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Endogenous opioids in systems neuroscience

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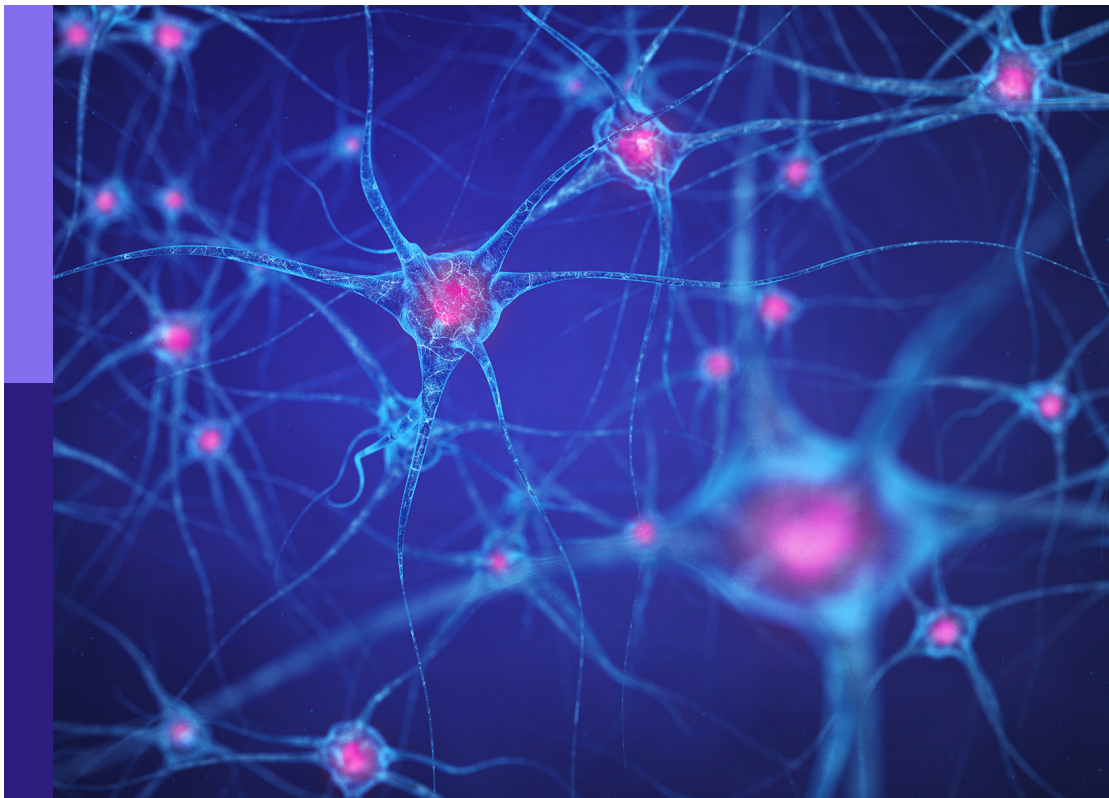

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Endogenous opioids in systems neuroscience

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Editorial: Broadening our conceptual understanding of endogenous opioids in systems neuroscience

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Editorial on the Research Topic
Broadening our conceptual understanding of endogenous opioids in systems neuroscience

Endogenous opioid peptides and their receptors are critical mediators of various physiological and psychological processes, including motivation, affect, pain processing, cognition, stress-responsivity, and autonomic function. These opioid systems are embedded in neuronal circuits that subserve specific aspects of such processes and play a pivotal role in finely tuning behavioral outcomes. This is of relevance as dysfunction in endogenous opioid systems has been implicated in a plethora of neuropsychiatric disorders characterized by alterations in several of symptom clusters. To date, opioid receptor systems remain a promising therapeutic target for a variety of neuropsychiatric disorders and are an area of on-going development.

As a field, we have achieved significant advancements in our understanding of the cellular and molecular underpinnings of endogenous opioids in regulating cellular activity and behavior. However, there remain critical knowledge gaps in our understanding of how endogenous opioid systems are embedded in neuronal circuits and how these systems regulate emergent properties of brain function. Recent advances in technology have permitted a thorough dissection of endogenous opioid systems with cell type, pathway, and subcellular resolution. Accordingly, progress has provided us with a better understanding of endogenous opioid system engagement in regulating neuronal systems that subserve the aforementioned physiological and psychological processes. Despite those critical breakthroughs, further efforts are necessary to unravel the precise granularity with which endogenous opioid systems dysregulation alters pathways necessary for the development and maintenance of pathophysiological states in mental health disorders.

With the present collection of articles in this Research Topic, we provide recent advancements in our understanding of endogenous opioids from receptor signaling to neuronal circuits and highlight future avenues and opportunities for research aiming at elucidating novel targets and approaches to treat neurological and psychiatric disorders.

Gamble et al. highlight recent advancements in uncoupling cellular mechanisms mediating beneficial pharmacological effects such as treatment of pain and receptor signaling that leads to various unwanted effects such as analgesic tolerance, physical dependence and activation of reward circuitry. They discuss the relatively unappreciated interaction

of mu opioid receptors (MOR) with receptor tyrosine kinases (RTKs), including RTKs transactivation of MOR, and the potential therapeutic implication for targeting RTK to enhance the safety and analgesic profile of MOR agonists. There is a strong case to be made that RTK inhibitors may be a co-treatment therapy that will allow opioid mediated analgesic properties with attenuated negative outcomes associated with chronic opioid pharmacotherapies.

Adhikary and Williams provide a synthesis of the recent advancement on cellular tolerance induced by chronic opioid receptor activation. In addition, they present insights into how tolerance can be manifested at the neuronal systems level. One highlight in their article is that tolerance is not mediated by a single regulatory mechanism but rather by various adaptations in cellular processes and circuits. In their review they discuss pre- and post-synaptic mechanisms underlying cellular tolerance to chronic morphine in multiple brain regions, and how the pharmacological properties of opioids, including their potency and efficacy, produce distinct adaptations that lead to tolerance.

The ventrolateral periaqueductal gray area (PAG) is well recognized for its importance in descending modulation of pain (pre-clinical; Lubejko et al.), or conditioned pain modulation (clinical). McPherson and Ingram review the importance of the opioid systems in descending pain modulation and how opioids contribute to the cellular and circuit diversity within the PAG implicated in pain transmission and opioid analgesia. The granularity on the description of circuit afferent, cell type specificity, and cellular signaling provides a cohesive summary of our current knowledge on this specific area of research. One of the important messages for future direction in this area is the potential for optimizing novel target drug development by taking advance of our knowledge of cellular heterogeneity in the PAG and other regions implicated in descending pain modulation.

The manuscript by Lubejko et al. further addresses the importance of opioid systems in analgesia and pain treatment. In this article, they emphasize the potential of neurostimulation as a novel treatment strategy that mitigates side effect profiles associated with small molecule MOR ligands. Various neurostimulation techniques have been proposed to treat pain including deep brain stimulation (DBS), spinal cord stimulators, vagal nerve stimulation and transcranial direct current stimulation (tDCS). This paper highlights evidence supporting the effectiveness of various neurostimulation techniques and how they may engage opioid systems to produce their beneficial effect in pain management. The authors also provide a comprehensive overview of the various brain regions and circuits where pain transmission is processed and modulated by opioids, as well as a future outlook of the next generation of safe, effective, and technologically-innovative clinical treatments.

Limoges et al. provide us with a detailed description of the architecture and function of the dynorphin kappa-opioid receptor system, specifically in amygdala circuits. This review presents evidence from current literature demonstrating that the dynorphin kappa-opioid receptor system plays a pivotal role in controlling many aspects of behavior, including aversive learning, pain-related, and alcohol and drug-seeking behaviors. Embedded in this article are comprehensive illustrations of the various neural circuit inputs onto dynorphin kappa opioid receptor expressing cells within the basolateral and central amygdala as well as targets of these neurons, providing a framework for how the kappa opioid system may contribute to neuropsychiatric disorders.

Adding to this article, Reeves et al. provide a comprehensive overview of the role of opioid systems in regulating synaptic transmission and intrinsic excitability across the nervous system. In their review they summarize the current literature to present a detailed description of how endogenous opioids finely tune information flow in discrete circuits, providing a cellular basis wherein opioid-mediated transmission may shape circuits at the systems level.

There is a pressing need to understand how opioids produce reinforcement and drug seeking behaviors, especially in the context of pain treatment, as chronic pain has been identified as a risk factor for developing an opioid use disorder. Higginbotham et al. provide a review on how pain or prolonged opioid exposure modifies reward circuitry and changes in opioid receptor function. There is strong evidence that persistent pain negatively impacts reward sensitivity and mood. Together, pain modulation of reward sensitivity and mood contributes to susceptibility in initial opioid misuse and the development of opioid use disorder. They provide a strong message that the ability to curb the opioid crisis will require more understanding of how pain and opioid-induced adaptations alter functional neurocircuitry. Indeed, much of this research is still in its infancy. Complementing this review is another by Rysztak and Jutkiewicz that comprehensively describe the mechanisms by which enkephalin peptides and enkephalin-expressing neuronal circuits mediate reward function, focusing on the modulation of mesolimbic dopamine circuitry. The authors further discuss alterations in those enkephalinergic systems in models of opioid use disorder.

Lastly, Maletz et al. share a compelling study in which they identify non-overlapping neuronal populations, expressing or lacking MOR, activated by morphine and hypoxia/hypercapnia in the Nucleus Tractus Solitarius. This original data report provides a deeper understanding of the circuits and neuronal populations involved in opioid-induced respiratory depression and may lead to further advancement for prevention and recovery of such events.

Overall, this compilation of papers recognized various future directions that need to be further explored. While there has been significant advancement in our understanding of cellular and synaptic level mechanisms that mediate opioid-regulated behaviors, there is much to be gained by furthering our understanding of how opioids regulate the activity of defined synapses and excitability to shape the activity of large-scale networks and emergent properties of the brain. Emerging research has shown that opioid receptor signaling is diverse and much more complex than what was previously appreciated (e.g. ligand-directed signaling or functional selectivity). However, how nuanced signaling by endogenous opioid systems regulate information flow in neuronal circuits is in large part unresolved. Further, future research is needed to develop a model that integrates opioid systems with other signaling modalities (e.g., RTKs, etc). This provides a basis for diversity of gene expression across cell types of the brain to influence not only where opioids may act, but also their function effect on circuits. Additionally, while the rapid rise in technology development has facilitated

swift progress in the neuroscience field, further tools are needed to precisely dissect the functional anatomy and activity of the opioid systems. Those will be critical to delineate the effects of endogenous opioids on circuits with high spatiotemporal resolution helping us to better understand and solve essential questions. For instance, revealing activity dynamics of not only neurons expressing opioid peptides, but subsequent opioid peptide release and receptor-mediated signaling in models of affective pain or analgesia tolerance will help elucidating potential steps where endogenous opioid system dysfunction occurs. Together, this will provide critical insight necessary to develop novel opioid therapeutics for safer treatment for both the sensory and emotional experiences associated with pain. Another exciting trajectory of the field will be to understand the role of opioids in the context of other treatment modalities such as neuromodulation (e.g., DBS, tDCS) or rapid-acting antidepressants, which may in part exert their function via endogenous opioids or can synergize with opioid-based treatments. In conclusion, here we present a collection of articles focused on opioid systems in the context of systems neuroscience, which highlights developments and provide insights to the future of opioid research in this setting.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Opioid Receptor-Mediated Regulation of Neurotransmission in the Brain

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Opioids mediate their effects *via* opioid receptors: mu, delta, and kappa. At the neuronal level, opioid receptors are generally inhibitory, presynaptically reducing neurotransmitter release and postsynaptically hyperpolarizing neurons. However, opioid receptor-mediated regulation of neuronal function and synaptic transmission is not uniform in expression pattern and mechanism across the brain. The localization of receptors within specific cell types and neurocircuits determine the effects that endogenous and exogenous opioids have on brain function. In this review we will explore the similarities and differences in opioid receptor-mediated regulation of neurotransmission across different brain regions. We discuss how future studies can consider potential cell-type, regional, and neural pathway-specific effects of opioid receptors in order to better understand how opioid receptors modulate brain function.

Keywords: opioid, synaptic plasticity, receptor signal transduction, neurotransmission, glutamate, GABA

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INTRODUCTION

Opioid drugs, which include both prescription painkillers, such as morphine and oxycodone, and illicit substances, such as heroin, are widely used and frequently misused (Kosten and George, 2002; Von Korff, 2013). An increase in prescription of opioid analgesics has precipitated an opioid crisis characterized by widespread opioid misuse, related complications, and opioid overdose (Kosten and George, 2002; Von Korff, 2013; Dahlhamer et al., 2016). This crisis presents a severe health exigency and makes salient a crucial scientific initiative to better understand the effects of opioid drugs and the mechanisms and opioid receptor systems on which these drugs act.

Classically, opioid receptors can be categorized into one of three subtypes: mu (MOR), delta (DOR), and kappa (KOR) (Le Merrer et al., 2009). Endogenous signaling peptides activate opioid receptors: endorphins (MOR), enkephalins (primarily DOR, MOR), and dynorphins (KOR). Opioid peptides or synthetic opioid peptide derivatives are often utilized as selective opioid receptor agonists and antagonists in research. The pharmacology of these diverse ligands is reviewed elsewhere (Rasakham and Liu-Chen, 2011; Gendron et al., 2016; De Neve et al., 2021). Some commonly studied opioid receptor agonists include DAMGO (MOR), DPDPE (DOR), U69,593 or U50,488 (KOR), and the endogenous opioid peptides, met-enkephalin (MetEnk), leu-enkephalin (LeuEnk) (DOR, MOR), and dynorphin (KOR). Commonly used opioid receptor antagonists include CTAP/CTOP (MOR), naltrindole (DOR), and nor-binaltorphimine (KOR) or less selective antagonists such as naloxone. Many opioid drugs, including morphine, fentanyl, and heroin primarily activate MORs (Pasternak, 2012). Opioid receptors are Class A G protein

coupled receptors (GPCRs) that couple to inhibitory $G_{i/o}$ proteins (**Figure 1**; Stein et al., 2003; Allouche et al., 2014). These receptors transduce extracellular messages using G protein ($G_{\alpha i}$ and $G_{\beta\gamma}$), mitogen-activated protein kinase (MAPK), and arrestin signaling pathways (Rosenbaum et al., 2009; Al-Hasani and Bruchas, 2011). Opioid receptors generally decrease neurotransmission through inhibiting voltage-gated calcium channels and activating inwardly rectifying potassium channels (Yamada et al., 1998; Al-Hasani and Bruchas, 2011). Opioid receptors can be located postsynaptically in neuronal soma and presynaptically in axon terminals (Olive et al., 1997). Postsynaptic opioid receptors inhibit neurotransmission by directly hyperpolarizing neurons, while presynaptic opioid receptors can indirectly reduce or enhance neural activity by reducing excitatory or inhibitory neurotransmission, respectively. The opioid receptors and their endogenous ligands are differentially expressed throughout the brain (Le Merrer et al., 2009; Erbs et al., 2015). Because of their widespread expression, opioid receptors are involved in a diverse array of physiological and behavioral functions, including nociception, drug reward and consumptive behavior, social memory, fear learning, stress and emotion, immune activation, and various physiological processes, such as respiration and gastrointestinal tract motility (Shippenberg et al., 1998; Drews and Zimmer, 2010; Van't Veer and Carlezon, 2013; Leroy et al., 2017; Eisenstein, 2019; Patel et al., 2019; Toubia and Khalife, 2019; van Steenbergen et al., 2019; Robble et al., 2020; Galaj and Xi, 2021).

The expanding understanding of opioid receptor functionality, distribution, and modulation of neurotransmission has demonstrated an important role for opioids in modulating neuroplasticity. Neuroplasticity refers to the ability of the brain to change structure and function across life and in response to experience (Voss et al., 2017). The phenomenon is multi-level and can occur across networks, isolated circuits, and amongst cell populations (Citri and Malenka, 2008; Voss et al., 2017). This manifests as changes in functional and structural connectivity, the formation, migration, and elimination of neurons and glia, alterations of neuronal processes, and through synaptic plasticity (Kays et al., 2012; Kelly and Castellanos, 2014). Synaptic plasticity may be persistent with activity-dependent strengthening (long-term potentiation, LTP) and weakening (long-term depression, LTD) of connections between neurons, although there are abundant forms of short-term plasticity as well (Citri and Malenka, 2008; Atwood et al., 2014a; Motanis et al., 2018). Activity-dependent neuroplasticity is mediated by endogenous neurotransmitter systems (Viveros et al., 2007; Bliss and Cooke, 2011; Pitchers et al., 2014). Exposure to exogenous substances (e.g., neurotransmitter receptor agonists, antagonists) can also induce "chemical" plasticity (Atwood et al., 2014a). Neuroplasticity underlies many crucial processes, including learning, cognition, and neurodevelopment, and is implicated in the development of neuropathology, including mood disorders, addiction, and neurodegenerative diseases. Therefore, it is important to elucidate the role of opioid receptors in neuroplasticity (Johansson, 2004; O'Brien, 2009; Kays et al., 2012; Schaefers and Teuchert-Noodt, 2016; Voss et al., 2017). Due to their ability to modulate different neurotransmitter systems, as well as directly influencing cellular function, opioid receptors are positioned to modulate both activity-dependent plasticity and opioid drug-induced chemical plasticity (Lüscher and Malenka, 2011; Beltrán-Campos et al., 2015; Hearing et al., 2018; Hearing, 2019; Puryear et al., 2020).

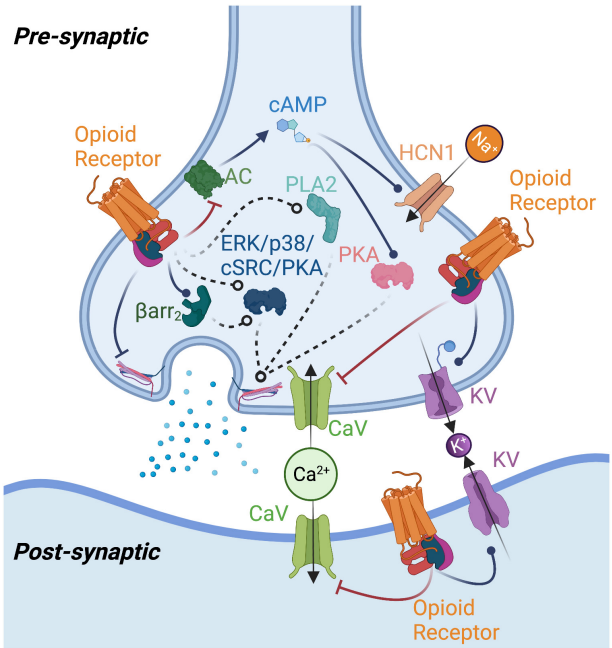

FIGURE 1 | Summary of potential mechanisms of opioid receptor-mediated modulation of neurotransmission. Opioid receptor activation enhances potassium channel (KV) and inhibits calcium channel (CaV) function, reducing neurotransmitter release or producing changes in postsynaptic excitability. Opioid receptors may modulate adenylyl cyclase (AC) function to reduce cAMP levels, thereby impacting protein kinase A (PKA) and type 1 hyperpolarization-activated cyclic nucleotide-gated (HCN1) channel activity. Beta-arrestin2 (Barr2), phospholipase A2 (PLA2), as well as kinases such as p38, ERK, protein kinase C (PKC), and cSrc have been implicated in mediating opioid receptor effects on neurotransmission. Opioid receptor-mediated G protein signaling could also directly affect neurotransmitter release machinery. Figure created with BioRender.com.

The goal of this review is to demonstrate how opioid receptors modulate neurotransmission. While opioid receptors modulate a variety of neurotransmitter systems, we have limited the scope of this review to excitatory (glutamatergic) and inhibitory (often GABAergic) transmission and postsynaptic modulation of neuronal excitability. We have focused on brain regions where much work on opioid receptor-mediated regulation of neurotransmission has been performed. A summary of the literature reviewed below is provided in **Table 1** and illustrated in **Figure 1** as a reference for the reader. **Figure 1** also illustrates how opioid receptors differentially impact neurotransmission pre- and postsynaptically. In this review, we focus on the role of opioid receptors themselves, rather than the impact of opioid drugs on general synapse and brain function. The studies reviewed herein utilized electrophysiology techniques in combination with pharmacological manipulation of opioid receptors. Studies investigating subpopulations within brain regions (i.e., input

regions, cell types, projection targets) have utilized many techniques, including targeted expression of optogenetic tools, tracing strategies, and reporter animal models. We will discuss potential generalizable principles regarding opioid receptor-mediated neuroplasticity, point out broad knowledge gaps, and suggest areas of future research to advance the field, especially as it relates to cell type- and synapse-specific explorations of opioid receptor function.

AMYGDALA

The amygdaloid complex is involved with emotional processing and consists of 13 nuclei, categorized as basolateral (basal, lateral, and accessory basal nuclei; BLA), cortical-like (cortical and lateral olfactory tract nuclei, periamygdaloid complex), centromedial [medial (CeM) and central nuclei (CeA)], bed nucleus of stria terminalis (BNST), or other (intercalated nuclei, anterior amygdala area, amygdalohippocampal area) (Sah et al., 2003). The amygdaloid complex has extensive connectivity across the brain, including local connectivity between amygdala nuclei (Pitkänen et al., 1997). MORs in the amygdala are involved with analgesia, fear and anxiety responses, and social behavior (Good and Westbrook, 1995; Wilson and Junor, 2008; Zhang et al., 2013; Lebow and Chen, 2016). Amygdala DORs play a role in modulating ethanol's effects; however, a functional role of amygdala DORs may not occur until after exposure to drugs of abuse, such as ethanol and morphine (Kang-Park et al., 2007; Bie et al., 2009a,b). Amygdala KORs are involved with anxiety and fear conditioning (Knoll et al., 2011). KOR activation in the amygdala increases anxiety-like behaviors and enhances the rewarding effects of nicotine, possibly due to nicotine's anxiolytic effect (Smith et al., 2012).

Basolateral Amygdala

The basolateral amygdala (BLA) is the primary input region of the amygdaloid complex and receives inputs from across the brain, including hippocampus, nucleus accumbens (NAc), prefrontal cortex (PFC), thalamus, and other amygdala nuclei (Huang et al., 2021). In the lateral nucleus, MORs hyperpolarize about 50% of neurons (Sugita and North, 1993). However, a later study found MORs do not directly hyperpolarize BLA neurons, but the activity of BLA neurons is modulated by presynaptic MORs (Blaesse et al., 2015). In the lateral nucleus, MORs and DORs presynaptically inhibit GABAergic input (Sugita and North, 1993). A later study found that MOR enhances voltage-gated potassium channel (Kv) 1.2 currents and enhances action potential (AP) spike adaptation *via* G protein PLA2 signaling in lateral amygdala (Faber and Sah, 2004). MetEnk inhibits GABAergic input to the BLA from intercalated cells, presumably through MORs (Gregoriou et al., 2019). It is unknown whether MORs regulate GABA transmission from other GABAergic inputs. MOR activation reduces GABAergic input to ∼75% of CeA-projecting BLA neurons *via* activation of Kv1.1/1.2 channels. Very few CeA-projecting BLA neurons have glutamate input that is inhibited by MOR activation (Finnegan et al., 2006). On the other hand, MOR activation produces a long-lasting depression of dorsal midline thalamic glutamatergic input to BLA neurons. MOR inhibition of midline thalamic input to BLA neurons is sufficient to reduce feedforward excitation of the CeM (Goedecke et al., 2019). These studies suggest MORs may primarily modulate BLA projections to the centromedial amygdaloid nuclei; however, additional studies are needed investigating MOR modulation of BLA projections to other regions.

Kappa activation in BLA enhances presynaptic GABA transmission in a tetrodotoxin (TTX)-sensitive manner with no effect on postsynaptic responses in adolescent, but not adult rats (Przybysz et al., 2017). KORs have no effect on glutamate transmission in BLA in rats. Further exploration of the effects of KOR activation on GABA transmission in adolescent rats showed that KOR activation has a variable effect on GABA transmission with subsets of cells showing potentiation, no responses, or depression (Varlinskaya et al., 2020). Further research is needed to determine if these subsets represent sub-populations with distinct afferents/efferents. In mice, KOR activation reduces synaptic transmission from the lateral amygdala to the BLA and blocks LTP induction in the BLA (Huge et al., 2009). Overall, these studies demonstrate KORs modulate neurotransmission in the BLA and these effects demonstrate species, age, input, and output specificity.

