with high-confidence MOR expression, marker genes defining *Oprm1*+ cell populations are indicated from both region-specific (yellow) and whole-brain (blue) scRNA-seq studies.

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