Bed Nucleus of the Stria Terminalis

MORs presynaptically inhibit GABAergic transmission to Ventral Tegmental Area (VTA)-projecting neurons in the ventrolateral BNST (Dumont and Williams, 2004). It is unknown whether MORs inhibit GABA transmission to non-VTA-projecting BNST neurons. MOR's effect on glutamate transmission in the BNST is also unknown. KORs presynaptically inhibit GABAergic input from CeA to BNST *via* extracellular signal-related kinases (ERK), but not p38 (Li et al., 2012). KOR activation induces presynaptic LTD *via* p38 (not PKA or MAPK) and calcium signaling in BNST at BLA, but not PFC inputs. Despite KOR-mediated inhibition of GABA transmission, the net effect of KOR activation is to reduce AP firing of BNST neurons. This may be caused by KOR-induced inhibition of glutamate transmission in the BNST. KOR inhibits glutamate onto both dynorphin-positive and dynorphin-negative neurons but has a larger effect on dynorphin-positive neurons (Crowley et al., 2016). Overall, presynaptic MORs and KORs modulate neurotransmission in the BNST; however, while KORs inhibit both GABA and glutamate transmission, MORs have only been shown to inhibit GABA transmission.

Centromedial Amygdala

MORs inhibit about 60% of CeA neurons, particularly those with bipolar morphology (Chieng et al., 2006). CeA neurons can be characterized as Type A or B based on the absence or presence of spike accommodation in response to prolonged depolarization current. MORs hyperpolarize a subset of Type A neurons through activation of potassium currents, whereas KORs only hyperpolarize Type B neurons (Zhu and Pan, 2004). Separate subpopulations of MOR-inhibited neurons were also inhibited by KORs or DORs. When the investigators looked at projection

TABLE 1 | Summary of effects of mu (MOR), delta (DOR), and kappa (KOR) opioid receptor activation on neuronal excitability (postsynaptic effects), presynaptic GABA release, and presynaptic glutamate release.

	Postsynaptic effects			Presynaptic GABA			Presynaptic Glutamate		
	MOR	**DOR**	**KOR**	**MOR**	**DOR**	**KOR**	**MOR**	**DOR**	**KOR**
Amygdala									
BLA	−			+		±	±		−
BNST				+		+			±
CeA	±	±	±	+	±	+	+	±	±
MICR	+						±	±	−
Brainstem/Midbrain									
DVM				±			+		
LC	+				+				+
MVN	−	+	−						
NTS	+	−	−				±	+	+
PAG	±		±	+	±	±	+	−	−
Pons	+	−	−	+	+	+	+	−	−
Raphe	±		±	+	−	−	+		+
RVM	±	±	±	±			±		±
SN			+	+	+	+			+
VTA/RMTg	±	±	±	+	±	+	+		+
Cortex									
ACC	±	±		−	+		±	+	
AIC				±	±	±			
mPFC	+				±			±	±
OFC				±					
S1	±	±	±						
Hippocampus									
CA1	±	±	±	±	±		−	−	
CA2					+				
CA3		±	±	+	−	−	±		+
DG	+	+	+	+	+	+	±	−	±
Hypothalamus									
AN	±	±	±	+	±	+	+	−	+
LH						+			
PO	+		+						
PVN				+			+		+
SON	±	±	±	±		±	+		±
VMH	+	−	−				+	−	+
Habenula									
LHb	±			±		+	±		+
Pallidum									
GP	+	−	±	+	±	+			−
EPN			+			+			
VP	+		+	+			+		+
Striatum									
DS	−	−		±	±		±	±	+
NAc				±	±	±	+	±	±
Thalamus									
Thalamus	+	−	±						

+, Identified effects of opioid receptor activation.
–, Identified null effect of opioid receptor activation.
±, Identified effects in a subpopulation of neurons or inconsistent results between studies.
Blanks indicate untested areas. Note that future studies may reveal heterogenous responses to opioid receptor activation where past studies have either observed widespread effects or null effects.

targets they found that MORs hyperpolarize parabrachial nucleus (PBN)-projecting neurons (Chieng et al., 2006). It is possible MOR-sensitive Type A neurons may specifically project to the CeA, although additional studies are needed to confirm this.

MORs appear to play a role in tonically inhibiting GABA release from synaptic terminals in the CeA. *In vivo* opioid exposure can also induce postsynaptic MOR-mediated inhibition of GABA current amplitudes (Kang-Park et al., 2009; Bajo et al., 2011). Specifically, periaqueductal gray (PAG)-projecting CeA neurons receive MOR-sensitive GABAergic input (Finnegan et al., 2005). Additional studies are needed to identify MOR-sensitive GABAergic inputs in the CeA. Like MORs, KOR activation inhibits GABA release in CeA in rats (Przybysz et al., 2017) and KORs may also tonically inhibit GABA release (Gilpin et al., 2014; Bloodgood et al., 2021; Khom et al., 2021). Similarly, DORs also inhibit GABA release in the CeA, but there is evidence for species differences. In one study in mice, DOR activation was shown to reduce GABA release; whereas, in another study in rats, DORs did not have an effect on GABA transmission under normal conditions, but gained the ability to do so in ethanol-treated rats (Kang-Park et al., 2007; Bie et al., 2009a). Similar to the CeA, MORs inhibit GABA transmission in the CeM; MetEnk, presumably through MORs, inhibits GABergic input from the nearby intercalated cell region of the amygdala (Gregoriou et al., 2019). MORs on the intercalated cells prevent feedforward inhibition from the BLA to the CeM (Blaesse et al., 2015). Future studies are needed to determine whether KORs or DORs inhibit GABA transmission in the CeM.

In contrast to opioid receptor-mediated effects on GABA transmission, MOR, but not DOR or KOR, activation reduces glutamate input in the CeA but not CeM (Zhu and Pan, 2005; Blaesse et al., 2015). Specifically, a small subpopulation of PAG-projecting neurons in the CeA receive MOR-sensitive glutamate input (Finnegan et al., 2005). A later study determined that MORs inhibit glutamate input to CeA neurons from the parabrachial nucleus and BLA (Kissiwaa et al., 2020), but another study found MORs do not inhibit BLA inputs to CeM neurons (Blaesse et al., 2015). Another study found MOR activation produces a transient depression of dorsal midline thalamic glutamatergic input to CeA neurons (Goedecke et al., 2019). Similar to some studies of CeA GABA transmission, DOR-mediated inhibition of glutamate release may be inducible (Bie et al., 2009a,b). A subset of BLA inputs are dually regulated by KORs and DORs, suggesting that there may be some CeA synapses that are sensitive to KORs and DORs that may not be distinguished when glutamate transmission is probed more broadly, as done previously (Zhu and Pan, 2005; Kissiwaa et al., 2020). In CeA neurons, direct parabrachial glutamatergic input to corticotropin-releasing factor (CRF) neurons is insensitive to KORs; however, KOR activation presynaptically inhibits local GABA neurons that receive parabrachial glutamatergic input, resulting in disinhibition of the CRF neurons (Hein et al., 2021).

Medial Intercalated Cell Region

GABAergic neurons of the medial island of intercalated cells send inhibitory projections to the BLA and CeM. MORs hyperpolarize these neurons in both rats and mice (Blaesse et al., 2015; Winters et al., 2017). In rats, both MOR and DOR, but not KOR, activation can reduce glutamate release from BLA inputs to intercalated neurons. Endogenous opioid peptide release in the intercalated cell region produces presynaptic inhibition of glutamate release *via* DORs and postsynaptic hyperpolarization *via* MORs (Winters et al., 2017). On the other hand, one study found that MORs do not inhibit glutamate input from BLA to the medial intercalated cell region in mice, suggesting possible species differences (Blaesse et al., 2015). MORs also inhibit GABA transmission to intercalated neurons in rats. Direct MOR activation *via* exogenous agonist application greatly decreases local GABA transmission, although endogenous opioid peptide release has only a minor effect on this inhibitory transmission (Winters et al., 2017).

BRAINSTEM AND MIDBRAIN

The brainstem connects the cerebrum to the spinal cord and cerebellum. It regulates respiration, consciousness, blood pressure, heart rate, and sleep (Angeles Fernández-Gil et al., 2010). The midbrain plays key roles in sensory and motor control and has received much attention for its role in reward processing and decision making (Ruchalski and Hathout, 2012). Brainstem and midbrain express the three opioid receptors (Mansour et al., 1987; Le Merrer et al., 2009) and play major roles in drug reward, pain, and respiration (Le Merrer et al., 2009; Dahan et al., 2018; Bagley and Ingram, 2020).

Dorsal Motor Nucleus of the Vagus

MOR activation presynaptically inhibits glutamate input, but not GABAergic input, consistent with MOR expression in terminals of glutamate, but not GABA neurons of the Dorsal Motor Nucleus of the Vagus (DVM) (Browning et al., 2002). Under normal conditions, opioid agonists fail to influence GABAergic input to these neurons; however, when cAMP signaling is engaged, MOR is trafficked to the synapse and inhibits GABA transmission. This is inhibited by disrupting cAMP and PKA signaling, suggesting that the cAMP-PKA pathway regulates trafficking of MORs into the cell surface of GABAergic nerve terminals (Browning et al., 2004). Conversely, another study found that MOR activation reduces both AP-dependent glutamate and GABA transmission in rat and mouse DVM GABA neurons. MOR activation reduces GABAergic input to DVM neurons from the nucleus of the solitary tract (NTS), potentially due to MORs on the NTS neurons (Glatzer et al., 2007). These data suggest that opioid actions may depend on the state of activation of vagal circuits.

Locus Coeruleus

The Locus Coeruleus (LC) has a long history of studies of the impact of opioid receptor-mediated regulation of cellular function due to its high expression of MORs that inhibit LC neuron excitability (Bird and Kuhar, 1977). Recording opioid effects on ion channel function in these neurons is a common methodology for exploring opioid receptor signaling and testing hypotheses regarding receptor desensitization and

opioid tolerance (for review, see Allouche et al., 2014). However, a detailed discussion of the many studies of opioid receptor desensitization and tolerance in the LC are beyond the scope of this review. In addition to MOR-mediated regulation of LC neuron excitability, KORs also function in the LC to inhibit glutamate input to LC neurons without affecting postsynaptic currents/membrane potential (McFadzean et al., 1987; Pinnock, 1992b). Local KORs within the LC are targeted by dynorphinergic neurons from other brain regions (Al-Hasani et al., 2013). LC neurons that project to the spinal cord are excited by DOR agonists *via* inhibition of presynaptic DORs on GABAergic inputs, but without an effect on glutamate input (Pan et al., 2002).

Nucleus of the Solitary Tract

MOR, but not KOR or DOR, agonists hyperpolarize neurons in the medial, dorsomedial and dorsolateral regions of the NTS through increasing potassium conductance (Rhim et al., 1993; Glatzer et al., 2007; Poole et al., 2007). In addition to increasing potassium conductance in these neurons, MORs are able to inhibit N- and P/Q-type voltage-gated calcium channels (VGCCs) in NTS neurons (Rhim et al., 1996; Endoh, 2006). While KORs were not found to hyperpolarize neurons, KORs and MORs were found to inhibit N- and P/Q-type, but not L-type VGCCs *via* $G_{\beta\gamma}$, but not PKA signaling (Rhim et al., 1993; Endoh, 2006). These data suggest that opioid receptors use different pathways to induce inhibition in the NTS.

MORs also inhibit synaptic transmission in the NTS. Presynaptic MORs reduce inhibitory input to NTS GABA neurons from solitary tract stimulation (Glatzer et al., 2007). Within the medial NTS, MOR activation blocks tonic GABA currents and reduces GABA release (Herman et al., 2012). Another study found that MOR-mediated local inhibition of GABA transmission was AP-dependent, suggesting MORs on cell bodies may modulate local GABA neurons (Glatzer and Smith, 2005).

Solitary tract glutamatergic input to NTS neurons is inhibited strongly by MOR and weakly by DOR and KOR agonists (Rhim et al., 1993; Glatzer and Smith, 2005; Poole et al., 2007; Boxwell et al., 2013). MOR inhibition is presynaptically localized (Glatzer and Smith, 2005). MORs equally inhibit solitary tract glutamate input to both GABAergic and non-GABAergic NTS neurons (Boxwell et al., 2013). Interestingly, MOR activation is less efficacious when GABA and glycine receptors are blocked (Boxwell et al., 2013). One study specifically recorded from NTS pro-opiomelanocortin (POMC) neurons and found that glutamate input was presynaptically regulated by MORs (Appleyard et al., 2005). On the other hand, in recordings from NTS neurons that project specifically to the PBN, DORs, but not MORs, inhibited solitary tract glutamatergic inputs (Zhu et al., 2009). One study specifically looked at tyrosine hydroxylase (TH)-positive and TH-negative neurons of the NTS (Cui et al., 2012). Like other studies they found that MORs presynaptically inhibited solitary tract input to both of these classes of neurons, but the effect was larger in TH-positive neurons. These data suggest that presynaptically expressed opioid receptors may differentially affect neurotransmitter release.

Periaqueductal Gray

The PAG is a hot spot for opioid signaling in the brain. MORs hyperpolarize and activate G protein-couple inwardly rectifying potassium channels (GIRKs) in a subpopulation of neurons within the PAG, mostly in lateral and dorsal regions of ventrolateral PAG (vlPAG) (Chieng and Christie, 1994; Vaughan and Christie, 1997; Chiou and Huang, 1999; Vaughan et al., 2003; Chen et al., 2016). Some report that KORs have no effect on GIRK in rat PAG, while the same group report that they do in mice (Chieng and Christie, 1994; Vaughan et al., 2003), suggesting that the animal model used for studying the opioid receptor effects is important. MOR inhibits about half of lateral rostral ventromedial medulla (RVM)-projecting PAG neurons and less than a quarter of RVM-projecting vlPAG neurons through activating an outward current (Osborne et al., 1996). An investigation of the specific responses within different types of PAG neurons shows that MOR activation hyperpolarizes ventral PAG GABA neurons and reduces AP firing (Chen et al., 2016). In serotonergic (5-HT) neurons however, MOR activation hyperpolarizes the neurons but enhances AP firing. In addition to their effects on GIRKs, MORs, but not DORs or KORs, inhibit calcium channels in PAG neurons (Kim et al., 1997; Connor et al., 1999). Some CeA inputs to ventrolateral PAG are sensitive to MOR and DOR activation, responding with both excitation (20% of responses) and inhibition (25% of responses). The identities and types of responses are not clear from this study (da Costa Gomez and Behbehani, 1995). This could be due to changes in neuronal excitability described above or changes in synaptic function described below.

MORs, but not KORs or DORs, presynaptically inhibit glutamate transmission to some degree in all regions of the PAG (Vaughan and Christie, 1997; Chiou and Huang, 1999). Looking at identified cellular targets, MOR decreases glutamate input to both GABA and 5-HT neurons (Chen et al., 2016). MORs presynaptically regulate GABA in all regions of the PAG to both GABAergic and 5-HT neurons, through presynaptic activation of potassium channels and PLA2 (Vaughan and Christie, 1997; Vaughan et al., 1997; Chen et al., 2016). MORs also inhibit GABA input to ventral PAG TH-expressing neurons that project to the BNST and co-release dopamine and glutamate (Li et al., 2016). Interestingly, this involves a short-term reduction in GABA release accompanied by a more persistent inhibition of GABA transmission *via* a postsynaptic mechanism. Regarding other opioid receptors that modulate GABA transmission, there may be species differences. In rats, only MORs inhibit GABA release; whereas, in mice, KORs, but not DORs, also inhibit GABA release (Vaughan et al., 2003; Li and Kash, 2019). MORs inhibit GABA input to a greater extent than glutamate input in the PAG. The greater inhibition of GABA input overcomes MOR's effects on glutamate input, as well as hyperpolarization, to increase AP firing of ventral PAG neurons (Chiou and Huang, 1999). The ability of DORs to inhibit GABA release in the PAG is plastic. DOR agonists have no effect on PAG GABAergic transmission in naïve mice but may be induced

to do so with chronic morphine treatment (Vaughan et al., 2003; Hack et al., 2005). DOR activation may also inhibit GABA reuptake *via* GABA transporter type 1 in the PAG (Pu et al., 2012). Overall, presynaptic opioid receptors modulate neurotransmission in the PAG; however, while MORs and KORs inhibit GABA, only MORs inhibit glutamate transmission. DORs have only been shown to inhibit GABA transmission, likely using a different mechanism.

Raphe Nuclei

There are two types of cells in the nucleus raphe magnus (NRM) that have differential responses to opioids. Primary 5-HT neurons are hyperpolarized *via* KOR-mediated GIRK activation (Pan et al., 1997; Li and Wang, 2001). Secondary GABAergic neurons are hyperpolarized by MORs also *via* GIRK activation (Pan et al., 1997; Li and Wang, 2001). MORs disinhibit primary cells through inhibiting GABA input to these KOR-sensitive cells (Pan et al., 1997). KORs also presynaptically inhibit glutamate input to both primary and secondary NRM cells (Bie and Pan, 2003).

MOR activation hyperpolarizes around 80% of non-5-HT DRN neurons and around 30% of 5-HT neurons, likely through enhancing potassium conductance. MOR activation reduces spontaneous GABA release and NMDA-induced activation of GABA release from local neurons, as well as neurons in the PAG onto 5-HT DRN neurons. As in the PAG, MORs also inhibit GABAergic input to DRN TH-expressing dopaminergic/glutamatergic neurons that project to BNST (Li et al., 2016). DOR and KOR activation have no effect on GABA transmission in these cells. One study found that MOR activation has no effect on glutamate input to 5-HT cells; whereas, a later study found that MORs are able to inhibit glutamate release and suggested this was due to experimental conditions (Pinnock, 1992a; Jolas and Aghajanian, 1997). In the positive study, they found that MORs were able to inhibit local glutamate release as well as glutamate input from the PAG (Jolas and Aghajanian, 1997). KORs are also able to inhibit glutamate input to DRN 5-HT neurons (Pinnock, 1992a). Therefore, MOR and KOR are capable of inhibiting both GABA and glutamate release, however up to the present time there is no evidence that DORs have a role in the Raphe nuclei.

Rostral Ventromedial Medulla

In RVM there are three different cell types that show differential responses to noxious stimuli: ON cells increase firing, OFF cells decrease firing, and NEUTRAL cells show no responses (Sikandar and Dickenson, 2011). MORs and DORs inhibit ON cell responses, increase activity of OFF cells, and have no effect on NEUTRAL cells (Cheng et al., 1986; Harasawa et al., 2000). MOR activation in RVM directly inhibits ON cells. In OFF cells, there are no effects of direct MOR agonist application, suggesting that opioid-mediated excitation of OFF cells is indirect (Heinricher et al., 1992, 1994).

In measures of direct cellular responses, there are two major cell types in RVM that respond to opioids: primary cells and secondary cells. Primary cells have a wider action potential, more negative resting membrane potential, and are not inhibited by MOR agonists. Secondary cells are generally presumed to be inhibitory interneurons that serve only to regulate the activity of the output neurons, have a shorter action potential, are often firing spontaneously, and are mostly hyperpolarized by MOR agonists (Pan et al., 2000; Cleary et al., 2008). Also, primary cells are responsive to KOR activation, producing outward currents (Pan et al., 1990, 2000). Subpopulations of secondary cells are responsive to MOR activation, also producing outward currents. Almost all spinally projecting RVM neurons respond to opioids in some fashion. Subpopulations of these neurons show outward current responses to either only MOR, only KOR, or both receptor activations (Marinelli et al., 2002). Interestingly, MOR responsive secondary cells are similar to ON cells *in vivo*, and KOR responsive primary cells are similar to OFF cells (Pan et al., 1990). Non-5-HT spinally projecting neurons are almost exclusively MOR responders; whereas, 5-HT neurons have equal proportions of MOR, KOR, and MOR/KOR responders (Marinelli et al., 2002; Zhang et al., 2006; Zhang and Hammond, 2010). About two thirds of TH-expressing and TH-negative bulbospinal neurons are hyperpolarized by MOR *via* GIRK activation (Hayar and Guyenet, 1998). DORs produce outward currents in subpopulations of RVM neurons (Marinelli et al., 2005). They specifically act in a subpopulation of MOR-regulated non-5-HT spinal cord-projecting neurons, as well as subpopulations of 5-HT spinal cord-projecting neurons that have differential sensitivities to MOR and KOR activation.

MOR activation reduces GABA, but not glutamate input to primary cells (Pan et al., 1990, 2000). MOR activation reduces GABA input likely *via* inhibition of presynaptic calcium channels, but not glutamate input to RVM neurons; however, it is not clear whether these are primary or secondary neurons due to the recording conditions (Vaughan et al., 2001). Glutamatergic input to secondary cells is presynaptically inhibited by KORs (Ackley et al., 2001). MORs inhibit GABA and glutamate input to bulbospinal TH-expressing and TH-negative neurons through presynaptic mechanisms (Hayar and Guyenet, 1998). In spinal cord-projecting rat RVM neurons MORs inhibit evoked glutamate inputs in ∼50% of cells, miniature excitatory postsynaptic currents (mEPSCs) in 55% of cells, evoked inhibitory inputs in about 70% of cells, and miniature inhibitory postsynaptic currents (mIPSCs) in 100% of cells (Finnegan et al., 2004). MORs agonists frequently activate output neurons in the brain *via* disinhibition. Thus, direct inhibition of "secondary cells" disinhibits "primary cells" or output neurons, allowing them to become active (Cleary et al., 2008).

Substantia Nigra

MORs, DORs, and KORs, have all been reported to modulate substantia nigra GABA release (Starr, 1985). KOR activation presynaptically inhibits glutamate transmission in Substantia Nigra (SN) pars reticulata (Maneuf et al., 1995). KORs can inhibit type-2 dopamine receptor (D2R)-mediated IPSCs in dopamine neurons of the SN pars compacta (Ford et al., 2007). The mechanism is unclear, given that KORs can both hyperpolarize and prevent IPSCs in the same neuron and this is not due to modulation of cAMP, kinases, calcium, or potassium channels. Overall, opioid receptors may play a role in regulating neurotransmitter release, however, more research

is needed to clarify the specific actions of each of the different opioid receptors.

Ventral Tegmental Area and Rostromedial Tegmental Nucleus

MORs hyperpolarize local GABA neurons within VTA, but not dopamine neurons, leading to greater excitation of dopamine neurons (Johnson and North, 1992). MORs can hyperpolarize secondary VTA cells, that are largely GABAergic as well as tertiary VTA cells that are NAc-projection neurons (Cameron et al., 1997). MOR-induced hyperpolarization of local GABAergic neurons rapidly desensitizes (Lowe and Bailey, 2015). In the Rostromedial Tegmental Nucleus (RMTg), also known as the tail of the VTA, neuron firing rate is reduced by MOR activation and RMTg neurons are hyperpolarized by MOR agonists, but not DOR or KOR (Lecca et al., 2011; Matsui and Williams, 2011). Contrary to other studies that find that MORs do not hyperpolarize dopamine neurons, there may be some dopamine neurons that express MORs. MORs can hyperpolarize some VTA dopamine neurons *via* increasing potassium conductance or exciting them *via* P/Q type calcium channel (Cav2.1) inhibition (Margolis et al., 2014, 2017). DPDPE-sensitive and deltorphin II-sensitive DORs are differentially expressed in different types of VTA neurons and produce a heterogeneous response: hyperpolarizing neurons *via* increasing potassium conductance or exciting neurons *via* Cav2.1, similar to MOR (Margolis et al., 2017). Interactions between the two different functional forms of DOR and MOR is not consistent between neurons, although receptor antagonist experiments reveal that functional interactions between the two different receptors do occur. KORs hyperpolarize VTA dopamine neurons *via* increasing potassium conductance (Margolis et al., 2003; Ford et al., 2007). Interestingly, only a subset of these neurons are disinhibited by MOR activation. KORs hyperpolarize VTA neurons that project to medial PFC (mPFC), but not to NAc (Margolis et al., 2006). Consistent with this, infusion of KOR agonist into VTA decreases dopamine levels in the mPFC, but not the NAc. Amygdala-projecting dopamine neurons within the VTA are also hyperpolarized by KOR activation (Margolis et al., 2008b). VTA dopaminergic neurons that project to NAc are more inhibited by KOR activation that produces outward currents (Ford et al., 2006). In contrast, VTA neurons that project to BLA (which are mostly dopaminergic) are more inhibited by MOR activation, also producing outward currents (Ford et al., 2006).

MORs reduce GABA transmission in VTA *via* inhibition of GABA release (Bergevin et al., 2002; Xiao and Ye, 2008; Matsui et al., 2014; Bull et al., 2017). MOR activation silences GABAergic VTA neuron firing and reduces evoked and spontaneous TTX-sensitive GABA release (Xiao and Ye, 2008). Knockout of MORs from NAc medium spiny neurons (MSN) reduces the ability of MORs to inhibit GABA input to local VTA GABA interneurons in VTA (Charbogne et al., 2017). Mechanisms for MOR-mediated GABA release inhibition implicate presynaptic potassium channels, beta-arrestin2, and proto-oncogene tyrosine-protein kinase Src (Bergevin et al., 2002; Bull et al., 2017). Contrary to postsynaptic MOR effects, presynaptic MORs on GABA terminals are resistant to desensitization, except when PKC is activated (Lowe and Bailey, 2015). MetEnk, presumably though MOR activation, reduces GABAergic input equally onto NAc- and BLA-projecting dopamine neurons (Ford et al., 2006). MOR regulates VTA GABAergic transmission at local interneuron synapses as well as at GABAergic inputs from the NAc, PAG, RMTg, and ventral pallidum (Matsui and Williams, 2011; Xia et al., 2011; Matsui et al., 2014; St Laurent et al., 2020). Comparing inputs to VTA dopamine neurons, one study found that MOR activation produces the greatest inhibition RMTg inputs, with very low inhibition of local interneuron input and moderate inhibition of NAc inputs (Matsui et al., 2014). A different study however concluded that MOR-modulated NAc inputs to VTA targeted VTA GABA neurons and not VTA dopamine neurons (Xia et al., 2011). MORs inhibit GABAergic input from the ventral pallidum onto both dopamine and non-dopamine neurons (Hjelmstad et al., 2013). Various forms of GABAergic plasticity occur at many of these synapses. Inhibitory LTD at RMTg-VTA dopamine neuron synapses occurs independently of MOR activation, however LTP at PAG-VTA neuron synapses is blocked by MOR activation (St Laurent et al., 2020). A variety of *in vivo* drug exposures and painful conditions shift the ability of MORs to regulate VTA GABA transmission (Shoji et al., 1999; Margolis et al., 2008a; Xiao and Ye, 2008; Guan and Ye, 2010; Madhavan et al., 2010; Graziane et al., 2013; Polter et al., 2014; Hipolito et al., 2015).

MORs and KORs non-occlusively reduce GABA input to VTA dopamine neurons (Shoji et al., 1999). GABAergic inputs from RMTg to VTA dopamine neurons are insensitive to KOR activation (Matsui and Williams, 2011). KOR activation has little effect on fast, $GABA_A$-mediated IPSCs recorded in NAc-projecting cells, but inhibits fast, $GABA_A$-mediated IPSCs in BLA-projecting cells (Ford et al., 2006). On the other hand, KOR activation inhibits $GABA_B$-mediated slow IPSCs: KORs inhibit GABAergic input to both BLA- and NAc-projecting cells, but this effect is stronger in NAc-projecting cells.

There is a minor role for DORs in regulating VTA GABA transmission under normal conditions, but as in other brain regions, DOR-mediated inhibition of GABA transmission is inducible by *in vivo* drug exposure (Margolis et al., 2008a; Mitchell et al., 2012; Bull et al., 2017). Following stress exposure, DORs gain the ability to produce postsynaptic insertion of $GABA_A$ receptors in a subset of neurons, *via* phosphoinositide 3-kinase (PI3K) and Akt signaling (Margolis et al., 2011). DORs do not regulate RMTg GABA synaptic inputs (Matsui and Williams, 2011).

Presynaptic MOR activation in VTA reduces glutamate transmission onto dopamine and non-dopamine neurons (Bonci and Malenka, 1999; Manzoni and Williams, 1999). In principal VTA neurons, which are primarily dopaminergic, KOR activation produces a small inhibition of glutamate input, whereas MORs produce a larger inhibition; these are non-occlusive indicating inhibition of separate populations of inputs (Margolis et al., 2005). In secondary neurons, KORs and MORs produce similar inhibition of glutamate input and the responses to each receptor activation are positively correlated. In tertiary neurons, of which a small percentage are dopaminergic, KOR and MORs similarly

inhibit glutamate input, but the magnitudes of inhibition are not correlated when dually tested in each cell. These effects are largely presynaptic, although neurons with postsynaptic KOR effects are more sensitive to MOR inhibition of glutamate input and vice versa (Margolis et al., 2005). MORs also inhibit glutamate input to RMTg neurons (Lecca et al., 2011). The LTP_{GABA} described above can be acutely blocked by glutamatergic presynaptic MOR activation, removing the glutamate necessary for plasticity induction (Nugent et al., 2007). The role of MOR-mediated regulation of glutamate as part of the local VTA microcircuit is important to not overlook. For example, in order for morphine to activate VTA dopamine neurons, there must be a VTA glutamatergic tone for MOR-mediated inhibition of RMTg inputs to have an effect (Jalabert et al., 2011).

Altogether, these studies indicate that opioid receptor activation has a broad effect on the VTA, targeting GABA, glutamate and dopamine transmission. Therefore, VTA opioid receptors have a key clinical relevance on the control of dopamine modulation. Although there has been much investigation of opioid receptor function in VTA, there is certainly more discover regarding the cell type- and synapse-specific function of the different opioid receptors in the VTA.

CORTEX

The cortex is involved with many higher functions, including planning, processing sensory information, memory, decision making, and emotional processing (Lamotte et al., 2021; Nadeau, 2021; Kolk and Rakic, 2022). All three opioid receptors are found in the cortex; the presence, modulation of neural activity, and behavioral role of cortical opioid receptors varies across different cortical areas, and these are involved with analgesia, morphine-induced locomotor sensitization, reducing anxiety, and with the rewarding and locomotor stimulation effects of opioids (Saitoh et al., 2018; Wang et al., 2020; Jiang et al., 2021).

Many early studies of opioid receptor responses in cortex failed to identify which specific cortical regions were being explored or looked across regions non-specifically. In rat cortical brain slices MOR, DOR, and KOR agonists inhibit evoked glutamate and GABA release (Bradford et al., 1986). In addition, extracellular recordings show that MOR, DOR, and KOR agonists reduce glutamate-evoked neuronal firing (Janiri et al., 1988). However, in contrast, potassium-evoked glutamate release in rat cerebral cortex brain slices is inhibited by MOR and KOR agonists, but not DOR agonists (Nicol et al., 1996). Cultured mouse neocortical neurons express postsynaptic MORs that co-localize with AMPARs (Liao et al., 2005). Activation of these MORs inhibits glutamate transmission and induces dendritic spine retraction. Similarly, morphine inhibits glutamate release from cortical synaptosomes *via* inhibition of voltage-gated calcium channels (Yang et al., 2004). GABAergic cortical interneurons are inhibited by MORs *via* membrane hyperpolarization through increased potassium conductance (Ferezou et al., 2007). Unlike cortical GABAergic interneurons, MOR mRNA was not found in pyramidal neurons and MOR activation had no postsynaptic effects in these neurons. There was nearly a complete overlap in interneurons that responded to DAMGO and to nicotinic acetylcholine receptor (nAChR) agonist, DMPP. nAChR activation induced AP firing in interneurons and IPSCs in pyramidal neurons that were both inhibited by MOR activation. nAChR-induced GABAergic input to pyramidal cells was multiphasic, with an initial increase in IPSCs and a subsequent decrease below baseline levels. The decrease was blocked by a MOR antagonist, suggesting that nAChR activation induces enkephalin release as a form of feedback control.

Anterior Cingulate Cortex

The Anterior Cingulate Cortex (ACC) is involved with emotion and reward processing, learning, and memory (Rolls, 2019). Met-Enk inhibits spontaneous, acetylcholine-evoked, and glutamate-evoked neuronal activity in the ACC (Palmer et al., 1978). In a subset of rat layer 5 ACC pyramidal neurons, DOR, but not MOR, activation produces direct hyperpolarization, presumably through a postsynaptic increase in potassium conductance (Tanaka and North, 1994). In comparison, MOR, but not DOR, activation hyperpolarizes a subset of non-pyramidal neurons. Met-Enk inhibits glutamate and GABA transmission in ACC neurons. This effect is mimicked by DOR, but not MOR agonist, suggesting the effect is mediated by DORs. However, a later study found MORs specifically inhibit midline thalamus inputs to layers 2/3 and layer 5 anterior cingulate cortex pyramidal neurons and parvalbumin (PV)-expressing interneurons. DORs inhibit interneurons that receive MOR-positive medial thalamic input to regulate feedforward inhibition to pyramidal neurons. Ultimately, DORs function to disinhibit thalamocortical circuits (Birdsong et al., 2019).

Insular Cortex

The insular cortex is involved with interoception, emotion, cognition, and motivation (Namkung et al., 2017). Anterior agranular insular cortex GABAergic neurons express KORs that function to disinhibit L5 pyramidal cell inputs to the SN (Pina et al., 2020). Dynorphin decreases GABA release, but increases glutamate release, leading to disinhibition. In L5 of rat insular cortex, paired recordings between nearby GABA neurons and other GABA neurons or pyramidal cells revealed the role of MOR in regulating these synapses (Yokota et al., 2016). MOR activation reduces fast-spiking interneurons (FSI) input to other FSIs, but not to pyramidal neurons. MOR activation also reduced GABAergic input to FSIs from non-FSI neurons. In contrast, DOR activation reduced FSI input to both other FSIs and pyramidal neurons but had no effect on inhibitory transmission from non-FSI GABA neurons. All inhibition is presynaptically localized. KOR activation has no impact on FSI inputs to other insular cortex neurons.

Medial Prefrontal Cortex

The Medial Prefrontal Cortex (mPFC) is involved with many cognitive functions and is comprised primarily of excitatory pyramidal neurons and a smaller population of inhibitory interneurons (Xu et al., 2019). MORs inhibit both non-pyramidal and pyramidal mPFC neurons, but through different

mechanisms. In non-pyramidal neurons, MORs inhibit sodium conductance through a G protein, PKA, and PKC pathway (Witkowski and Szulczyk, 2006). In pyramidal neurons, MORs inhibit N-type VGCCs through a cAMP-PKA pathway (Rola et al., 2008). DORs can both inhibit and disinhibit pyramidal neuron activation. Presynaptic DORs inhibit prelimbic mPFC principal neurons through inhibiting glutamate release onto these neurons (Yamada et al., 2021). On the other hand, DORs increase GABA transmission from somatostatin-expressing interneurons to PV-expressing interneurons, which disinhibits pyramidal neurons, which MORs do not do (Jiang et al., 2021). KORs also inhibit neurotransmission in mPFC. KOR activation reduces glutamate release onto mPFC pyramidal neurons (Tejeda et al., 2013). Specifically, BLA glutamatergic inputs to mPFC are inhibited by KOR activation in *in vivo* extracellular recordings in anesthetized rodents (Tejeda et al., 2015).

Orbitofrontal Cortex

MORs presynaptically inhibit GABA release onto pyramidal neurons of the rat ventrolateral Orbitofrontal Cortex (OFC) (Qu et al., 2015), consistent with identified expression of MOR in these GABA cells (Huo et al., 2005). MOR-LTD of presynaptic FSI PV-expressing neurons inhibit GABAergic input to pyramidal neurons of medial, but not lateral OFC. Stimulating cAMP production shifts MOR activation to produce short-term depression rather than LTD. Endogenous opioid LTD can be induced *via* moderate frequency stimulation in the presence of peptidase inhibitors, but not low frequency stimulation (Lau et al., 2020).

Sensorimotor Cortices

MetEnk and LeuEnk inhibit a subset of sensorimotor cortical neurons, some of which are hyperpolarized by MOR agonists (Stanzione et al., 1989). In the somatosensory cortex, MORs and DORs inhibit spontaneous neuronal firing and glutamate-induced firing activity. In a subset of cells, dynorphin inhibits firing and in some recordings where dynorphin had little effect alone, it attenuated the effects of MOR and DOR activation (Janiri et al., 1988).

Overall, these studies indicate opioid receptor effects on neurotransmission and neural activity within cortical areas show great diversity across region, cell type, and neural pathways. As discussed, in some cortical regions, opioid receptor effects have been shown to occur *via* different mechanisms than in other regions. Additional studies are needed to evaluate circuit-specific opioid receptor regulation of neurotransmission throughout the cortex in order to more fully understand the impact of opioids on higher brain function.

HIPPOCAMPUS

The hippocampus is a brain region crucial to facilitating memory, learning, and spatial processing (Bird and Burgess, 2008). All three opioid receptors are heterogeneously distributed throughout the entire hippocampus and are regulated by the endogenous opioids dynorphin and enkephalin (Simmons and Chavkin, 1996).

CA1

In measures of population spike (PS) amplitudes in CA1, both MOR and DOR enhance amplitudes in CA1 (Lee, 1978; Dunwiddie et al., 1980; French and Zieglgansberger, 1982; Valentino and Dingledine, 1982; Dingledine et al., 1983; Bostock et al., 1984; Vidal et al., 1984; Dunwiddie and Su, 1988; Neumaier et al., 1988; Moudy et al., 1989; Wimpey et al., 1989; Pieretti et al., 1994). Morphine increases hippocampal activity in CA1 in slice and in freely moving animals (Linseman and Corrigall, 1982). MORs, but not DORs or KORs, increase the duration of CA1 field potentials (Pieretti et al., 1994). The timing of MOR activation can also determine whether it can enhance CA1 function. MOR activation prevents the inhibitory effects of temporo-ammonic pathway stimulation on Schaffer collateral inputs to CA1 when the timing of stimulation of the two pathways was further apart than one theta cycle, but had no effect when timing was less than one theta cycle (McQuiston, 2011).

The effects of MOR and DOR activation are likely not due to effects on pyramidal cells themselves, although KORs might have some effects on pyramidal cell potassium currents (Madamba et al., 1999). Rather, opioid receptor-induced enhancement of population spike amplitudes is due to disinhibitory mechanisms (Zieglgansberger et al., 1979; Corrigall and Linseman, 1980; Dunwiddie et al., 1980; Neumaier et al., 1988; Lupica and Dunwiddie, 1991; Miller and Lupica, 1994; McQuiston, 2007, 2008; Tian et al., 2015). Specifically, opioids hyperpolarize GABAergic interneurons within CA1 and reduce GABA input to pyramidal neurons (Madison and Nicoll, 1988; Lupica and Dunwiddie, 1991; Lupica et al., 1992; Lupica, 1995; Capogna et al., 1996; Lafourcade and Alger, 2008; Krook-Magnuson et al., 2011; Banghart et al., 2018; Fan et al., 2019). Although DORs can inhibit GABA transmission, they do not appear to be the primary mediators of these effects (Watson and Lanthorn, 1993; Lupica, 1995). MORs can reduce feedforward and feedback inhibition, whereas DORs do not. However, both MORs and DORs are able to inhibit spontaneous GABA transmission, but not monosynaptic inhibitory postsynaptic potentials (IPSPs) (Lupica et al., 1992). However, some of the complexity may be attributable to how MORs and DORs individually regulate GABA transmission in local circuits. In CA1, MORs inhibit interneuron input to the soma, whereas DORs inhibit input to dendrites of pyramidal neurons (Svoboda et al., 1999). In support of this, one study showed that MORs inhibit FSI GABA, but not regular spiking GABA basket cell input to CA1 pyramidal neurons (Glickfeld et al., 2008; Shao et al., 2020). However, a very recent study showed that both MORs and DORs independently activate GIRK in PV neurons as well as inhibit GABA release on to pyramidal neurons (He et al., 2021). MORs hyperpolarize FSI basket cell neurons, but not regular spiking basket cell neurons. FSIs typically synapse on to somas, whereas regular spiking neurons synapse on to dendrites (Straub et al., 2016). MORs also inhibit neuropeptide Y (NPY)-expressing neurogliaform interneurons through membrane hyperpolarization (Krook-Magnuson et al.,

2011). In addition, MOR specifically reduces tonic firing of the Ivy class of neurogliaform cells in CA1, reducing GABAergic input to pyramidal neurons (Krook-Magnuson et al., 2011).

The ability of MORs and DORs to disinhibit CA1 pyramidal cell function can be pathway and layer specific and may explain some of the confusing results regarding DOR activation and the broader effect of MOR activation. MORs, but not DORs, mediate feedforward inhibition from Schaffer collateral input (Rezai et al., 2013). However, DORs are expressed in interneurons within CA1 that receive input from the temporo-ammonic pathway, but not the Schaffer collateral pathway. Both MOR and DOR mediate feedforward inhibition from the temporo-ammonic pathway. While MOR enhances excitatory transmission in all layers, it is most effective at enhancing propagation through CA1 output layers (McQuiston, 2007, 2008). Stimulating CA2 pyramidal neuron input to CA1, MOR activation prevented feedforward inhibition of CA1 pyramidal neurons in deep layer and excitatory radiatum giant cells layers, but not pyramidal neurons in superficial layers through its inhibition of FSI interneurons (Nasrallah et al., 2019). MORs can also enhance excitation of pyramidal cells through enhancing excitatory responses to acetylcholine receptor activation (Kearns et al., 2001). Along with this, MOR activation can inhibit cholinergic receptor-induced cholecystokinin-expressing basket cell-mediated theta oscillations in CA1 (Nagode et al., 2014).

Opioid receptors can also have an inhibitory effect on CA1 function. LTD in CA1 is blocked by naloxone and enhanced by MOR, but not DOR or KOR activation (Francesconi et al., 1997; Wagner et al., 2001). Prior fentanyl exposure enhances LTD expression in CA1 as well (Tian et al., 2015).

CA2

KOR and DOR activation in CA2 increases the PS following stratum radiatum stimulation (Vidal et al., 1984). Presynaptic DORs produce GABAergic LTD at FSI PV-expressing basket cell inputs to pyramidal neurons of CA2, but only short-term depression in CA1 (Piskorowski and Chevaleyre, 2013). The DOR effects enable long-lasting potentiation of CA2 transmission following high frequency stimulation of Schaffer collateral inputs that prevents the strong feedforward inhibition of CA3-CA2 transmission through DOR-mediated inhibitory LTD (iLTD) (Nasrallah et al., 2015). DOR-mediated iLTD acts as a gate for feedforward inhibition in CA2 to allow for greater activation of CA2 pyramidal neurons in response to both distal and proximal glutamatergic synaptic drive (Nasrallah et al., 2017). DOR antagonists block input timing-dependent plasticity in CA2, likely preventing the iLTD of PV-expressing inputs to pyramidal neurons (Leroy et al., 2017).

CA3

Mossy fiber stimulation induces a potentiation of glutamate transmission in stimulated pathway of guinea pig CA3, but inhibition of nearby mossy fiber synapses (Weisskopf et al., 1993). Dynorphin presynaptically inhibits these other mossy fiber pathways; inhibiting KOR signaling allows for LTP induction in this other pathway. Dynorphin is more effective at inhibiting synapses that had undergone LTP induction than those that did not. KOR effects on CA3 LTP are mediated by a non-voltage-gated channel, calcium-dependent process (Castillo et al., 1996). KORs inhibit NMDAR-mediated currents in CA3 of guinea pig hippocampus, but DORs and MORs do not (Caudle et al., 1994). KOR modulation of mossy fiber signaling within CA3 does not occur in Sprague-Dawley rats, but does occur in other rodents. MORs equally inhibit mossy fiber transmission in rats and guinea pig (Salin et al., 1995). Species differences could be due to differential KOR expression. KOR activation enhances the voltage-dependent potassium current known as the M-current [I(M)] in rat CA3 pyramidal neurons, whereas DOR activation reduces I(M) (Moore et al., 1994). DOR antagonists inhibit IPSCs in CA3, but do not block LTP (Krug et al., 2001; Leroy et al., 2019).

MOR activation has no effect on excitatory postsynaptic potentials, but instead reduces IPSPs (Capogna et al., 1993). Activation of DORs and KORs does not inhibit IPSPs. MOR-mediated presynaptic inhibition of GABA transmission produces disinhibition that is G protein mediated and blocked by PKC activation but does not involve potassium or calcium conductance changes (Capogna et al., 1993, 1996). Later studies show that opioid analgesics that activate MORs can inhibit glutamate transmission in CA3, contrary to earlier studies (Lu et al., 2020, 2021).

Dentate Gyrus

Morphine increases hippocampal activity in dentate gyrus in slice and in freely moving animals (Linseman and Corrigall, 1982). Within the dentate gyrus, MOR activation enhances LTP induction and naloxone prevents LTP induction of the lateral, but not medial perforant pathway (Bramham et al., 1991; Xie and Lewis, 1991; Sagratella et al., 1996; Ito et al., 2001). Interestingly, electrophysiological studies of MOR knockout mice demonstrated an inability to form LTP in the DG but not in CA1, indicating that MOR activation was crucial to LTP in the DG, but not in CA1 (Matthies et al., 2000). LTP of synaptic transmission is blocked by a DOR antagonist, without affecting potentiation of the population spike (Bramham et al., 1991; Krug et al., 2001). Perforant pathway stimulation-induced opioid peptide release with a resultant MOR- and DOR-mediated disinhibition is crucial to facilitating LTP in the dentate gyrus (Bramham and Sarvey, 1996; Ito et al., 2001). In contrast to their lack of effect in CA1, KOR activation in dentate gyrus prevents LTP induction, in contrast to MOR-induced enhancement of LTP (Sagratella et al., 1996).

MORs and DORs hyperpolarize granule cells in the dentate gyrus (Piguet and North, 1993). A study showed that activation of KORs in dentate gyrus produces hyperexcitable granule cells through a postsynaptic G protein-Kv4.2 A-type potassium current mechanism, but without a change in resting membrane potential or input resistance (McDermott and Schrader, 2011).

As in CA1–CA3 areas of hippocampus, opioid receptors in the dentate gyrus also produce disinhibition *via* their actions on GABAergic neurons; although, it appears that this disinhibition has less of an effect on LTP induction at dentate gyrus synapses. Consistent with this, MOR, DOR, and KOR activation enhance excitatory transmission in dentate gyrus granule cells, likely

due to disinhibition. MOR activation is the most efficacious (Neumaier et al., 1988). MORs and DORs inhibit GABA transmission in the dentate gyrus (Piguet and North, 1993). In granule cells, MORs inhibit $GABA_A$ and $GABA_B$-mediated IPSCs (Shao et al., 2020). Dentate gyrus population spikes are potentiated by morphine through disinhibition, but morphine does not affect LTP induction itself (Akaishi et al., 2000).

While some studies show that KORs can enhance excitatory transmission in dentate gyrus, other studies demonstrate that KOR has more of an inhibitory effect due to effects on glutamate transmission (Neumaier et al., 1988). In guinea pig dentate gyrus, KOR activation reduces PS amplitude, while DOR and MOR had no effect. KOR activation inhibits glutamate transmission from perforant path inputs, without affecting GABA transmission (Wagner et al., 1992). A combination of brain slice electrophysiology, pharmacological probing, and anatomical lesioning revealed that KOR activation in dentate gyrus presynaptically inhibits glutamate release (Simmons et al., 1994). Activation of KORs inhibits LTP formation between the perforant path and granule cells of the guinea pig dentate gyrus (Terman et al., 1994). KORs inhibit hilar mossy fiber collateral-based LTP of guinea pig dentate gyrus granule cells, the latter of which likely occurs in a $GABA_A$-dependent mechanism (Terman et al., 2000). A recent study showed MORs can inhibit glutamate transmission in dentate gyrus, specifically, NMDAR-mediated, but not AMPAR-mediated, EPSCs (Shao et al., 2020).

HYPOTHALAMUS

The hypothalamus coordinates the neuroendocrine system (Swaab et al., 1993) and regulates metabolism, reproduction, and parental behavior (Travaglio and Ebling, 2019; Evans et al., 2021; Orikasa, 2021). Hypothalamic neurons release several neurotransmitters and peptides, including GABA, glutamate, dopamine, growth hormone-releasing hormone, gonadotropin-releasing hormone, oxytocin, and vasopressin (Kim et al., 2020). All three opioid receptors are expressed in the hypothalamus (Tavakoli-Nezhad and Arbogast, 2010; Chu Sin Chung and Kieffer, 2013).

Arcuate Nucleus

In the Arcuate Nucleus (AN), MORs most likely inhibit only oxytocin cells, not vasopressin cells (Wakerley et al., 1983). MOR activation hyperpolarizes a subset of neurons by inducing outward current with inward rectification with no effect of TTX. Some of these MOR-sensitive cells are POMC neurons (Loose et al., 1991; Pennock and Hentges, 2011). MOR activation induces outward potassium currents in POMC neurons within the AN (Ibrahim et al., 2003). MORs act as autoreceptors, having direct effects and reducing AP firing within the recorded neuron, but can have similar effects in non-POMC neurons (Kelly et al., 1990, 1992; Lagrange et al., 1994). MORs also inhibit gonadotropin-releasing hormone-expressing neurons (Lagrange et al., 1995). DORs specifically hyperpolarize non-POMC AN neurons, while KORs do not appear to hyperpolarize AN neurons (Loose and Kelly, 1990; Pennock and Hentges, 2011). Interestingly, POMC neurons are directly inhibited by dynorphin A through activation of potassium conductance (Zhang and van den Pol, 2013; Pennock and Hentges, 2014). Previously it was considered that was due to KOR activation (Zhang and van den Pol, 2013). However, follow up studies found that this was likely due to actions of dynorphin A on MORs (Pennock and Hentges, 2014). Later studies determined KORs do hyperpolarize a subset of AN neurons, specifically NPY neurons (Zhang and van den Pol, 2013). In the AN, KOR activation reduces AP firing of neurons that express dynorphin, indicating that these receptors serve as autoreceptors (Ruka et al., 2013, 2016). Looking at synaptic transmission, in AN, MORs and KORs, but not DORs, presynaptically reduce glutamate input (Emmerson and Miller, 1999). Presynaptic MORs and KORs inhibit glutamate and GABA input to POMC neurons (Pennock and Hentges, 2011; Zhang and van den Pol, 2013). In comparison, a DOR agonist was unable to inhibit evoked GABA release but had a modest inhibitory effect on basal GABA transmission; although, it was not clear what the cause of this discrepancy was (Pennock and Hentges, 2011). MOR-mediated inhibition of GABA input is more sensitive than that of postsynaptic hyperpolarization, suggesting there may be opioid peptide concentration-dependent local circuit dynamics at play (Pennock and Hentges, 2011).

Preoptic Hypothalamus

The preoptic hypothalamus plays a role in thermoregulation, where the neurons can be characterized by their thermosensitivity (impulses s–1°C–1) by the thermal coefficient (TC). Preoptic area neurons are hyperpolarized by MOR activation (Wagner et al., 2000). MOR activation-induced hyperpolarization reduces tonic firing activity of all types of neurons and reduces the temperature sensitivity of warm-sensitive neurons (neurons with a TC $\geq$ 0.8 impulses s–1°C–1) (Yakimova, 2006). In the ventrolateral preoptic area, morphine reduces the firing rate and hyperpolarizes sleep-promoting neurons (as assessed by sensitivity to norephinephrine treatment) but has no effect on non-sleep-promoting interneurons (Wang et al., 2013). The investigators found that this was due to dually activated MORs and KORs.

Paraventricular Nucleus

In the Paraventricular Nucleus (PVN), LTD of glutamate input to vasopressin neurons is induced by paired stimulation that combines metabotropic glutamate receptor (mGluR) 1/5 activation with postsynaptic activity to cause somatodendritic dynorphin release that acts at presynaptic KORs (Iremonger et al., 2011). Presynaptic KOR activation mediates synaptic depression *via* inhibition of glutamate release downstream of calcium channel opening that the investigators predict is due to actions on release machinery (Iremonger and Bains, 2009). PVN parvocellular neurons can undergo LTD of GABAergic input *via* mGluR5-driven L-type calcium channel-dependent somatodendritic enkephalin release to act on presynaptic MORs. This iLTD requires ongoing MOR activation, as it is reversible by naloxone (Wamsteeker Cusulin et al., 2013). The released enkephalin can spread to other nearby GABA and glutamate synapses to produce pathway-independent LTD as well.

Supraoptic Nucleus

KORs inhibit both oxytocin and vasopressin neurons of the Supraoptic Nucleus (SON), whereas MORs and DORs primarily inhibit oxytocin neurons (Inenaga et al., 1990). KORs inhibit neuron function by limiting calcium entry to reduce AP firing (Inenaga et al., 1994). In magnocellular neurons of the SON, MORS, but not KORs or DORs, inhibit postsynaptic N- and P/Q-type voltage-gated calcium channels (Soldo and Moises, 1998). In oxytocin neurons of the SON, naloxone treatment increases post spike excitability *in vivo*, suggesting an endogenous MOR tonic activation. The authors discovered that morphine treatment likely engages potassium conductances that are relieved during naloxone-precipitated opioid withdrawal, resulting in hyperexcitable oxytocin neurons, with no effects in nearby vasopressin neurons (Brown et al., 2005). MOR effects on magnocellular neurons are weak, due to inhibition of glutamate input (presynaptic), with no effects on GABA or postsynaptic effects (Liu et al., 1999). Glutamatergic and GABAergic input to magnocellular neurons is decreased presynaptically by MOR activation, with no apparent postsynaptic effects. MOR-mediated inhibition appears to be independent of inhibition of calcium channels or activation of potassium channels. KORs are also able to inhibit GABAergic input to a subpopulation of magnocellular neurons (Honda et al., 2004). Vasopressin magnocellular SON neurons were recorded in organotypic slice cultures to measure rhythmic firing patterns. KOR-mediated inhibition of glutamate release is part of the mechanism that governs the rhythmic firing of these neurons (Israel et al., 2010). This is supported by *in vivo* measures that show that KOR activation influences rhythmic firing of vasopressin, but not oxytocin, neurons of the SON (Brown et al., 1998). Dynorphin is co-released with vasopressin from the dendrites of these neurons (Brown and Bourque, 2004).

The hypothalamus is a region of great cell-type heterogeneity across hypothalamic nuclei. Both presynaptic and postsynaptic MORs and KORs have been shown to regulate hypothalamus neurons; although, the effect and mechanism varies across nuclei and cell-type. The role of DORs in the hypothalamus is less clear, as studies have found conflicting results. This may be due to a limited effect of DORs in subpopulations of hypothalamic neurons, but additional studies are needed to understand how DORs regulate neurotransmission in the hypothalamus. Most research of opioid receptor regulation of neurotransmission in the hypothalamus has focused on only a handful of hypothalamic nuclei, leaving much to be discovered. Interestingly, MORs and KORs have been shown to act as autoreceptors in multiple hypothalamic nuclei. Future studies will reveal if these opioid receptors also act as autoreceptors in other hypothalamic nuclei.

LATERAL HABENULA

The lateral habenula (LHb) regulates reward, aversion, motor and cognitive function, sleep and circadian rhythms, pain, navigation, and maternal behaviors (Hu et al., 2020). It is not clear if DOR is expressed in this area, however, MORs and KORs are expressed, suggesting a role in reward, analgesic and stress responses (Gardon et al., 2014; Simmons et al., 2020). In the LHb, MOR activation has subpopulation effects: some neurons show hyperpolarization, some neurons show reduced glutamate synaptic input, and some neurons show reduced GABA input (Margolis and Fields, 2016). KOR activation in LHb presynaptically inhibits glutamate transmission, but has both inhibitory and enhancing effects on GABA transmission (Simmons et al., 2020). The net impact of KOR on regulating glutamate and GABA transmission produces KOR-mediated hyperexcitability of neurons that express hyperpolarization-activated cation currents (Ih) and decreases the excitability of Ih-negative neurons. Additional studies are needed to identify which specific LHb inputs are regulated by MORs and KORs.

PALLIDUM

The pallidum is composed of the globus pallidus, entopeduncular nucleus, and ventral pallidum. Together, the pallidum has important roles in hedonic actions, motivation, and cognition (Smith et al., 2009; Saga et al., 2017). All three opioid receptor are highly expressed in the pallidum (Le Merrer et al., 2009).

Globus Pallidus

Presynaptic MORs inhibit GABA input from dorsal striatum and from local GABAergic neurons (Stanford and Cooper, 1999). In contrast, DORs inhibit evoked local GABA transmission, but do not inhibit striatal inputs. DOR activation has no effect on AP-dependent spontaneous IPSCs, but inhibits mIPSCs. MORs, but not DORs or KORs, postsynaptically inhibit N-type VGCCs in dissociated Globus Pallidus (GP) neurons (Stefani et al., 2001). Similar to MOR, KOR activation in GP hyperpolarizes about 25% of cells and presynaptically inhibits GABAergic input from striatum and local GABAergic collaterals (Ogura and Kita, 2000). KORs have no effect on glutamate transmission, and it is unknown if DORs or KORs regulate glutamate transmission in GP.

Entopeduncular Nucleus

A subpopulation of Entopeduncular Nucleus (EPN) neurons were hyperpolarized by dynorphin-mediated KOR activation *via* increasing potassium conductance. Electrical stimulation of the (GP) evokes GABA release from striatal and pallidal inputs to the EPN. Dynorphin equally inhibited IPSCs from both sources (short- and medium-latency IPSCs) presynaptically. Dynorphin released from striatal inputs could be an autofeedback mechanism, heterosynaptic (targeting pallidal input), or directly inhibit EPN neurons (Ogura and Kita, 2002).

Ventral Pallidum

MORs hyperpolarize a subpopulation of Ventral Pallidum (VP) neurons, presumably through activation of potassium currents (Napier and Mitrovic, 1999). Looking at specific regional targets of VP neurons, MORs hyperpolarize GABAergic VP neurons that project to the VTA (Hjelmstad et al., 2013). *In vivo* electrophysiological recordings reveal that MOR activation reduces inhibitory GABAergic input, and excitatory substance P input from the NAc within the VP and enhances glutamate

input from amygdala (Napier and Mitrovic, 1999). MOR activation produces LTD of GABA release in VP (Kupchik et al., 2014). In *in vivo* electrophysiological recordings, stimulation of VTA inputs to VP reduces firing of VP neurons. KOR and MOR activation block this, either due to direct inhibition of dopamine inputs or inhibition of non-dopaminergic VTA input (Napier and Mitrovic, 1999; Mitrovic and Napier, 2002). MORs also antagonize NAc-induced inhibitory transmission in VP (Chrobak and Napier, 1993). KORs postsynaptically inhibit GABAergic transmission from both direct pathway MSN (dMSN) and indirect pathway MSN (iMSN) inputs to VP GABA neurons. KORs generally increase GABAergic input to VP vGluT2-expressing neurons, but they could not determine if this was pre- or postsynaptically mediated and did not test specific GABAergic synaptic inputs (Inbar et al., 2020).

In summary, subpopulations of pallidal neurons are hyperpolarized by postsynaptic MORs and KORs. Presynaptic opioid receptors also modulate neural activity of pallidal neurons by inhibiting GABA release from striatal terminals and local GABAergic collaterals; although, the effect varies across opioid receptor and neurocircuit. Excitatory neurotransmission in VP is regulated by MORs and KORs, but excitatory transmission in other pallidal areas has not been shown to be modulated by opioid receptors. Most studies investigated circuit and subpopulation effects of opioid receptors in pallidum have focused on VP, therefore future studies are needed to identify specific subpopulation effects in GP and EPN.

STRIATUM

The striatum is divided into dorsal and ventral regions. The dorsal striatum (DS) is heavily involved in motor control, learning, reward, and decision making (Balleine et al., 2007). The dorsal striatum is further divided into the dorsolateral (DLS) and dorsomedial striatum (DMS). The DMS is involved with goal-directed behaviors, while the DLS is involved with habitual behaviors (Lovinger, 2010; Corbit and Janak, 2016). The ventral striatum, also known as the nucleus accumbens (NAc) plays a critical role in establishing reward-associated memories to the effects of drugs and natural cues (Hyman et al., 2006). All 3 opioid receptors are highly expressed in the striatum and regulate synaptic plasticity (Le Merrer et al., 2009; Atwood et al., 2014b).

Dorsal Striatum

Aside from an early study of opioid effects on neuronal function in dorsal striatum, there is very little indication that opioid receptors alter membrane properties of the principal dorsal striatal MSNs. One early study found that MORs slightly hyperpolarize a subset of MSNs (Jiang and North, 1992). They also found that DORs hyperpolarize a subset of non-MSN, tonically active neurons, ablating AP firing. Later studies suggest that these are likely tonically active interneurons that release acetylcholine and glutamate and their firing is inhibited by both MORs and DORs (Ponterio et al., 2013; Laurent et al., 2014). MORs reduce the firing of these cholinergic interneurons through postsynaptic G protein signaling (Ponterio et al., 2013, 2018). MOR modulation of these neurons may be circadian (Jabourian et al., 2005).

It was initially thought that opioid receptors do not inhibit GABA release in dorsal striatum(Jiang and North, 1992). However, later work found opioid receptors regulate GABA transmission in a subregion and synapse-specific manner that could be missed using more non-specific measures. MORs only inhibit GABAergic transmission within striosome subcompartments. MOR-mediated inhibition of GABA transmission within striosomes is mediated by presynaptic cAMP-PKA signaling, likely modulating presynaptic potassium channel function, and MOR inhibition is enhanced by PKC inhibition (Miura et al., 2007; Inoue et al., 2012). MORs inhibit spontaneous and TTX-insensitive GABAergic inputs in both cell types (dMSN and iMSN) (Ma et al., 2012). An elegant dissection of specific GABAergic synapses within striosomes that MORs and DORs regulate found that MORs inhibit dMSN and iMSN input to dMSNs, although inhibition of dMSN-dMSN transmission is stronger than iMSN-dMSN transmission (Banghart et al., 2015). DORs selectively inhibit iMSN input to dMSNs. Neither MOR nor DOR inhibit somatostatin-expressing interneuron input to dMSNs. DOR-mediated disinhibition of dMSNs is slightly more efficacious than MOR. MOR and DOR have little effect on GABA transmission in matrix of dorsal striatum. DOR activation produces iLTD at FSI-MSN synapses (Patton et al., 2016).

It has been known for some time that MORs and DORs inhibit glutamate release in dorsal striatum (Jiang and North, 1992). Despite MORs being enriched in striosome subcompartments of striatum, MORs equally inhibit glutamate transmission in both striosomes and matrix (Miura et al., 2007). One study that explored differences in MOR effects in dMSNs and iMSNs found that MORs reduce spontaneous glutamate release onto iMSNs in DLS, but not dMSNs (Ma et al., 2012). However, these data do not align with data from other laboratories that found more widespread MOR-mediated inhibition of glutamate release (Atwood et al., 2014b). They also reported that MORs have minimal effect on TTX-insensitive glutamate transmission in either type of MSNs in the DLS (Ma et al., 2012). MOR and DOR activation in the DLS and DMS produce antagonist-irreversible LTD in young rats and mice as well as adult mice (Atwood et al., 2014b; Fritz et al., 2018; Munoz et al., 2018, 2020). In the DLS, MOR and DOR LTD are not mutually occlusive, indicating that they inhibit different inputs. In the presence of peptidase inhibitors, electrical stimulation of glutamate release produces opioid receptor antagonist-sensitive LTD that is mGluR5 dependent. Antagonists for both MOR and DOR each partially prevent this LTD, while naloxone fully prevents this LTD. KORs may also play a role in this LTD (see below). Others have found that antidromic stimulation within the globus pallidus induces opioid peptide release (presumably enkephalins) within dorsal striatum that is sufficient to inhibit glutamate input from cortex. This was mediated by MORs, but not DORs. Paired recordings showed MSN firing could

produce corticostriatal inhibition in a nearby MSN with a subpopulation showing reciprocal inhibition of cortical input (Blomeley and Bracci, 2011).

More recent work has attempted to dissect which specific glutamate synapses in the dorsal striatum are sensitive to MOR and DOR activation. In the DLS, the only cortical input that is sensitive to MOR activation are those that arise from anterior insular cortex in a mechanism that involve the activation of presynaptic HCN1 channels (Munoz et al., 2018, 2021). MORs also produce LTD in the DMS, but in this subregion the LTD is mediated by inputs from BLA, mPFC, and ACC (Munoz et al., 2020). In contrast, another recent study concluded that MORs do not inhibit ACC or mPFC inputs to DMS MSNs (Birdsong et al., 2019). The two studies were both done in mice, so it is not clear why the results are not aligned. DOR inhibits prelimbic mPFC input to DMS MSNs and motor cortex inputs to DLS MSNs (Atwood et al., 2014b; Birdsong et al., 2019). There has not been an exhaustive study of DORs effects on other cortical inputs to date. Interestingly, MORs also produce LTD of glutamate release from tonically active "cholinergic" interneurons in the DLS (Munoz et al., 2018). MORs also inhibit glutamatergic inputs from thalamus, albeit with a transient suppression rather than LTD in both DLS and DMS (Atwood et al., 2014b; Munoz et al., 2018; Birdsong et al., 2019; Reeves et al., 2021). It does not appear that DORs inhibit glutamate input from thalamus (Atwood et al., 2014b; Birdsong et al., 2019).

The early study of opioid effects on neurotransmission in dorsal striatum concluded that KORs have no effect on glutamate release (Jiang and North, 1992). However, a more recent study found that KORs can inhibit glutamate release in brain slices from young rats, specifically in dorsolateral striatum (DLS) (Atwood et al., 2014b). Activation of KORs produces an irreversible, long-lasting synaptic depression, which is similar to plasticity produced by DORs, but not MORs, in dorsal striatum. A KOR antagonist could also fully block endogenous opioid LTD in DLS, similar to the effects of naloxone, whereas MOR and DOR antagonists individually only partially blocked LTD (Atwood et al., 2014b). Given that KORs also inhibit dopamine release in dorsal striatum, it will be important to disambiguate in the future if this is due to direct activation of KORs on glutamate terminals or due to its actions on dopamine terminals which could account for the KOR antagonist on endogenous opioid LTD (Schoffelmeer et al., 1997; Szabo et al., 1999; Mamaligas et al., 2016; Hawes et al., 2017). For example, activation of Pdyn-containing dMSNs in DMS induces release of dynorphin that acts on presynaptic KORs on dopamine terminals to prevent theta burst stimulation-induced glutamatergic LTP in MSNs (Hawes et al., 2017). Similar mechanisms could account for the effects of KOR on inhibiting glutamate transmission under certain conditions.

Ventral Striatum (Nucleus Accumbens)

The NAc can be subdivided into shell and core regions. Many studies specifically state whether measures were made in shell or core, and some provide even greater specificity. However, plenty of other studies make no distinction. Therefore, in this section where there is no specific subregion mentioned we are only able to generalize the role of opioid receptors on the specific measures discussed. There is very little evidence that opioid receptors have postsynaptic effects that influence AP firing in NAc, but much evidence that they do modulate synaptic transmission (Yuan et al., 1992; Martin et al., 1997).

As in dorsal striatum, there are some discrepant data regarding the role of MOR in regulating GABA transmission in NAc. One report demonstrates that MORs inhibit GABAergic transmission in both NAc shell and core, however, MORs have a larger effect on GABA transmission in the shell (Brundege and Williams, 2002). Another study shows that MORs inhibit GABA release in NAc shell equally in control and in forskolin-enhanced GABA release conditions (Chieng and Williams, 1998). A third study shows that MORs inhibit spontaneous GABA release similarly in D1 and D2 MSNs of the NAc core and NAc shell. Measures of TTX-insensitive GABA release show that GABA input is only inhibited in D1 MSNs in the core and D2 MSNs of the shell (Ma et al., 2012). However, a different study of the NAc shell showed that MOR activation has no effect on GABAergic input (Hoffman and Lupica, 2001). In contrast, about 50% of MSNs received input that was presumably regulated by DOR as a mixed DOR/MOR agonist was effective at blocking GABA transmission, but a MOR agonist was ineffective in these neurons. KOR activation strongly inhibits GABAergic output from D1 MSNs, but more weakly inhibits GABA output from D2 MSNs (Tejeda et al., 2017). KORs also inhibit GABA release, but with a different mechanism. KOR-mediated inhibition of GABA release is at the level of calcium entry through N-type VGCCs (Hjelmstad and Fields, 2003). Potassium channel blockade had no effect on KOR actions. Due to KOR expression on VTA dopamine neuron inputs, KORs could theoretically inhibit CIN-driven GABA co-release from VTA dopamine inputs (Britt and McGehee, 2008; Nelson et al., 2014).

MORs presynaptically inhibit glutamate release in NAc core and shell (Martin et al., 1997; Hoffman and Lupica, 2001; Brundege and Williams, 2002; Hoffman et al., 2003; James et al., 2013). Postsynaptic MOR activation was reported to enhance NMDAR, but reduce AMPAR currents (Martin et al., 1997). Regarding spontaneous glutamate release, MORs equally inhibit glutamate input to D1 MSNs in NAc core and NAc shell, but have a much larger effect on D2 MSNs of the NAc shell. MORs inhibit TTX-insensitive glutamate inputs to D1 and D2 MSNs in NAc core and NAc shell, although the effect is most robust in D1 MSNs of the shell (Ma et al., 2012). MOR's effects on glutamate transmission in NAc may not always be neuronal in origin. MOR activation on astrocytes in NAc core induces glutamate release, producing slow inward currents *via* extrasynaptic NMDARs in nearby neurons (Corkrum et al., 2019). DOR has a minor effect on inhibiting glutamate transmission in NAc shell, perhaps only in a subset of glutamate inputs (Brundege and Williams, 2002). KORs presynaptically inhibit glutamate release on to MSNs of NAc shell without having any postsynaptic effects (Hjelmstad and Fields, 2001). KOR inhibition of glutamate transmission persist in the presence of N- and P/Q-type calcium and potassium

channel blockers (Hjelmstad and Fields, 2003). Strong PFC input to NAc can produce heterosynaptic inhibition of weaker ventral hippocampal inputs, a process that is in part mediated by KORs (Brooks and O'Donnell, 2017). KOR inhibits glutamate input from BLA, but not ventral hippocampus, to D1 MSNs of the NAc shell and core, but not D2 MSNs (Tejeda et al., 2017). This effect was stronger in NAc shell than core, but was independent of D1 MSN projection target. The net effect of KOR activation at the GABA and glutamate synapses allows for KORs to decrease D1 MSN firing and disinhibit D2 MSN firing in response to BLA input. In contrast, KOR has no effect on ventral hippocampal drive of D1 MSNs, but still allows for disinhibition of D2 MSNs. The authors conclude that KOR acts as a pathway-specific filtering mechanism for BLA versus ventral hippocampal control of NAc function. KOR inhibition of glutamate transmission in NAc MSNs is lost in animals with 5 days of repeated cocaine exposure with at least up to 2 weeks of withdrawal (Mu et al., 2011). KORs also regulate glutamatergic input to PV-expressing FSIs in NAc, however, this is specific to thalamic, but not cortical inputs (Coleman et al., 2021). In addition, activation of KORs produces a postsynaptic LTD plasticity, wherein AMPARs are internalized *via* a PKA-calcineurin signaling pathway.

Altogether, these studies indicate that opioid receptor activation has little effect on membrane properties of both dorsal and ventral striatal neurons, with the exception of cholinergic interneurons. A growing body of evidence indicates that each type of opioid receptor is capable of inhibiting glutamate transmission and MORs and DORs regulate GABA transmission, although not universally at all striatal synapses. The biological relevance of synapse- and opioid receptor subtype-specific regulation of striatal excitatory and inhibitory transmission is currently unclear. Refined approaches for manipulating the expression of these receptors at specific synapses will help decipher the interplay between receptors in controlling striatal-mediated behaviors and circuit function.

THALAMUS

The thalamus acts as a relay hub for cortical sensory and motor functions, controlling perception, action and mentation (Schmitt and Halassa, 2017). MORs are highly expressed, but DORs and KORs are sparsely expressed, throughout the thalamus (Le Merrer et al., 2009; Chu Sin Chung and Kieffer, 2013; Chen et al., 2015; Bengoetxea et al., 2020).

In the thalamic reticular nucleus there are two predominant types of neurons that are both GABAergic, but display different firing properties (bursting and non-bursting). MOR activation, but not DOR or KOR, hyperpolarizes subpopulations of each class of neurons, revealing further subpopulations of neurons in this nucleus. The mechanism of hyperpolarization is due to increased potassium conductance (Brunton and Charpak, 1997). MOR activation hyperpolarizes dorsal midline thalamus neurons that project to the BLA and CeA (Goedecke et al., 2019). In the centrolateral thalamus, MOR activation, but not DOR or KOR, hyperpolarized neurons *via* increased GIRK function, independent of synaptic input. The investigators explored MOR hyperpolarization of other thalamic neurons (principal relay, midline, and intralaminar nuclei) and found widespread MOR-mediated inhibition of thalamic neurons, suggesting that the thalamus is a highly sensitive region to MOR-mediated neuronal hyperpolarization (Brunton and Charpak, 1998).

In contrast to MORs, KOR effects in the thalamus appear to be restricted to specific thalamic nuclei. KOR activation produces direct hyperpolarization of anterior paraventricular thalamic neurons through GIRKs that peak around the ages of puberty and then decrease at later ages. MOR activation hyperpolarizes these neurons; although, the effect of the MOR agonist desensitizes and produces heterologous desensitization of KOR responses (KOR responses do not desensitize independent of MOR activation) (Chen et al., 2015). Additional studies are needed to investigate potential KOR effects in other thalamic nuclei.

OTHER REGIONS

In the above sections, we opted to review brain regions that have received the most attention. However, in our survey of the literature there are, in the context of opioid receptor-mediated regulation of neurotransmission, some other less studied brain regions or subregions that deserve further investigation and we briefly review them here.

Lateral Hypothalamus

In the Lateral Hypothalamus (LH), local GABA neurons within the perifornical region inhibit the activity of orexin neurons. KOR activation specifically reduces this GABAergic input, as revealed by optical probing of these local GABA neurons (Ferrari et al., 2018). No studies to date have investigated MOR or DOR effects on neurons in the LH.

Medial Vestibular Nucleus

Also known as the nucleus of Schwalbe is located in the brainstem (Highstein and Holstein, 2006). DORs, but not MORs or KORs inhibit Medial Vestibular Nucleus (MVN) neurons. DOR inhibition is *via* activation of an outward potassium current (Sulaiman and Dutia, 1998).

Pons

Located in the brainstem, parabrachial nucleus neurons are hyperpolarized by MORs, but not DORs or KORs, likely through enhancing potassium currents (Christie and North, 1988; Cramer et al., 2021). Pontine Kölliker-Fuse nucleus neurons are hyperpolarized by MOR activation *via* GIRK activation (Levitt et al., 2015; Levitt and Williams, 2018). MORs and KORs inhibit GABA release in PBN, but only MORs regulate glutamate release (Cramer et al., 2021).

Ventromedial Hypothalamus

Very few studies have investigated the role of opioid receptors in modulating neural function in the Ventromedial Hypothalamus

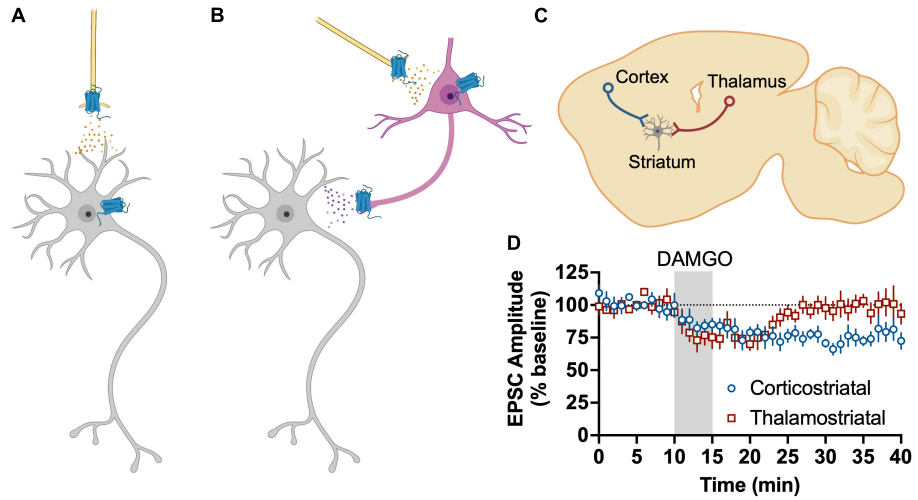

FIGURE 2 | Summary of opioid receptor-mediated modulation of neurotransmission. Opioid receptor activation-mediated modulation of neurotransmission can have differential effects on neurocircuit function depending on the localization of the receptors. **(A)** Opioid receptors found on glutamatergic terminals will reduce glutamate release upon activation, thus inhibiting a postsynaptic neuron. Opioid receptors on postsynaptic neurons will generally reduce neuronal excitability. **(B)** Opioid receptors found on inhibitory neuron (e.g., GABAergic) terminals or postsynaptically will reduce inhibitory transmission, disinhibiting a postsynaptic neuron. Alternatively, opioid receptors on glutamate neurons that impinge on inhibitory neurons will reduce excitatory drive of these neurons, thus reducing inhibitory transmission and producing disinhibition through a polysynaptic mechanism. **(C)** Opioid receptors localized to different synaptic terminals can produce differential outcomes upon activation. As an example from our own work, MORs are localized to cortical and thalamic glutamatergic inputs to dorsal striatum (DS). **(D)** Upon activation by the MOR agonist, DAMGO, MORs reduce the amplitude of glutamate-mediated excitatory postsynaptic currents (EPSCs). Activation MORs on glutamate inputs from cortex produces a long-lasting reduction in EPSC amplitudes. However, activation of MORs on thalamic inputs only produces a transient reduction, despite also being a glutamatergic input to the same neurons that express the long-lasting reduction in glutamate transmission from cortical inputs. Adapted from data from Munoz et al. (2018). Figure created with BioRender.com.

(VMH) or LH. In the VMH, MORs hyperpolarize neurons to reduce cellular excitability, including those that express the leptin receptor, *via* enhancing GIRK currents (Emmerson and Miller, 1999). DORs and KORs do not appear to hyperpolarize neurons in the VMH. Presynaptic MORs strongly inhibit glutamate input to VMH neurons, whereas DORs have no effect, and KORs have only a small effect on glutamate release (Emmerson and Miller, 1999; Devidze et al., 2008). It is unknown which glutamatergic inputs to the VMH are modulated by MORs and KORs.

GENERAL PRINCIPLES, KNOWLEDGE GAPS, AND FUTURE DIRECTIONS

Across brain regions opioid receptors play major roles in regulating glutamate and GABA release through presynaptic mechanisms and neuronal excitability through postsynaptic mechanisms. There is heterogeneity in the precise mechanisms whereby opioid receptors regulate neurotransmitter release, even within any given brain region (**Figure 1**). At some synapses this appears to involve inhibition of calcium channels, while at others it involves activating potassium channels. There is also evidence that diverse kinase signaling pathways may be involved at distinct synapses. These divergent mechanisms do not appear to be due to the specific identity of the opioid receptors, but rather due to the specific synaptic terminals on which the receptors are expressed. On the other hand, all three opioid receptor types appear to generally modulate neuronal excitability through their actions on potassium channels, such as GIRKs. However, local circuit effects must be considered when deciphering pre- versus postsynaptic localization of opioid receptor actions, as postsynaptic hyperpolarization can reduce local circuit neurotransmitter release (**Figures 2A,B**).

In order to better understand how opioid receptors modulate neurocircuit function, there is a need to identify the specific cell types that express these receptors and the subcellular localization of the receptors. Conditional knockout and fluorescent reporter transgenic mice are useful for identifying the cell types that express the various opioid receptors and how the expression of receptors within those cell types affects neurotransmission (Gaveriaux-Ruff et al., 2011; Weibel et al., 2013; Ehrich et al., 2015; Erbs et al., 2015; Chen et al., 2020). Another important consideration is identifying specific circuits that are modulated by opioid receptors. In many brain regions, opioid receptor effects on neurotransmission differ according to localization within the region, projection targets, or input regions. Optogenetic methods are increasingly accessible and are useful for identifying the specific synapses at which opioid receptors reside and how they specifically modulate neurotransmission.

It is not common for assessments of long-term opioid receptor-mediated synaptic plasticity to be performed. For many investigators, it is sufficient to determine whether a synapse is regulated by opioid receptors. However, there are missed opportunities to observe the diversity in ways in which opioid receptors modulate neurotransmission. At some synapses, activation of opioid receptors produces long-lasting effects

on neurotransmission that persist even once opioid receptor antagonists are applied, which argues against persistent receptor activation. At other synapses, opioid receptor activation only produces transient responses, only lasting while the receptors are engaged (**Figures 2C,D**). Opioid receptors display desensitization at some synapses, while other synapses appear to be resistant to receptor desensitization. Whether a particular type of receptor in a given synapse or cell type produces long-lasting or short-term effects upon activation or desensitizes or not is a fascinating area of study that will yield rich insights into how opioids affect cognition, behavioral output, and physiological functions. Comparisons between mechanisms of synapse- and cell type-specific opioid receptor modulation of neurotransmission could also reveal novel opportunities for targeted combinatorial therapeutics. There is clearly much left to discover regarding how opioid receptors can utilize such a diverse array of mechanisms to precisely modulate neurotransmission.

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REVIEW
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Cellular Tolerance Induced by Chronic Opioids in the Central Nervous System

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Opioids are powerful analgesics that elicit acute antinociceptive effects through their action the mu opioid receptor (MOR). However opioids are ineffective for chronic pain management, in part because continuous activation of MORs induces adaptive changes at the receptor level and downstream signaling molecules. These adaptations include a decrease in receptor-effector coupling and changes to second messenger systems that can counteract the persistent activation of MORs by opioid agonists. Homeostatic regulation of MORs and downstream signaling cascades are viewed as precursors to developing tolerance. However, despite numerous studies identifying crucial mechanisms that contribute to opioid tolerance, no single regulatory mechanism that governs tolerance in at the cellular and systems level has been identified. Opioid tolerance is a multifaceted process that involves both individual neurons that contain MORs and neuronal circuits that undergo adaptations following continuous MOR activation. The most proximal event is the agonist/receptor interaction leading to acute cellular actions. This review discusses our understanding of mechanisms that mediate cellular tolerance after chronic opioid treatment that, in part, is mediated by agonist/receptor interaction acutely.

Keywords: opioid, tolerance, electrophysiology, kinases, withdrawal

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INTRODUCTION

Mu opioid receptor (MOR) ligands are the first choice for the treatment of acute, post-surgical or trauma. There are however side effects that limit their utility including respiratory depression, constipation sedation, dizziness, and nausea; chronic: abuse potential, dependence (Paul et al., 2021). Treatment with opioids have limited value for long-term treatment of most chronic pain. The development of analgesic tolerance is one component limiting the value of chronic treatment with opioids. A second component results from the development of opioid use disorder. Given the widespread distribution of MORs in the central nervous system (CNS), it is not surprising that multiple systems level actions are associated with both the acute and chronic actions of opioids. MOR expressing areas directly involved in the pain pathway include primary afferent, dorsal horn, and thalamic neurons (reviewed, Corder et al., 2018). There are also multiple pain associated regions that express MORs, such as the parabrachial area, periaqueductal gray, and rostral ventromedial medulla (reviewed, Corder et al., 2018). Additionally, actions of MORs in limbic areas such as the ventral tegmental area, nucleus accumbens, medial striatum, and rostromedial tegmental nucleus underlie the initial processes in the development of tolerance and consequently

opioid abuse disorder (reviewed, Williams et al., 2013). The effects of opioids therefore result from actions in multiple areas at both the pre- and postsynaptic levels. Further complicating this, the downstream receptor-dependent signaling cascades vary across brain regions. Receptor actions are defined in part by expression levels, the complement of downstream effectors and the efficiency of receptor/effector coupling. Postsynaptic actions include inhibition mediated by an increase in potassium conductance and an inhibition of voltage dependent calcium channels and adenylyl cyclase (reviewed, Williams et al., 2001). In addition, receptors located on presynaptic terminals act to inhibit transmitter release. Opioid receptor dependent inhibition of GABA and glutamate results in the modulation of postsynaptic neurons through disinhibition and inhibition, respectively. Recent interest in agonist bias of G protein verses arrestin activation across different neurons has added another important layer in the understanding of the downstream actions of opioids (Gillis et al., 2020; Stahl and Bohn, 2021). A substantial component of opioid tolerance results from the downstream adaptations that result from continued MOR signaling during chronic treatment. These processes counteract continued MOR signaling also underlie the withdrawal that results following termination of opioid treatment. This review will discuss two levels of tolerance, namely receptor dependent and systems dependent tolerance.

Although the acute actions of opioids are established in multiple CNS areas, there are few areas where the mechanisms that underlie tolerance and the adaptive mechanisms that result from chronic treatment have been examined. It is also important to distinguish acute desensitization from long-term tolerance. Opioid signaling is disrupted in both processes, but there are distinct differences. Acute desensitization is most often induced with high concentrations of agonist that results in a reduction of signaling. Acute desensitization develops in minutes and recovers in 10's of minutes upon agonist removal (reviewed, Williams et al., 2013; Birdsong and Williams, 2020). It is established that phosphorylation of the C-terminus of MOR is a necessary step in the induction of acute desensitization. Recent work indicates that acute desensitization is largely blocked by inhibition of GRK2/3 adding to work indicating a key role for protein kinase C (PKC) (Bailey et al., 2006) and c-Jun N-terminal Kinase (JNK) (Melief et al., 2010).

Tolerance to opioids, on the other hand, requires treatment with agonist for hours or days and is not associated with measurable change in MOR mRNA or protein expression (Ammon-Treiber and Hollt, 2005; Dang and Christie, 2012). The recovery from tolerance is very slow (days–months). Further the time course of this recovery is dependent on what measure is used to determine tolerance implying that tolerance is cell type and/or pathway selective (reviewed, Williams et al., 2013). Similar to acute desensitization, components of long-term tolerance are also dependent on phosphorylation of the C-terminus, in that cellular tolerance is blocked with the expression of receptors where phosphorylation sites in the C-terminus are mutated to alanine (Arttamangkul et al., 2018; Kliewer et al., 2019). There are however downstream adaptive mechanisms at the cellular level that result from the continued activation of receptors that persist in the absence of C-terminus phosphorylation (Kuhar et al., 2015; Leff et al., 2020; Adhikary et al., 2022a). The goal of this review is to summarize what is known about the development of tolerance in single neurons induced by chronic treatment with opioids. This cell-centric view of opioid actions is the basis of circuit and systems level outcomes following chronic opioid treatment and a full-appreciation of cellular changes across relevant brain regions will be critical in the search for ligands that can provide efficacious analgesia over extended periods without untoward actions on other circuits.

AREAS WHERE NEURONS HAVE BEEN EXAMINED FOLLOWING CHRONIC MORPHINE TREATMENT

Morphine, a partial MOR agonist, remains a gold-standard for acute pain management despite a number of averse potential outcomes. Postsynaptic tolerance measured in brain slices from animals exposed to chronic morphine treatment has been examined using several protocols. The classical method to describe receptor tolerance requires concentration response curve in preparations from opioid naïve and treated animals. Early work in brain slices of rat locus coeruleus (LC) determined the concentration response to [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO) and normorphine by measuring the activation of potassium conductance (Christie et al., 1987). In slices from morphine treated animals there was a twofold rightward shift in the DAMGO, a full agonist, concentration response curve and the maximum current induced by normorphine, a partial agonist, was decreased to ∼50% of that measured in slices from untreated animals (Christie et al., 1987). Additionally, the decreases in response to both DAMGO and normorphine was long lasting (6 h). The interpretation of this result was that a reduction in receptor reserve caused the decrease in potassium conductance induced by a saturating concentration of ligand in preparations from morphine treated animals compared to naïve animals. Thus, measuring potassium conductance using partial agonists provide a sensitive assay to determine the decrease in receptor reserve. More recent results found two components to cellular tolerance induced by chronic morphine treatment. One transient form of cellular tolerance declined as the circulating concentration of morphine (1 μM) washed out of the brain slice over 60–90 min and was sensitive to inhibition of PKC (Bailey et al., 2009; Levitt and Williams, 2012). The transient decrease in signaling was considered to be a form of desensitization that recovered with the removal of morphine. Long-term tolerance as previously reported persisted for at least 6 h following preparation of the brain slice (Christie et al., 1987; Levitt and Williams, 2012). The results carried out in the LC from mice were similar but not identical in that the degree of tolerance induced by morphine in the mouse LC is qualitatively less than that measured in rat (Quillinan et al., 2011). There is also a difference in signs of acute withdrawal between mice and rat indicating a marked species difference in the adaptive processes following chronic treatment with opioids (Uddin et al., 2021).

Recent results carried out in rat brain slices in the Kölliker-Fuse (KF) – a region involved with respiratory control – indicate that the degree of tolerance induced after chronic (6–7 days, 80 mg/kg/day) morphine treatment was very small (Levitt and Williams, 2018). Conversely, in dissociated PAG and primary afferent neurons from mouse, the action of DAMGO to inhibit voltage dependent calcium current was reduced in preparations from morphine treated animals (Connor et al., 2015). This result differs from those obtained in brain slices of the LC where the maximum current induced by DAMGO, a potent and efficacious agonist, was the same in preparations from control and morphine treated animals (Christie et al., 1987; Connor et al., 2015). The difference between these results can be explained by the differences in receptor reserve (as defined by the number of receptors and/or the receptor/effector coupling efficiency) in the LC versus dissociated PAG neurons. The activation of potassium conductance in acutely isolated LC neurons, where the dendritic arbor was eliminated, was reduced relative to that in brain slice preparations (Ingram et al., 1997). Further, morphine was unable to activate the potassium current and blocked the current induced by [Met]5enkephalin (ME) or DAMGO. The interpretation was that morphine occupied MORs, but the receptor/effector coupling was reduced to the point that potassium channels were not activated. Distinct differences between cell types have also been characterized in experiments using AtT20 cells (Miess et al., 2018). With the combination of whole cell or perforated patch recordings to examine acute morphine dependent desensitization, there was no desensitization with whole cell recordings that was present with perforated patch recordings. The interpretation was that whole cell recording resulted in a washout of a soluble cellular component that was necessary for acute desensitization (Miess et al., 2018). Thus, the measurement of opioid induced tolerance is highly dependent on both the cell under study and the method used to obtain the results even when examining the same cell type. Despite these complexities, the data generated to date suggests that the degree of cellular tolerance measured using the activation of potassium conductance or the inhibition of voltage gated calcium channels in single neurons is species, cell type, and brain region dependent.

POSTSYNAPTIC ADAPTIVE MECHANISMS

Beyond the decrease in downstream effector activation, chronic morphine treatment also affects MOR-regulating processes. For example, acute desensitization of the MOR is more pronounced after chronic morphine and methadone treatment (Dang and Williams, 2004, 2005; Quillinan et al., 2011; Arttamangkul et al., 2018). Additionally, the recovery from acute desensitization of MORs is prolonged following chronic treatment with morphine and the kinase regulation of G protein coupled receptors (GPCRs) is altered (Quillinan et al., 2011; Arttamangkul et al., 2018; Leff et al., 2020).

Opioid induced acute desensitization in the LC has been shown to be primarily homologous, in that desensitization of the MOR does not affect signaling of another GPCR on the same cell (Harris and Williams, 1991; Bailey et al., 2003, 2009; Dang et al., 2011). A decrease in sensitivity following chronic opioid treatment is also restricted to MORs and not to other GPCRs that couple to the same effectors (Christie et al., 1987; Connor et al., 1999; Bailey et al., 2009), suggesting specific actions on MOR and not on downstream effectors such as G-protein activated inwardly rectifying potassium channels that carry the described potassium conductance. However, multiple $G_{i/o}$ coupled GPCR in the LC share signaling components, and there is evidence for heterologous desensitization to the α_2 adrenergic receptor after MOR activation in mouse LC based on a more sensitive assay (Dang et al., 2012). In that study the current induced by a low concentration of noradrenaline was compared before and following acute desensitization induced by ME and a component of heterologous desensitization was detected. Furthermore, heterologous desensitization of the α_2 adrenergic receptor was also shown in rats less than 20 days old in the LC (Llorente et al., 2012). Finally recent work found that chronic morphine treatment disrupted the ability of the GPCR kinase G protein coupled receptor kinase (GRK2/3) blocker, compound 101, to inhibit the recovery from MOR desensitization as well as the acute desensitization of the somatostatin receptor (Leff et al., 2020).

The mechanism underlying increased desensitization and slowed recovery from desensitization after chronic morphine treatment is phosphorylation dependent. In animals expressing total phosphorylation deficient (TPD) MORs, acute desensitization of MORs is blocked and the recovery from desensitization is faster compared to WT animals chronically treated with morphine (80 mg/kg/day, Arttamangkul et al., 2018). The kinase that is mainly responsible for acute desensitization of MOR is the GPCR kinase, GRK2/3, and blockade of GRK2/3 can nearly abolish desensitization (Doll et al., 2012; Lowe et al., 2015; Miess et al., 2018). However, inhibition of GRK2/3 after chronic morphine treatment was no longer sufficient to block desensitization or recovery from desensitization (Leff et al., 2020). Additionally, inhibitors of kinases including GRK2/3, PKC, and JNK were required to block desensitization, suggesting that chronic morphine treatment led to adaptations that induced functional adaptations of other kinases (Leff et al., 2020).

PRESYNAPTIC ADAPTIVE MECHANISMS

Tolerance to opioids measured at the presynaptic level has been examined for decades beginning with early studies with the guinea pig ileum and mouse vas deferens. Following chronic morphine treatment there was a rightward shift in the concentration response curve that resulted from a reduction in MOR receptor reserve (Chavkin and Goldstein, 1984). This study was the prelude to others that indicated that a reduction in receptor reserve may be a common mechanism that underlies cellular tolerance. Although it is possible that there is a reduction in receptor number, a more likely explanation is that there is a decrease in the receptor/effector coupling.

Acute regulation of MORs upon agonist binding in the presynaptic terminal region of neurons is generally thought to utilize different mechanism than postsynaptic receptors. One key difference is that no acute desensitization was detected following application of a saturating concentration of agonist (Blanchet and Lüscher, 2002; Fyfe et al., 2010; Pennock et al., 2012; Jullié et al., 2020). The mechanisms underlying this lack of desensitization are not fully known, but recent work using single particle tracking has demonstrated that presynaptic MORs are phosphorylated and internalized, but are rapidly replaced at sites of transmitter release by lateral diffusion of extrasynaptic axonal receptors (Jullié et al., 2020). The extrasynaptic MORs were not subject to phosphorylation or internalization such that they are poised to replenish receptors at the sites of transmitter release.

One hallmark of downstream adaptive mechanism following long-term opioid exposure is the compensatory upregulation of adenylyl cyclase (Sharma et al., 1975; Terwilliger et al., 1991; Avidor-Reiss et al., 1995). The functional consequence of the increase in adenylyl cyclase activity is an over recovery of cAMP-dependent processes that remained in the continued presence of opioids (Sharma et al., 1975). Upon removal of morphine there was a marked overshoot in the production of cAMP and was implicated in a cellular form of acute opioid withdrawal. The role of adenylyl cyclase following chronic morphine treatment has been examined at multiple synapses (Bonci and Williams, 1996, 1997; Chieng and Williams, 1998; Ingram et al., 1997; Vaughan et al., 1997; Shoji et al., 1999). The increase in cAMP production has two downstream consequences. First is increased activation of PKA that augments transmitter release (Bonci and Williams, 1996, 1997; Chieng and Williams, 1998; Ingram et al., 1998) and through the addition of opioid sensitive adenylyl cyclase increased the inhibition mediated by opioids upon withdrawal (Ingram et al., 1998). Second, cAMP is metabolized in the extracellular space to adenosine (Brundege et al., 1997). The increase in extracellular adenosine then acts on adenosine A1 receptors to decrease transmitter release (Matsui et al., 2014). This modulation of adenosine by opioids is synapse specific (Brundege and Williams, 2002a) and could be dependent on the location of adenosine release in a given synapse (Adhikary et al., 2022b). Additionally, chronic morphine treatment can also increase the sensitivity of adenosine to A1 receptors (Brundege and Williams, 2002b). The increase in transmitter release in the continued presence of opioids is viewed as an adaptive mechanism that counters opioid induced inhibition of release and represents a form of cellular tolerance. Upon withdrawal of opioids the rise in extracellular adenosine to depress transmitter release is thought to represent a mechanism that reduces the signs of acute opioid withdrawal.

ADAPTIVE MECHANISMS FOLLOWING CHRONIC TREATMENT WITH AGONISTS OF VARYING POTENCY AND EFFICACY

It is established that different agonists induce distinct patterns of analgesic tolerance *in vivo*. By reducing the number of functional receptors with an irreversible antagonist of MORs (β-chlornaltrexamine, β-CNA), the analgesic efficacy in the whole animal was determined measuring the antinociceptive effect a number of opioids after partial irreversible antagonism. High-efficacy agonists require fewer receptors to produce antinociception and are therefore less affected by partial irreversible block with β-CNA, than the antinociceptive response for low-efficacy agonists (Kumar et al., 2008; Madia et al., 2009; Sirohi et al., 2009). These studies have found that fentanyl has the greatest relative efficacy, followed by etorphine, methadone and morphine, hydromorphone, oxycodone, and lastly hydrocodone. Relative efficacy also correlated with analgesic tolerance with low-efficacy agonists like morphine and oxycodone inducing greater tolerance more rapidly than high-efficacy agonists like etorphine and fentanyl (Walker and Young, 2001; Grecksch et al., 2006; Pawar et al., 2007; Kumar et al., 2008). Additionally, high dose etorphine, but not morphine or oxycodone, induced a substantial upregulation of dynamin-2, leading to downregulation of MORs (Pawar et al., 2007).

The mechanism underlying agonist specific *in vivo* tolerance is largely unknown, however, work in brain slice experiments from LC neurons have found that opioid agonists with different potencies and efficacies exert unique their effects on the MOR activation and regulation (Virk and Williams, 2008; Quillinan et al., 2011; Adhikary et al., 2022a). In rats treated with morphine, the acute decline of peak current by ME and morphine was facilitated and recovery from desensitization was reduced compared to untreated animals (Dang and Williams, 2004, 2005). The enhancement of desensitization suggests that after chronic treatment a subsequent desensitizing stimulus causes a greater uncoupling of MORs from its effectors compared to untreated animals. Rats chronically treated with methadone also had increased desensitization, and the concentration-response curve of ME was right-shifted twofold, but the recovery from desensitization was the same as in untreated animals (Quillinan et al., 2011). In experiments with rats chronically treated with oxycodone there was no rightward shift in the concentration-response curve to ME or oxycodone. There was also no change in the extent of desensitization or the rate of recovery from desensitization (Adhikary et al., 2022a). There was a rightward shift in the concentration response to ME in rats treated with fentanyl and in increase in the extent of desensitization (Adhikary et al., 2022a). These data support a critical role of agonist efficacy in mediating cellular tolerance after chronic treatment.

It is important to note that, the induction of tolerance to morphine on single cells in the LC required sustained treatment. Animals treated for 1 day with morphine did not exhibit any form or tolerance nor was the recovery from acute desensitization affected (Quillinan et al., 2011). In addition, the decrease in the rate and extent of recovery from acute desensitization in slices taken from morphine treated animals was not dependent on the dose of morphine applied using the osmotic mini pump (Quillinan et al., 2011). The conclusion is that continued signaling, even at a low level, was required to induce tolerance to morphine. Although the same result was not induced by chronic treatment with methadone, unlike treatment with morphine, it is possible that tolerance to methadone requires more than one week.

Cellular tolerance as measured by upregulation of second messengers also is agonist specific. Chronic morphine treatment resulted in a functional upregulation of PKC and JNK, resulting in these kinases contributing to desensitization of MOR and Somatostatin receptors (Leff et al., 2020). Curiously, even without inducing changes to MOR desensitization and tolerance, chronic oxycodone treatment (30 mg/kg/day) resulted in changes in the kinase dependence of somatostatin receptor desensitization (Adhikary et al., 2022a). Thus the continued signaling of MOR by oxycodone induced an adaptation downstream that altered the desensitization of somatostatin receptors. One possible explanation is that persistent MOR signaling with agonists that do induce desensitization or internalization had cellular effects unrelated to receptor dependent tolerance. This observation was based on experiments where animals were treated with fentanyl, a highly efficacious internalizing agonist. In those experiments, tolerance was measured by measuring a rightward shift in the concentration response curve to ME and demonstrated that chronic treatment with fentanyl (1.5 mg/kg/day) induced tolerance at the receptor level but did not cause an alteration in the kinase regulation of the somatostatin (SST) receptor (Adhikary et al., 2022a).

The role of phosphorylation of the C terminus induced by fentanyl after chronic treatment was examined with experiments using the expression of MORs where all phosphorylation sites on the C terminus were mutated to alanine (TPD-MORs). Treatment of animals with fentanyl expressing the TPD-MORs resulted in an altered kinase regulation of the somatostatin receptor, unlike experiments with wild-type MORs (Adhikary et al., 2022a). The experiment supported the role of continued signaling as a key mechanism that underlies the regulation of kinase dependent desensitization of GPCRs. Equally possible is that receptor dependent desensitization and internalization prevents the induction of altered kinase regulation of GPCRs by a downstream mechanism unrelated to acute signaling. The precise mechanisms that underly the induction of receptor dependent tolerance and adaptations that affect downstream processes at the cellular level are not known. It is however clear that the two processes are agonist dependent in the LC.

CONCLUSION

Ultimately, how different agonists mediate regulation of MORs after chronic treatment, and therefore, the combination of receptor and cellular dependent tolerance are not fully understood. It is also not known if agonist efficacy or some other regulatory property of an agonist plays a role in mediating tolerance at the level of the receptor. For example, tolerance is induced by chronic treatment with morphine in spite of the fact that it is relatively inefficient at inducing desensitization and internalization. The idea that cellular tolerance is dependent on internalization was suggested by experiments where, morphine-bound MORs on the plasma membrane were phosphorylated and presumed desensitized (Zhang et al., 1998; Koch et al., 2001, 2005). Therefore, one theory of tolerance postulates that the lack of internalization, and consequently reduced recovery from desensitization, contributes to tolerance. A second theory states that the lack of internalization induced by morphine leads to continuous and persistent signaling, resulting in counter regulatory adaptations (Whistler and von Zastrow, 1998; Whistler et al., 1999). It is possible that both persistent signaling and decoupling of MORs from effectors contribute to cellular tolerance but it is clear that tolerance measured at the cellular level is only one component of the tolerance that is measured in living animals. The future understanding of tolerance will require work that connects cellular tolerance at the cellular and synaptic level in single neurons with whole animal work. What is known in the LC is a start but a complete understanding can only be accomplished through cellular and synaptic work in multiple areas of the CNS. Synapse specific effects of acute opioid actions are underway (Birdsong et al., 2019), but there is much to be done.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Nucleus Tractus Solitarius Neurons Activated by Hypercapnia and Hypoxia Lack Mu Opioid Receptor Expression

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Impaired chemoreflex responses are a central feature of opioid-induced respiratory depression, however, the mechanism through which mu opioid receptor agonists lead to diminished chemoreflexes is not fully understood. One brainstem structure involved in opioid-induced impairment of chemoreflexes is the nucleus of the solitary tract (NTS), which contains a population of neurons that express mu opioid receptors. Here, we tested whether caudal NTS neurons activated during the chemoreflex challenge express mu opioid receptors and overlap with neurons activated by opioids. Using genetic labeling of mu opioid receptor-expressing neurons and cFos immunohistochemistry as a proxy for neuronal activation, we examined the distribution of activated NTS neurons following hypercapnia, hypoxia, and morphine administration. The main finding was that hypoxia and hypercapnia primarily activated NTS neurons that did not express mu opioid receptors. Furthermore, concurrent administration of morphine with hypercapnia induced cFos expression in non-overlapping populations of neurons. Together these results suggest an indirect effect of opioids within the NTS, which could be mediated through mu opioid receptors on afferents and/or inhibitory interneurons.

Keywords: opioid, nucleus of the solitary tract, respiratory depression, hypercapnia, hypoxia

INTRODUCTION

The primary cause of death from illicit opioid use is respiratory depression caused by the activation of mu opioid receptors (MORs) in various brainstem respiratory nuclei (Dahan et al., 2001; Bateman et al., 2021). Opioid-induced respiratory depression presents with slow and irregular breathing due to inhibition in rhythmogenic and pattern-modulating respiratory nuclei (Palkovic et al., 2020; Bateman et al., 2021; Ramirez et al., 2021). This decrease in ventilation leads to decreased blood concentrations of O_2 and increased levels of CO_2 (Macintyre, 2001; Pattinson, 2008). Additionally, opioids also affect the hypoxic and hypercapnic chemoreflexes due to the activation of MORs, which further exaggerates opioid effects on breathing (Weil et al., 1975; Dahan et al., 2001; May et al., 2013).

Several opioid-sensitive respiratory nuclei have been implicated in the hypoxic and hypercapnic ventilatory chemoreflex responses, including the nucleus of the solitary tract (NTS) (Coates et al., 1993; Nattie and Li, 2002, 2008; Zhang et al., 2011; Zhuang et al., 2017). The NTS contains CO_2-sensitive neurons (Dean et al., 1989; Nichols et al., 2009a) and is also the site where chemoreceptor afferents from oxygen-sensitive carotid bodies first synapse before this information is relayed from second-order neurons to upstream respiratory regions (Andresen and Kunze, 1994; Kline et al., 2010; King et al., 2012; Zoccal et al., 2014). Hypercapnia and hypoxia elicit expression of the immediate early gene, cFos, as an indicator of recent neural activity in the NTS (Jansen et al., 1996; Teppema et al., 1997; Ohtake et al., 2000; Tankersley et al., 2002; King et al., 2012).

The NTS abundantly expresses MORs (Mansour et al., 1994; Zhuang et al., 2017). Microinjection of the MOR agonist DAMGO in the caudomedial portion of the NTS inhibits both the hypercapnic and hypoxic ventilatory response in rats, which is blocked by the selective MOR antagonist CTAP (Zhang et al., 2011; Zhuang et al., 2017). Whether this inhibition is caused by somatodendritic MORs or presynaptic MORs on afferent terminals is unknown. In addition, systemic administration of morphine has been shown to induce cFos expression in the NTS, indicating possible activation of NTS neurons by opioids (Hammond et al., 1992; Grabus et al., 2004; Salas et al., 2013).

Despite significant advances in our understanding of opioid-induced respiratory depression, the mechanisms through which MOR agonism leads to impaired chemoreflexes are not well-understood. Here, we sought to assess whether NTS neurons activated during chemoreflex ventilatory responses express MORs and whether morphine would reduce hypercapnia-mediated activation. We examined the overlap between chemoreflex-sensitive neurons and opioid-sensitive neurons in the NTS by measuring cFos expression as a proxy for neuronal activation following exposure to moderate hypercapnia, hypoxia, or morphine in mice with fluorescently tagged MOR-expressing neurons. Our results imply that while a small portion of MOR-expressing neurons is activated by hypercapnia and hypoxia, the majority of chemoreflex-activated NTS neurons are not directly opioid-sensitive.

METHODS

Animals

All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida and were in agreement with the National Institutes of Health "Guide for the Care and Use of Laboratory Animals." Homozygous $Oprm1^{Cre/Cre}$ mice (Liu et al., 2021) (Jackson Labs Stock #035574) were crossed with homozygous Ai9-tdTomato Cre reporter mice (Jackson Labs Stock #007909) to generate $Oprm1^{Cre/tdT}$ mice. $Oprm1^{Cre/tdT}$ mice (male and female, 2–6 months old) and wild-type C57BL/6J mice (male and female, 2–7 months old) were used for all experiments. Experimental groups were counterbalanced for age and sex. No apparent age or sex-dependent differences were observed, so data were pooled. All mice were bred and maintained at the University of Florida animal facility. The mice were group-housed in standard-sized plastic cages and kept on a 12-h light/dark cycle, with water and food available *ad libitum*.

Drugs

Morphine sulfate was obtained from the National Institute on Drug Abuse Supply Program (RTI International, Research Triangle Park, NC).

Chemoreflex and Morphine Challenges

Chemoreflex challenges were performed in two phases in separate cohorts of mice. The first phase utilized Buxco whole-body plethysmography chambers (Buxco Electronics Ltd., NT, United States). In this phase, $Oprm1^{Cre/tdT}$ mice were acclimated to the chambers ventilated (0.5 L/min) with standard compressed room air for 1 h per day for 3 consecutive days prior to experimentation. On experiment day, mice were placed in the chambers and given a 15-min acclimation period with standard compressed air. Following this acclimation period, mice were exposed to either standard compressed air, a hypoxic challenge (10% O_2, 90% N_2), or a hypercapnic challenge (7% CO_2, 21% O_2, 72% N_2), for 60 min. Animals were then removed from the chambers and placed in their home cage for 60 min prior to perfusion.

The second phase of chemoreflex challenges was conducted using vivoFlow whole-body plethysmography chambers (SCIREQ Inc, Montreal, QC, Canada). The mice were handled and exposed to the whole-body plethysmography system ventilated (0.5 L/min) with standard air for 1 h a day for 3 consecutive days immediately prior to experimentation. $Oprm1^{Cre/tdT}$ and C57BL/6J wild-type mice were grouped into one of four conditions: saline injection (10 μl/g, i.p.) with standard air, saline injection with hypercapnic air (7% CO_2, 21% O_2, 72% N_2), morphine injection (30 mg/kg, i.p.) with standard air, or morphine injection with hypercapnic air. On experimentation day, the mice were acclimated to the chambers for 15 min, injected with saline or morphine and exposed to standard air or hypercapnic air for 60 min. The mice were then returned to their home cages for 30 min prior to perfusion.

Plethysmography

During the second phase of challenges, recordings of respiratory frequency and estimated tidal volume were collected using vivoFlow whole-body plethysmography and IOX2 software (SCIREQ Inc, Montreal, QC, Canada) 10–30 min post-saline or morphine (30 mg/kg) injection in mice breathing standard air or hypercapnic air. To calibrate volume changes, 10 ml of air was injected into the chambers using a 10 ml syringe prior to each recording session, in accordance with the manufacturer's instructions. Tidal volume was calculated from the integral of the inspiratory time. It is important to note that body temperature was not recorded, so tidal volume measurements are estimates. Since tidal volume is used in the calculation of minute ventilation, these measurements are also estimates. The chambers were ventilated with a constant airflow of 0.5 L/min of standard air or hypercapnic air (7% CO_2), as described above. All plethysmography experiments were conducted at room

temperature without thermoregulatory compensation. Potential breaths were rejected if the ratio of inspiratory to expiratory volume was below 70%.

Immunohistochemistry

Following experimental exposures, the mice were deeply anesthetized using isoflurane and transcardially perfused with PBS followed by 10% formalin. Brains were removed and post-fixed overnight. Brains were then cryoprotected in 10% sucrose/PBS solution, followed by 20% sucrose/PBS solution. Free-floating coronal sections (40 μm) containing caudal NTS [-7.56 to−7.76 mm caudal to bregma (Franklin and Paxinos, 2008)] were prepared with a cryostat and stored in PBS at 4°C until staining.

The free-floating sections were washed and permeabilized with PBS-T (0.3% TritonX-100), blocked in 3% NGS in PBS-T for 1 h and incubated in primary antibody (rabbit anti-cFos [Abcam, ab190289] 1:2000 in blocking buffer) overnight. The sections were then washed in PBS-T and incubated in secondary antibody (goat anti-rabbit AlexaFluor 488 [Invitrogen, A11008] 1:500) for 2 h. The sections were washed and rinsed once in ddH_2O before mounting with Flouromount-G DAPI (ThermoFisher) mounting medium. The sections were imaged using a confocal laser scanning microscope (Nikon A1R) with a 10X objective (N. A. 0.3).

Image Processing and Cell Counting

Image processing was performed in FIJI (Schindelin et al., 2012) and the interactive machine learning software Ilastik (Berg et al., 2019). For each image, maximum intensity projections were generated using FIJI and imported into Ilastik. In Ilastik, a segmentation algorithm was manually trained on the set of images. Under this framework, all images analyzed with a particular algorithm receive identical treatment.

Ilastik was used to calculate features related to pixel intensity, edges, and texture for each pixel at seven different radii (0.3, 0.7, 1.0, 1.6, 3.5, 5.0, 10.0). The features calculated included: Gaussian Smoothing (intensity), Laplacian of Gaussian, Gaussian Gradient Magnitude, and Difference of Gaussians (edge detection), and Structure Tensor Eigenvalues and Hessian of Gaussian Eigenvalues (texture). The segmentation algorithm was trained on the complete set of max-intensity projections for a given region, using experimenter annotations to label a subset of pixels in each image as "cell" or "background." A parallel random forest (VIGRA) algorithm predicts the probability that the remaining pixels are "cell" or "background" based on these annotations and the 42 calculated features.

After training, the algorithm exports a probability map for each image, representing the likelihood that each pixel constitutes part of a cell. The max-intensity projections and corresponding probability maps were then loaded into an Ilastik Object Classification Workflow, where probability thresholding and size filters were used to identify cells. Random images and the corresponding binary images were reviewed by a blinded experimenter observer to verify the accuracy of the algorithm.

The number of cFos+, tdTomato+ or co-labeled cells was determined in the caudal NTS (-7.56 to−7.76 mm caudal to bregma) for each section (2–6 sections/mouse) and averaged to determine the mean # of cFos+, tdTomato+ or co-labeled cells/section for each mouse. N values reported in Results represent the number of mice per group.

Statistics

All statistical analyses were performed in GraphPad Prism 8. Error bars represent the standard error of the mean (SEM). Data with $n > 7$ were tested for normality with D'Agostino and Pearson normality test. For normally distributed data and data with $n \leq 7$, comparisons between two groups were made using unpaired Student's two-tailed *t*-test. Comparisons between three or more groups were made using two-way ANOVA followed by Holm-Sidak *post hoc* test.

RESULTS

The Hypercapnic Ventilatory Response Is Suppressed by Morphine

Opioids attenuate the hypercapnic ventilatory response (HCVR) mediated by the NTS (Zhang et al., 2011; Zhuang et al., 2017), but whether this inhibition is caused by somatodendritic MORs or presynaptic MORs on afferent terminals is unknown. To begin to answer this question, wild-type mice were exposed to standard air or hypercapnia (7% CO2) following an injection of saline or morphine (30 mg/kg). The ventilatory effects of morphine and hypercapnia were verified using whole-body plethysmography (**Figure 1**). In the mice exposed to standard air ($n = 11$), morphine significantly reduced breathing frequency (saline: 264 ± 8 bpm vs. morphine: 152 ± 11 bpm, $p < 0.0001$ by two-way ANOVA and Holm-Sidak *post hoc* test, **Figure 1A**), but not tidal volume or minute ventilation (tidal volume = saline: 6.7 ± 0.5 ml/kg vs. morphine: 8.3 ± 0.5 ml/kg, $p = 0.212$ by two-way ANOVA and Holm-Sidak *post hoc* test, **Figure 1B**; minute ventilation = saline: 1.8 ± 0.1 ml/min/g vs. morphine: 1.2 ± 0.1 ml/min/g, $p = 0.124$ by two-way ANOVA and Holm-Sidak *post hoc* test, **Figure 1C**). Hypercapnia-induced increases in minute ventilation, breathing frequency, and tidal volume were all significantly depressed by morphine (minute ventilation in saline 4.7 ± 0.4 ml/min/g vs. morphine 2.5 ± 0.3 ml/min/g, $p < 0.0001$ by two-way ANOVA and Holm-Sidak post-test; frequency in saline 358 ± 9 bpm vs. morphine 251 ± 3 bpm, $p < 0.0001$ by two-way ANOVA and Holm-Sidak post-test; tidal volume in saline 13.0 ± 1.1 ml/kg vs. morphine 10.1 ± 1.0 ml/kg, $p = 0.0407$ by two-way ANOVA and Holm-Sidak post-test; $n = 10$ mice), consistent with established effects of morphine on the HCVR in mice and humans (Weil et al., 1975; Dahan et al., 2001).

NTS cFos Expression Induced by Hypercapnia

We next examined the expression of cFos, as a proxy for neuronal activation, in the NTS of WT mice exposed to standard air or hypercapnia. The NTS of saline-treated, standard air-exposed mice ($n = 6$ mice, 5-6 sections/mouse) contained a low number of cFos-expressing cells, scattered throughout the region (9 ± 3 cFos+ cells/section; **Figures 2A,E**). The number of cFos+ cells was significantly increased in saline-treated mice that underwent

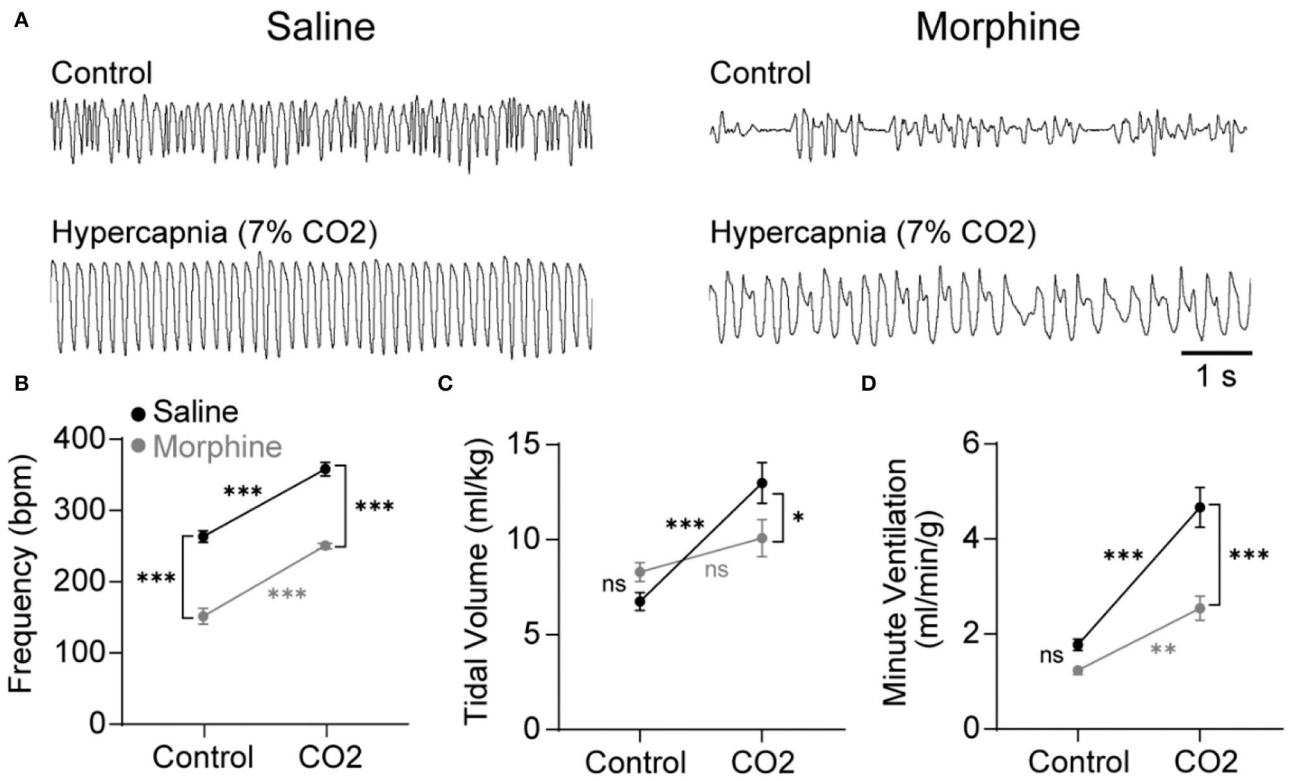

FIGURE 1 | Morphine suppresses breathing during control and hypercapnic conditions in wild-type mice. Respiration was measured during control conditions (standard room air) or hypercapnia challenge (7% CO_2) using whole-body plethysmography following an injection of saline (black symbols) or morphine (30 mg/kg; gray symbols). **(A)** Representative plethysmography traces during each of the four conditions. Scale bar applies to all traces. Inspiration is downward. **(B–D)** Saline control $n = 11$, morphine control $n = 11$, saline hypercapnia $n = 10$, morphine hypercapnia $n = 10$. Data are graphed as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.0001$, ns $p > 0.05$ by two-way ANOVA and Holm-Sidak multiple comparison's test.

a hypercapnic challenge ($n = 7$ mice, 2-6 sections/mouse: 17 ± 2 cFos+ cells/section, $p = 0.037$, unpaired t-test; **Figures 2B,E**), consistent with existing literature (Jansen et al., 1996; Teppema et al., 1997; Tankersley et al., 2002).

NTS cFos Expression Induced by Acute Morphine

In mice exposed to standard air, morphine significantly increased the number of cFos-expressing cells in the NTS relative to saline treatment ($n = 6$ mice, 2-6 sections/mouse, 101 ± 6 cFos+ cells/section, $p < 0.0001$ by unpaired t-test, **Figures 2C,F**). These data indicate that the NTS contains a large proportion of neurons that express cFos in response to morphine administration. These neurons may or may not play a role in the HCVR. We next measured cFos expression in morphine-injected mice that were exposed to hypercapnia ($n = 9$ mice, 4–6 sections/mouse). Hypercapnia further increased the number of cFos+ cells in morphine-treated mice compared to morphine treatment in standard air (125 ± 7 cFos+ cells/section, $p = 0.026$ by unpaired t-test, **Figures 2D,F**). The results imply that hypercapnia recruits an additional population of NTS neurons that do not overlap with those activated under the influence of morphine alone.

Hypercapnia Induces cFos Expression in MOR-Negative Cells

To determine whether cells activated by hypercapnia express MORs, we crossed $Oprm1^{Cre/Cre}$ mice with Ai9 tdTomato Cre-reporter mice to generate $Ai9^{tdT/+}$::$oprm1^{Cre/+}$ mice (hereby referred to as $Oprm1^{Cre/tdT}$ mice) which express tdTomato in MOR-expressing cells. We exposed $Oprm1^{Cre/tdT}$ mice to standard air or hypercapnia (7%) and identified cFos+, tdTomato+, and co-labeled cells in the NTS (**Figure 3**). There was no significant difference in the average number of tdTomato+ neurons in the NTS of hypercapnia-exposed ($n = 8$ mice, 5-6 sections/mouse) and standard air-exposed ($n = 7$ mice, 2-6 sections/mouse) mice (hypercapnia: 218 ± 39 cFos+ cells/section vs. standard air: 179 ± 20 cFos+ cells/section, $p = 0.40$ by unpaired t-test). In both groups, tdTomato expression in the NTS occurred in both neuronal cell bodies and neurites (**Figures 3A,C**), indicating MOR expression in afferents in the NTS, as well as NTS neurons themselves. Consistent with results from wild-type mice, there were significantly more cFos+ cells in the NTS of hypercapnia-exposed mice relative to standard room air-exposed mice (hypercapnia: 18 ± 3 cFos+ cells/section vs. control: 8 ± 3 cFos+ cells/section, $p = 0.030$ by unpaired t-test, **Figure 3D**). There was also a significantly

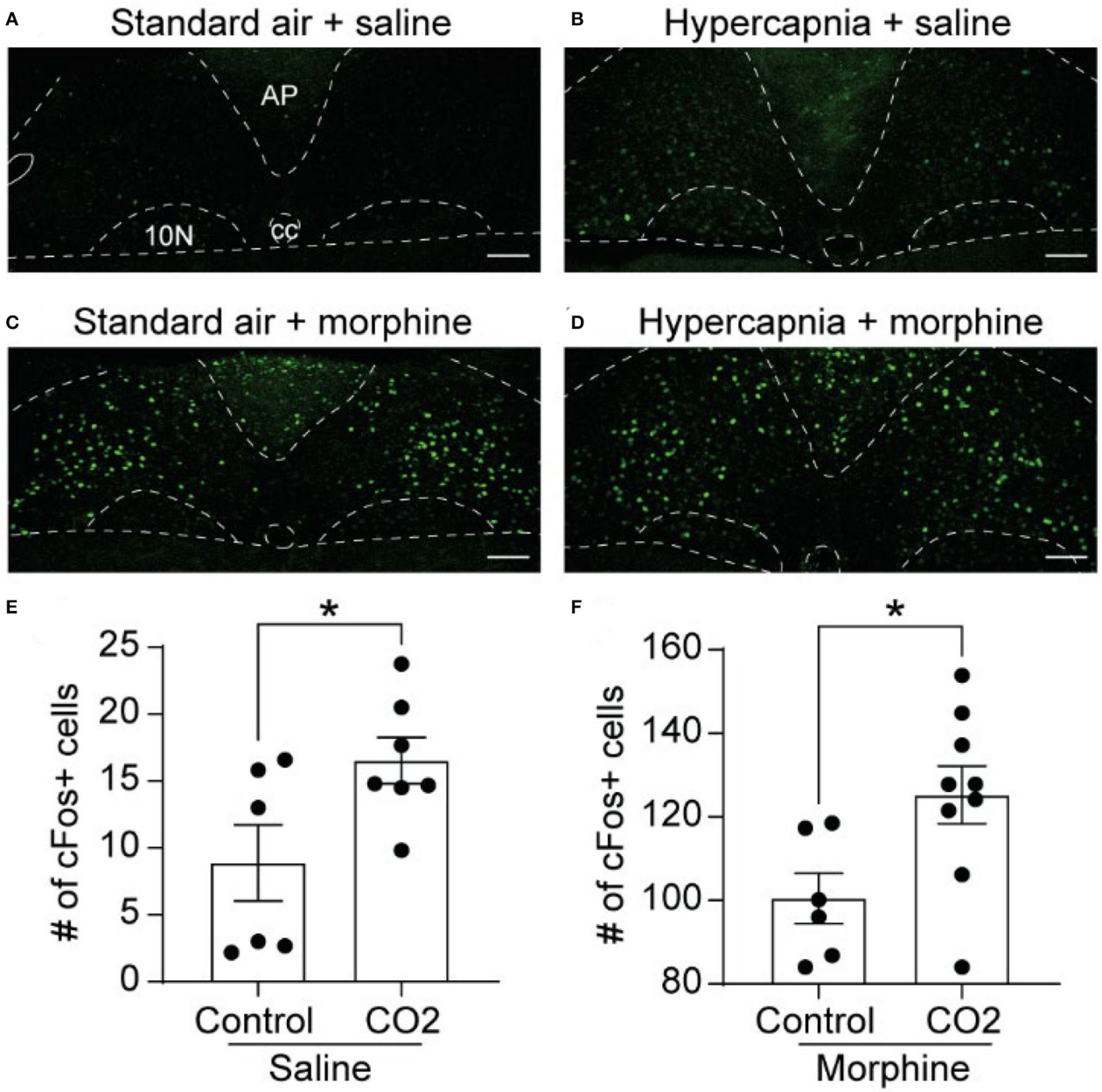

FIGURE 2 | Hypercapnia and morphine activate non-overlapping populations of NTS neurons. Wild-type mice were exposed to standard room air (Control) or hypercapnia (7% CO_2) following an injection of saline or morphine (30 mg/kg). **(A–D)** Example images of cFos immunolabeling (green) in NTS sections from mice exposed to each of the four treatment groups. Scale bar = 100 μm. AP, area postrema; cc, central canal; 10N, dorsal motor nucleus of vagus. **(E,F)** Summary data of the average number of cFos+ NTS cells per section (n = 6–9 mice/group, 2–6 sections/mouse). Hypercapnia increased the number of cFos+ cells in saline **(E)** and morphine **(F)** treated mice. Each data point represents the average # of cFos+ cells per section for an individual mouse. Bar and error are group mean ± SEM. *p < 0.05 by unpaired t-test.

higher number of cFos+/tdTomato+ co-labeled cells in the hypercapnia group relative to the control group (hypercapnia: 1.3 ± 0.4 co-labeled cells/section vs. control: 0.4 ± 0.1 co-labeled cells/section, p=0.044 by unpaired t-test, **Figure 3E**), indicating that some MOR-expressing NTS neurons participate in the HCVR. However, the number of cFos+/tdTomato+ co-labeled cells was very low in both groups. Only 7.6% of cFos+ cells were MOR+, and fewer than 1% of MOR+ cells were cFos+. Taken together, these data suggest that very few of the cells activated by hypercapnia are MOR+, implying that

opioid inhibition of the hypercapnic ventilatory response at the level of the NTS is likely mediated by presynaptic, rather than somatodendritic inhibition.

Hypoxia Induces cFos Expression in MOR-Negative Cells

Opioid-induced hypoventilation produces oxygen desaturation, in addition to the accumulation of CO2. Activation of MORs in the NTS can also significantly impair the hypoxic ventilatory response (Zhang et al., 2011; Zhuang et al., 2017). To determine whether cells activated by hypoxia express MORs, we exposed Oprm1$^{Cre/tdT}$ mice to standard air or hypoxia (10%) and identified cFos+, tdTomato+, and co-labeled cells in the NTS (**Figure 4**, $n = 6$ mice/group, 4-6 sections per mouse). Consistent with prior studies (Teppema et al., 1997; Ohtake et al., 2000; King et al., 2012), there was a significantly higher number of cFos+ cells in the NTS of hypoxia-exposed mice relative to standard air-exposed mice (hypoxia: 35 ± 4 cFos+ cells/section vs. control: 11 ± 7 cFos+ cells/section, $p = 0.017$ by unpaired *t*-test, **Figure 4D**). However, there was no significant difference in the number of cFos+/tdTomato+ co-labeled cells between the hypoxia and control groups (hypoxia: 0.6 ± 0.2 cFos+ cells/section vs. control: 0.2 ± 0.2 cFos+ cells/section, $p = 0.244$ by unpaired *t*-test, **Figure 4E**). The number of co-labeled cells was very low in both standard air and hypoxia-exposed mice. Fewer than 5% of cFos+ cells were MOR+, and fewer than 1% of MOR+ cells were cFos+. Taken together, these data suggest that very few of the cells activated by hypoxia are MOR+, implying that opioid inhibition of the hypoxic ventilatory response at the level of the NTS is also likely mediated by presynaptic, rather than postsynaptic inhibition.

Morphine Induces cFos Expression in MOR-Negative and MOR-Positive Cells

Since activation of MORs by morphine could directly lead to cFos expression independent of neuronal activation (Shoda et al., 2001), we next tested if NTS cells activated by morphine treatment expressed MORs ($n = 2$ mice, 3-4 sections/mouse). Only 6.2% of tdTomato+ cells was cFos+, while 15.1% of the cFos+ cells was tdTomato+ indicating they expressed MORs (**Figure 5**, 299 ± 92 tdTomato+ cells/section; 101 ± 37 cFos+ cells/section; 15 ± 5 co-labeled cFos+/MOR+ cells/section). This finding that morphine administration induces cFos expression in both MOR-positive and MOR-negative cells, suggests at least two potential pathways by which morphine can induce cFos expression in NTS cells. While some cells may express cFos due to direct signaling pathways from co-expressed postsynaptic receptors, the vast majority of cFos expression induced in the NTS by morphine is indirect, and likely due to neuronal activation.

Since Oprm1$^{Cre/tdT}$ mice lose a functional copy of MOR (Liu et al., 2021), it was important to determine if morphine is still effective in these mice. In Oprm1$^{Cre/tdT}$ mice, morphine (30 mg/kg) did cause respiratory depression compared to saline-treated controls ($n = 4$/group). Morphine reduced minute ventilation (saline-treated: 2.1 ± 0.1 ml/min/g vs. morphine-treated: 1.0 ± 0.1 ml/min/g, $p = 0.003$ by unpaired *t*-test) due to a significant reduction in breathing frequency (saline-treated: 280 ± 9 bpm vs. morphine-treated: 172 ±7 bpm, $p < 0.0001$ by unpaired *t*-test) and tidal volume (saline-treated: 7.6 ± 0.5 ml/g vs. morphine-treated: 5.7 ± 0.5 ml/g, $p = 0.029$ by unpaired *t*-test). Importantly, baseline respiration in Oprm1$^{Cre/tdT}$ mice was similar to wild-type mice (minute ventilation $p = 0.121$, frequency $p = 0.271$, tidal volume $p = 0.327$, unpaired *t*-tests). Morphine-induced respiratory depression in Oprm1$^{Cre/tdT}$ mice also manifests similarly to wild-type mice (minute ventilation $p = 0.162$, frequency $p = 0.306$, unpaired *t*-tests).

DISCUSSION

While our knowledge about the mechanisms and cellular basis of opioid-induced respiratory depression has significantly increased in recent years (Bateman et al., 2021; Ramirez et al., 2021), the mechanisms through which opioids lead to impaired chemoreflexes are not well-understood. The goal of this study was to determine the amount of overlap between chemoreflex-sensitive neurons and opioid-sensitive neurons in the NTS by measuring cFos expression as a proxy for neuronal activation in mice with fluorescently tagged MOR-expressing neurons. We hypothesized that hypercapnia would activate MOR-expressing neurons, and that morphine would reduce this hypercapnia-mediated activation. On the contrary, our results indicate that although MORs are expressed in neurons and neurites in the NTS, most neurons that are activated by hypercapnia do not express MORs. Similarly, most NTS neurons that are activated by hypoxia also do not express MORs. Thus, opioid effects on hypercapnic and hypoxic ventilatory responses in the NTS (Zhang et al., 2011; Zhuang et al., 2017) are indirectly mediated.

Morphine Activation of NTS Neurons

Opioid receptors are inhibitory G protein-coupled receptors that inhibit neuronal activity through hyperpolarization and inhibition of neurotransmitter release. Despite this, morphine significantly increased cFos expression in the NTS, consistent with previous studies (Hammond et al., 1992; Grabus et al., 2004; Salas et al., 2013). There are multiple mechanisms by which this could occur. First, opioids can excite neurons by disinhibition (i.e., inhibition of tonic GABA release) (Johnson and North, 1992; Lau et al., 2020). The NTS contains numerous GABAergic interneurons and receives GABAergic afferent projections from other areas (Fong et al., 2005; Bailey et al., 2008). The mu opioid agonist endomorphin-1 inhibits spontaneous GABAergic neurotransmission in the NTS and hyperpolarizes a portion of GABAergic NTS interneurons (Glatzer et al., 2007). In addition, endomorphin-1 inhibits solitary tract stimulation-evoked glutamate release onto GABAergic neurons in the NTS (Glatzer et al., 2007), which could also decrease GABAergic interneuron activity. Thus, disinhibition is a likely mechanism by which morphine increased cFos expression in the NTS and could lead to impairments in hypoxic ventilatory responses (Tabata et al., 2001; Chung et al., 2006). Second, MOR-coupled intracellular signaling cascades can lead to the

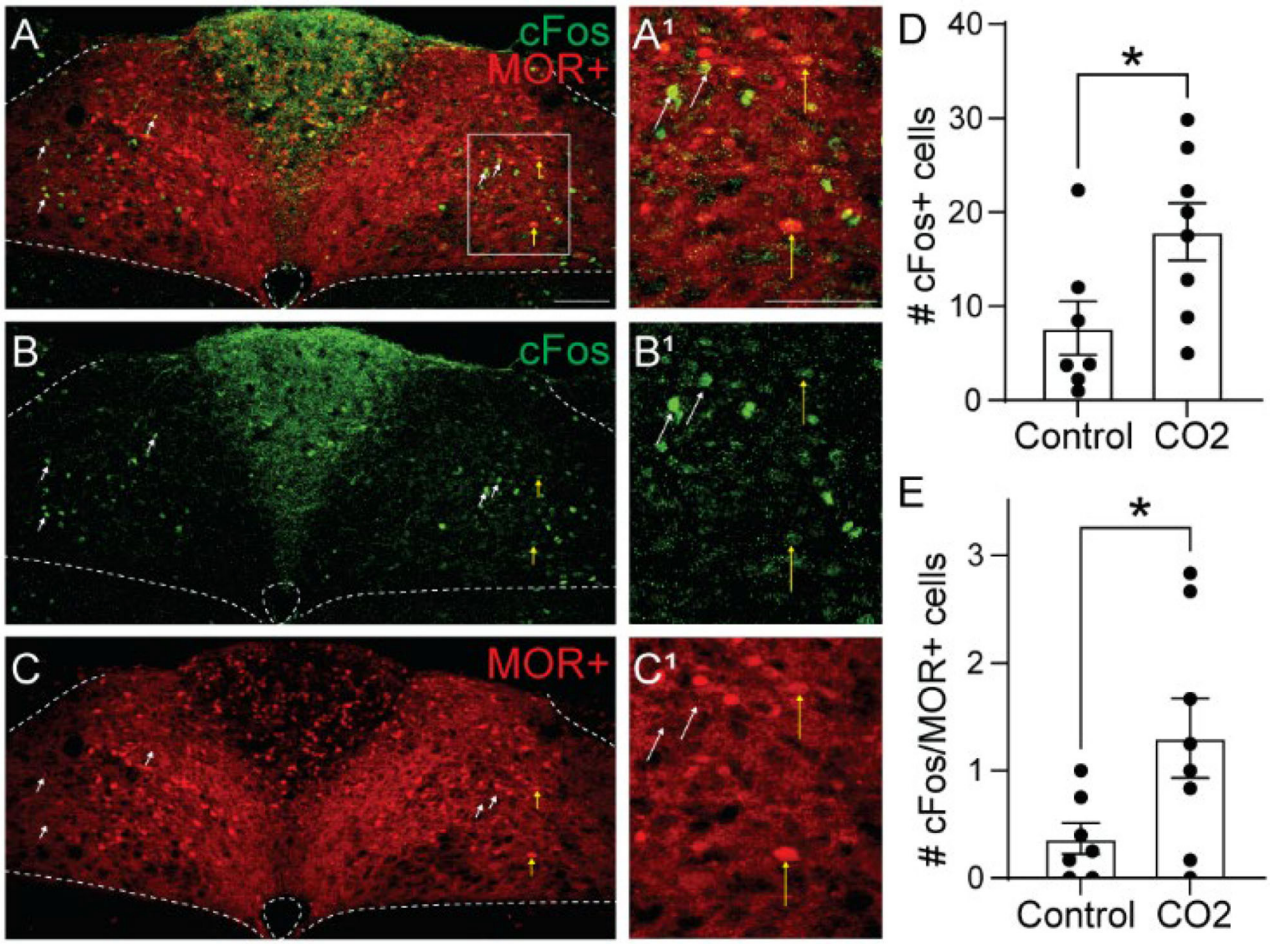

FIGURE 3 | Hypercapnia activates MOR-negative neurons. Identification of cFos immunolabeling and tdTomato expression, as an indicator of MOR expression, in the NTS of Oprm1$^{Cre/tdT}$ mice that were exposed to standard air (Control) or hypercapnic air (7% CO2). **(A–C)** Example images of cFos immunostaining (green) and MOR+ tdTomato expression (red) from a mouse exposed to hypercapnia. Very few cells co-expressed cFos and tdTomato (indicated by yellow vertical arrows). White diagonal arrows are pointing at example cells that are cFos+, but do not express MORs. **(A^1–C^1)** are zoomed in views of the boxed region in **(A–C)**. Scale bar = 100 μm. **(D,E)** Summary data of the average number of cFos+ NTS cells per section **(D)**, or the average number of co-labeled cFos+/MOR+ NTS cells per section **(E)** (n = 7–8 mice/group, 2–6 sections/mouse). Each data point represents the average # of cFos+ cells per section for an individual mouse. Bar and error are group mean ± SEM. *$p < 0.05$ by unpaired t-test.

induction of cFos expression in the absence of neuronal activation (Shoda et al., 2001). This signaling mechanism would only induce cFos expression in neurons that express MORs. Since only a small percentage of cFos expressing cells co-expressed MORs, this is likely a minor mechanism by which morphine-induced cFos expression in the NTS. Finally, since morphine reduces ventilation, which can lead to hypoxemia and accumulation of CO_2, morphine could have activated neurons through chemoreflex pathways. There were more cFos expressing neurons in the NTS from mice that received morphine and a hypercapnic challenge. Morphine exacerbation of hypercapnia may recruit additional non-opioid-sensitive CO_2-sensitive NTS neurons. Since morphine also induces hypoxemia, the recruitment of additional hypoxia-sensitive neurons is also possible. Interestingly, withdrawal from chronic morphine treatment also induces cFos expression in the NTS (Stornetta et al., 1993; Laorden et al., 2002; Mannelli et al., 2004; Benavides et al., 2005). Presumably, the neurons activated by acute morphine and morphine withdrawal should be distinct populations, but this remains to be determined.

Presynaptic MORs in the NTS

Our findings that neurons activated by hypercapnia and hypoxia do not express MORs suggest that the effects of opioid agonist in the NTS (Zhang et al., 2011; Zhuang et al., 2017) are indirect and possibly mediated by presynaptic MORs on axon terminals. The NTS contained a significant amount of MOR-expressing neurons and neurites compared to the surrounding area, consistent with previous reports (Mansour et al., 1994; Aicher et al., 2000; Zhuang et al., 2017), and a substantial amount

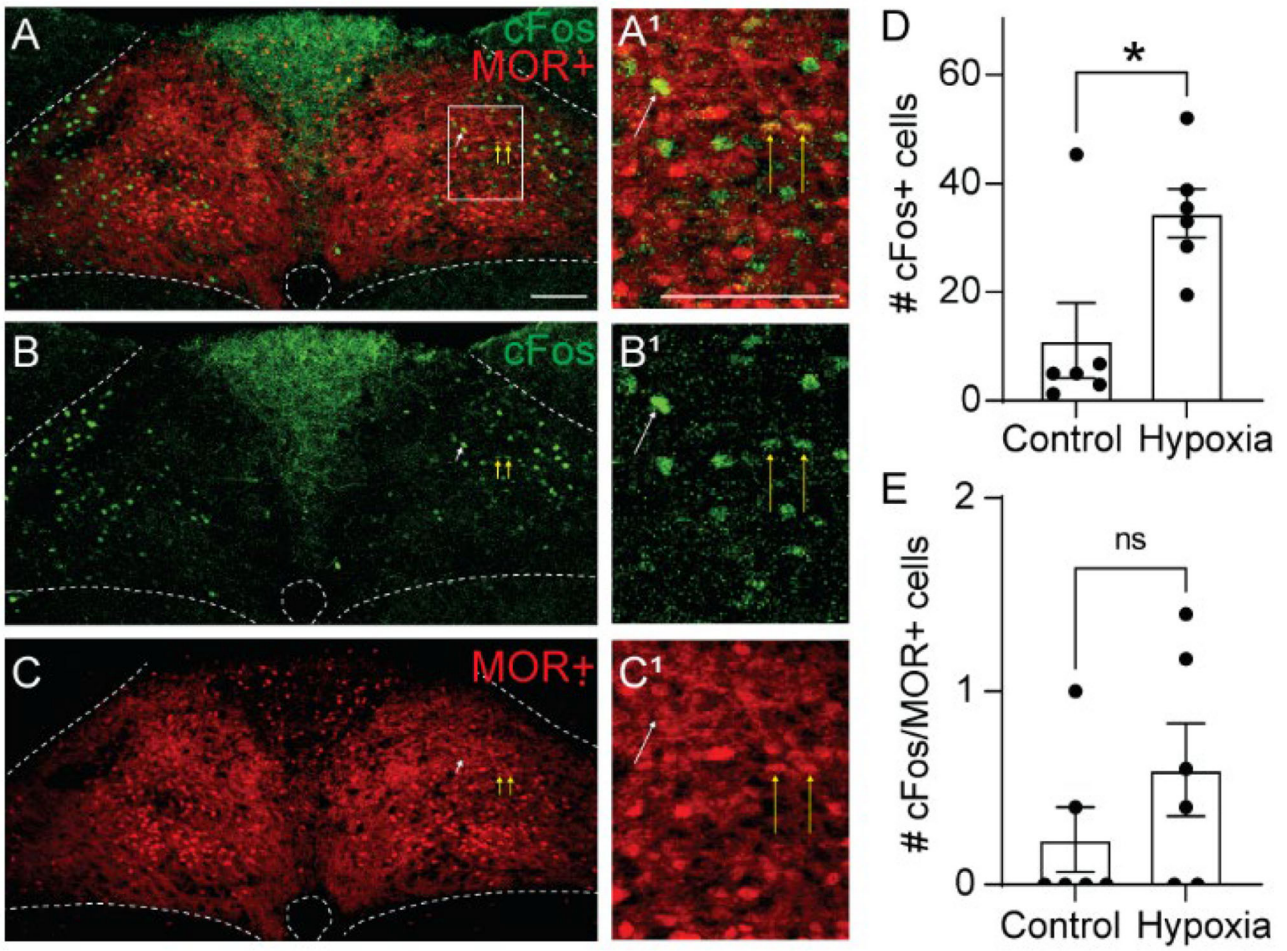

FIGURE 4 | Hypoxia activates MOR-negative neurons. Identification of cFos immunolabeling and tdTomato expression, as an indicator of MOR expression, in the NTS of Oprm1$^{Cre/tdT}$ mice that were exposed to standard air (Control) or hypoxic air (10% O2). **(A–C)** Example images of cFos immunostaining (green) and MOR+ tdTomato expression (red) from a mouse exposed to hypoxia. Very few cells co-expressed cFos and tdTomato (indicated by yellow vertical arrows). White diagonal arrows are pointing at example cells that are cFos+, but do not express MORs. **(A^1–C^1)** are zoomed in views of the boxed region in **(A–C)**. Scale bar = 100 μm. **(D,E)** Summary data of the average number of cFos+ NTS cells per section **(D)**, or the average number of co-labeled cFos+/MOR+ NTS cells per section **(E)** ($n = 6$ mice/group, 4–6 sections/mouse). Each data point represents the average # of cFos+ cells per section for an individual mouse. Bar and error are group mean ± SEM. *$p < 0.05$; ns, $p > 0.05$ by unpaired t-test.

of MOR expression in the NTS is in afferents (Aicher et al., 2000). The NTS is the first relay for several cardiorespiratory afferents, including lung and airway vagal afferents and carotid body chemoreceptor afferents (Kubin et al., 2006). Ultrastructural microscopy identified MORs on vagal afferent terminals in the medial NTS, which primarily synapsed onto non-MOR-expressing NTS neurons, suggesting that MORs modulate NTS neurons either presynaptically or postsynaptically, but not both (Aicher et al., 2000). The mu opioid agonist endomorphin-1 inhibits solitary tract stimulation-evoked glutamate release onto GABAergic neurons in the NTS, supporting the functional expression of MORs on solitary tract axon terminals (Glatzer et al., 2007). Presynaptic MORs also inhibit GABAergic and glutamatergic neurotransmission in the NTS (Rhim et al., 1993; Glatzer et al., 2007). MOR agonist injection into the NTS also inhibited bronchopulmonary C-fiber-induced reflexes in the NTS (Zhuang et al., 2017). The relative contribution of these afferent-specific presynaptic MORs in the hypercapnic and hypoxic ventilatory response remains to be determined.

Other Brain Areas Involved in Opioid Suppression of Chemoreflexes

Opioid suppression of hypercapnic and hypoxic ventilatory responses could be due to activation of MORs in other chemosensitive areas outside the NTS as well. Injection of opioid into the caudal medullary raphe suppresses the hypoxic and hypercapnic ventilatory responses in anesthetized rats (Zhang et al., 2007, 2009). In addition, locus coeruleus neurons are involved in the hypercapnic ventilatory response (Biancardi et al., 2008; Oliveira et al., 2017; Magalhães et al., 2018) and are inhibited by MORs (North and Williams, 1985; Levitt and Williams, 2012). One area that is unlikely to mediate opioid

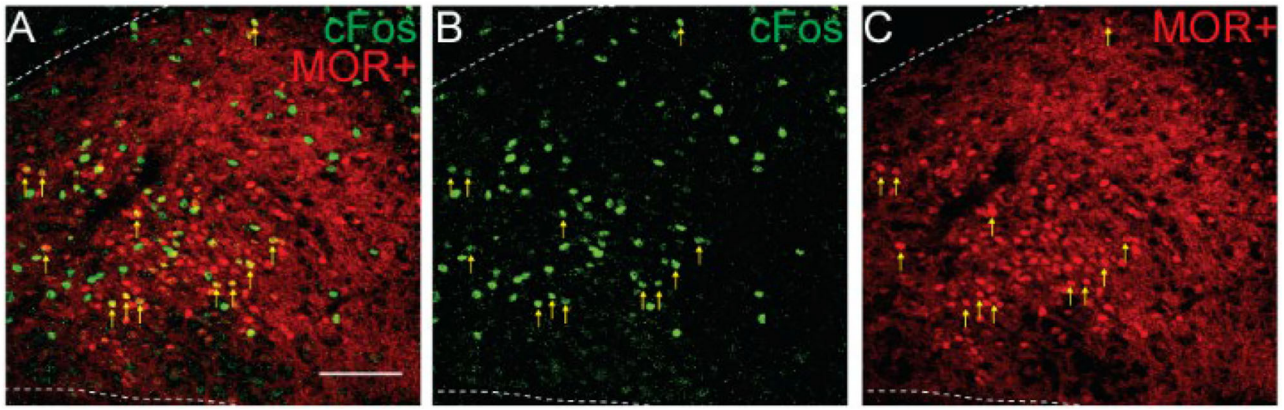

FIGURE 5 | Morphine induces cFos expression in MOR-positive and MOR-negative NTS neurons. Identification of cFos immunolabeling and tdTomato expression, as an indicator of MOR expression, in the NTS of Oprm1$^{Cre/tdT}$ mice that were injected with morphine (30 mg/kg). **(A–C)** Example images of cFos immunostaining (green) and MOR+ tdTomato expression (red). Scale bar = 100 μm. Cells that co-expressed cFos and tdTomato are indicated by yellow vertical arrows.

impairment of chemoreflexes is the carotid bodies. Although MORs are expressed in the carotid bodies, transection of the carotid sinus nerve enhanced (rather than reduced) morphine-induced suppression of the hypoxic and hypercapnic ventilatory response (Baby et al., 2018).

NTS Endogenous Opioids in Physiological Responses

Ventilation is enhanced in mice lacking mu opioid receptors (Dahan et al., 2001), suggesting endogenous opioids influence the control of breathing. The NTS is a potential source of endogenous opioids. The endogenous opioid endomorphins are abundantly expressed in the caudal NTS (Pierce and Wessendorf, 2000; Greenwell et al., 2007), and endomorphin-2 containing axon terminals oppose dendritic MORs in the NTS (Silverman et al., 2005). Selective stimulation of proopiomelanocortin (POMC) neurons in the NTS suppresses breathing, which is blocked by the opioid antagonist naloxone (Cerritelli et al., 2016), suggesting the release of opioid peptide from these neurons could modulate breathing. Furthermore, vagal afferents into the NTS contain endomorphin-2 (Silverman et al., 2005), and stimulation of the NTS or the vagus nerve is analgesic (Lewis et al., 1987; Kirchner et al., 2006) implicating the NTS as an endogenous integrator of both pain and breathing (Boscan et al., 2002). Presumably, endogenous opioids in the NTS could also modulate hypoxic and hypercapnic responses, and perhaps adaptations that occur in these responses during chronic hypoxia (Chung et al., 2006; Powell, 2007; Nichols et al., 2009b).

CONCLUSIONS

Here, we identified that NTS neurons activated by hypercapnia and hypoxia do not express MORs, ruling out the direct effects of opioids on these neurons. More likely presynaptic MORs on axon terminals and/or MORs on inhibitory interneurons predominantly mediate opioid suppression of chemoreflexes in the NTS. The specific afferents and synaptic target suppressed by MORs remain to be elucidated. Furthermore, the role of endogenous opioids and adaptations that could occur in these afferent-specific synapses during chronic opioid or altered chemoreception states are unexplored future directions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at the University of Florida.

AUTHOR CONTRIBUTIONS

EL and AV designed research. SM and BR performed experiments and analyzed data. SM, AV, and EL prepared figures and drafted the manuscript. All authors revised and approved the final version of the manuscript.

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The role of enkephalinergic systems in substance use disorders

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Enkephalin, an endogenous opioid peptide, is highly expressed in the reward pathway and may modulate neurotransmission to regulate reward-related behaviors, such as drug-taking and drug-seeking behaviors. Drugs of abuse also directly increase enkephalin in this pathway, yet it is unknown whether or not changes in the enkephalinergic system after drug administration mediate any specific behaviors. The use of animal models of substance use disorders (SUDs) concurrently with pharmacological, genetic, and molecular tools has allowed researchers to directly investigate the role of enkephalin in promoting these behaviors. In this review, we explore neurochemical mechanisms by which enkephalin levels and enkephalin-mediated signaling are altered by drug administration and interrogate the contribution of enkephalin systems to SUDs. Studies manipulating the receptors that enkephalin targets (e.g., mu and delta opioid receptors mainly) implicate the endogenous opioid peptide in drug-induced neuroadaptations and reward-related behaviors; however, further studies will need to confirm the role of enkephalin directly. Overall, these findings suggest that the enkephalinergic system is involved in multiple aspects of SUDs, such as the primary reinforcing properties of drugs, conditioned reinforcing effects, and sensitization. The idea of dopaminergic-opioidergic interactions in these behaviors remains relatively novel and warrants further research. Continuing work to elucidate the role of enkephalin in mediating neurotransmission in reward circuitry driving behaviors related to SUDs remains crucial.

KEYWORDS

opioid, enkephalin, substance use disorder, reward, circuitry

Introduction

Substance use disorders (SUDs; also known as drug addiction) are characterized by an inability to control drug use, continuing drug use despite adverse consequences, and relapse even after long periods of abstinence. Multiple risk factors contribute to vulnerability for developing of a SUD, such as genetic and environmental factors (for review, see: Volkow and Li, 2005). Due to its chronic relapsing nature, long-term treatment and abstinence is difficult. Research into the neurobiological substrates of SUDs may reveal mechanistic insight into the development of and relapse to SUDs and provide potential targets for therapeutics.

Current theories of the mechanisms underlying SUDs emphasize the role of the mesolimbic dopamine system. "Classic" drugs of abuse, such as psychostimulants, opioids, and nicotine, that maintain self-administration behavior in both animal models and humans, induce a characteristic elevation in dopamine in the nucleus accumbens (NAc) after administration (for review, see: Di Chiara et al., 2004). This can occur *via* stimulation (or disinhibition) of dopamine neurons in the ventral tegmental area (VTA) projecting to the NAc and/or by inhibiting the reuptake of dopamine in the NAc and is thought to be the critical mechanism underlying the primary reinforcing effects of drugs of abuse. Drug-paired cues, one important factor contributing to relapse, can also lead to increased dopamine in the NAc, which further supports other frameworks explaining the role of dopamine in various aspects and stages of SUDs, such as the opponent process and incentive salience theories (for reviews, see: Berridge, 2007; Trigo et al., 2010). In addition to dopamine, numerous neurotransmitter and receptor systems have been implicated in the adaptations caused by drugs of abuse and in the transition from recreational use to SUDs. The endogenous opioid system, comprised of multiple opioid receptor types and endogenous ligands, is highly expressed in reward circuitry and has been proposed to be a crucial modulator of SUDs (for review, see: Trigo et al., 2010).

Positron emission tomography (PET) imaging in human subjects have suggested a potential role of endogenous opioids in the effects of drugs of abuse. For example, oral administration of amphetamine in male subjects reduced binding of [^{11}C]carfentanil, a radiolabeled molecule that binds to mu opioid receptors, in the basal ganglia, frontal cortex, and thalamus after amphetamine administration, suggesting that endogenous opioid peptides were released and displaced carfentanil (Colasanti et al., 2012; Mick et al., 2014). Further evidence in human subjects also supports a potential role for endogenous opioid systems in SUDs (Chan et al., 2020). Administration of non-selective opioid receptor antagonists, such as naltrexone or naloxone, may be effective in treating psychostimulant use disorder (Comer et al., 2013) and may reduce cigarette consumption and the satisfaction during *ad libitum* smoking (Covey et al., 1999), although these results are not consistent across all studies (Sutherland et al., 1995). Generally, these reports suggest that the endogenous opioid system plays a role in modulating the effects of drugs of abuse and SUDs, warranting further investigation into the role of opioids.

Most opioid receptor types (mu, delta, kappa, and ORL1) and endogenous opioid peptides (β-endorphin, enkephalins, dynorphins, and others) have been implicated, to some extent, in the neuroadaptations that occur following administration of different drugs of abuse as well as in reward-related behaviors. For many years, each opioid peptide was thought to be primarily selective for one opioid receptor type; however, more recent studies indicate that opioid peptides bind to and activate all three of the canonical opioid receptors, albeit with different affinities and efficacies (Gomes et al., 2020). Previous reports have reviewed the potential role of β-endorphin (Roth-Deri et al., 2008; Le Merrer et al., 2009) or dynorphin (Banks, 2020; Karkhanis and Al-Hasani, 2020; Koob, 2020; Best et al., 2022; Ragu Varman et al., 2022) in SUDs. Therefore, this review will focus specifically on the role of the endogenous enkephalinergic system (e.g., enkephalin peptides and receptors they bind to) in modulating the reward pathway and reward-related behaviors because (1) there is widespread synthesis and release of enkephalins in the reward pathway and (2) the receptor targets of enkephalin are also widely distributed throughout the reward circuitry, namely the mesolimbic and nigrostriatal pathways (for reviews, see Akil et al., 1984; Shippenberg et al., 2008; Le Merrer et al., 2009; Trigo et al., 2010).

It is important to note that studies rarely evaluate the *exclusivity* of enkephalins and enkephalin-induced opioid receptor activation in the neurobiological mechanisms of SUDs. It is also possible that enkephalins always act in conjunction with other opioid peptides and simultaneously at multiple opioid receptor types to produce their effects. Interestingly, there is still much unknown about endogenous enkephalins. In many instances, the sites of enkephalin synthesis and release are not fully appreciated but are thought to be released in response to drugs of abuse and likely play a role in regulating certain behaviors (described below). On the other hand, β-endorphin is synthesized primarily in the arcuate nucleus and nucleus of the solitary tract with fibers projecting to many brain regions, including parts of the reward pathway such as the VTA and NAc, as well as released from the pituitary gland into circulation (Lee and Wardlaw, 2007; Roth-Deri et al., 2008). Therefore, both of these endogenous opioid peptides are likely involved in SUDs and potentially have overlapping, or possibly redundant, roles. For the purposes of this review, we consider the enkephalinergic system to be comprised of enkephalins, enkephalin-hydrolyzing enzymes, and the receptors activated by enkephalins as described below. Hopefully, by combining knowledge from different studies, we will eventually understand the function of endogenous opioidergic systems in reward, motivation, and SUDs.

Basic biology

There are three primary opioid peptide gene families: proopiomelanocortin (*POMC*), proenkephalin (or preproenkephalin; *PENK*), or prodynorphin (*PDYN*). These genes are translated into prepropeptides (*POMC*, proenkephalin A, and *PDYN*, respectively) before being cleaved into the final functional peptides, β-endorphin, enkephalin, and dynorphin. The primary peptides share a common amino acid N-terminal sequence Tyr-Gly-Gly-Phe-X (Met/Leu for enkephalin).

A fourth family of opioid peptide, nociceptin, is derived from prepronociceptin.

Proenkephalin A is cleaved into six copies of met-enkephalin and one copy of leu-enkephalin (Akil et al., 1984; Mclaughlin, 2006). Leu-enkephalin can also be derived from *PDYN* (Akil et al., 1984). Therefore, met-enkephalin may be a more specific marker of proenkephalin activity. Enkephalins are inactivated by two membrane-bound (or soluble) metallopeptidases: neutral endopeptidase (NEP) and aminopeptidase N (APN) (Roques et al., 1980; Ramírez-Sánchez et al., 2019). These peptidases are found near synapses (Ramírez-Sánchez et al., 2019) and are located in brain regions also containing enkephalins, such as the caudate putamen, globus pallidus, substantia nigra, and spinal cord (Waksman et al., 1986). While commonly referred to as enkephalinases, these peptides can also contribute to the formation and degradation of other peptides and/or peptide fragments. While this has brought about the renaming of some of these enzymes, such as enkephalinase to neprilysin (Bayes-Genis et al., 2016), we will refer to enzymes that cleave enkephalin as enkephalinases; however, we recognize that this nomenclature does not include the breadth of activity of these enzymes.

Opioid peptides bind to opioid receptors, which are G-protein coupled receptors (GPCRs). These receptors are coupled to the Gi/o proteins, leading to inhibition of cAMP, inhibition of Ca^{2+} channels, activation of inwardly rectifying K^{+} channels and MAP kinase pathway, which ultimately inhibits neuronal activation and neurotransmitter release (Law et al., 2000). Each receptor is encoded by separate genes, MOR: *Oprm1,* DOR*: Oprd1,* KOR*: Oprk1,* and ORL1*: Oprl1*. Canonically, it is believed that β-endorphin, met-/leu-enkephalin, and dynorphin preferentially bind to the mu opioid receptor (MOR), delta opioid receptor (DOR) and kappa opioid receptor (KOR), respectively. Nociceptin/orphanin FQ binds to the nociceptin opioid peptide receptor [NOPR; or opioid receptor-like 1 (ORL1)]. Enkephalins bind with high affinity to DOR and MOR [with slightly greater affinity (10-fold) for DOR than MOR; measured under non-physiological conditions] (Raynor et al., 1994), but more recently, all opioid peptides have been shown to bind to each of the opioid receptors to some extent (Gomes et al., 2020). For example, β-endorphin, met-enkephalin, and dynorphin have been shown to be full agonists at MOR and partial agonists at DOR. Shorter forms of β-endorphin, generally thought to have limited activity at opioid receptors, are agonists at MOR (Gomes et al., 2020). Therefore, focusing on enkephalin-DOR or enkephalin-MOR interactions in studies investigating SUDs may be overlooking important interactions of other endogenous opioid peptides and receptor types. Overall, while the studies described here implicate enkephalin in multiple aspects of SUDs, there are likely distinct and overlapping roles of other endogenous opioid peptides as well.

Anatomy & distribution in reward circuitry

Some primary regions of enkephalin release occur within the reward pathway, specifically in the NAc, VTA, and pallidum [comprised of the ventral pallidum (VP) and globus pallidus (GP)]. Interestingly, it is unclear where enkephalin in the NAc comes from, with some studies suggesting that it comes from projection neurons (e.g., dorsal raphe nucleus to NAc shell; Castro et al., 2021) and/or from local release within the NAc (Al-Hasani et al., 2018). On the other hand, the source of enkephalin release in the VP is likely from dopamine D2 receptor-expressing medium spiny neurons (MSNs) in the NAc projecting to the VP (Kalivas et al., 1993; Heinsbroek et al., 2017), but it is unknown whether or not these projections are the only source of enkephalin in the VP. These D2-expressing MSNs projecting from the NAc to the VP presumably release enkephalin as well as GABA, and are considered part of the "indirect" pathway (Zahm et al., 1985), while D1 MSNs (expressing dynorphin) are part of the "direct" pathway, regulating motor function, movement, and reward (Yager et al., 2015). Enkephalin-containing cell bodies seem to be present in the VTA (Johnson et al., 1980; Khachaturian et al., 1983), and are presumably the source of enkephalin release in this brain region, yet this has not been directly tested. Without having a better understanding of sites of enkephalin synthesis and the projection of enkephalin-containing neurons, our knowledge of enkephalinergic circuitry in mediating aspects of SUDs will be limited. Further work needs to be done to better identify the source of enkephalin peptide synthesis and release within the reward circuitry.

Significantly more is known about the expression of both MOR and DOR in reward circuitry. Both opioid receptor types are highly expressed in the same regions with PENK mRNA (Mansour et al., 1993, 1994), including the NAc, caudate putamen, and amygdala. MOR and DOR expression in the mesolimbic circuitry of the rodent brain have been confirmed by autoradiography as well as with expression of fluorescently labeled opioid receptors (GFP-labeled DOR and mCherry-labeled MOR; Erbs et al., 2015). Furthermore, their exact localization on neurons informs us how these receptors regulate neurotransmitter release and/or neuronal activation.

MORs are thought to be located pre- and postsynaptically on neurons in mesolimbic areas (for review, see: Shippenberg et al., 2008) and more specifically on dendrites or dendritic spines in the NAc, amygdala, and VTA near terminals expressing and, presumably, releasing enkephalin (Svingos et al., 1996; Herman et al., 2022). On D2-expressing MSNs, MORs are expressed both postsynaptically in the NAc (Castro and Berridge, 2014) and in the VP, capable of regulating GABA release in the VP (Heinsbroek et al., 2017). Presumably, activation of MORs on D2 MSN terminals in the VP should

also regulate enkephalin release; however, this has not been directly tested. MORs have also been identified postsynaptically on pallidal cell bodies (Olive et al., 1997). MORs located in the VTA are present on GABAergic interneurons, such that MOR activation (either *via* exogenous or endogenous ligands) leads to disinhibition of VTA dopamine neurons projecting to the NAc (Johnson and North, 1992).

DORs are thought to be located primarily on axons and axon terminals, on both enkephalin and non-enkephalin releasing neurons (Svingos et al., 1998). In axons and axon terminals, DORs may not always be expressed only on the cell surface, but also located intracellularly and trafficked to the surface under certain conditions (Wang et al., 2008). Within the NAc, DORs have been found on terminals of glutamate neurons projecting from the prefrontal cortex (PFC) (Svingos et al., 1999; Castro and Berridge, 2014; Mongi-Bragato et al., 2018), on cholinergic interneurons (in addition to MORs; Castro and Berridge, 2014; Laurent et al., 2014) and (to a lesser extent) on dopamine terminals (Svingos et al., 1999). Similarly, in the VTA, DORs are expressed presynaptically on GABAergic terminals and can modulate GABA release (Margolis et al., 2008). In contrast, other studies have shown that DORs are expressed postsynaptically on D2 MSNs in the NAc (Castro and Berridge, 2014) and on cell bodies in the VP (Olive et al., 1997).

The presence of enkephalin in the primary cell type of the NAc and prevalence of DORs and MORs throughout the reward pathway further implicates its central role in modulating reward-related neurotransmission. However, the widespread distribution of enkephalin and overlapping MOR and DOR expression in many brain regions and cell types begins to highlight the complexity (and possible redundancy) of the endogenous opioid system in mediating SUDs.

Methods used to evaluate enkephalin

There are different methods and techniques to evaluate enkephalinergic involvement in reward-related pathways and behaviors. Methods for measuring enkephalin release are limited (for review, see: Conway et al., 2022); therefore, studies often measure enkephalin concentrations in various brain regions as indirect measures of releasable peptide or a releasable pools. Peptide expression and release are likely related, such that if there is increased peptide synthesized, packaged in vesicles, and available for release (intracellular expression), then more peptide is actually released (either tonically or during stimulated release).

Enkephalin peptide concentration can be measured using highly sensitive radioimmunoassay (RIA). Antibodies used in these assays that bind to enkephalin peptide have limitations in selectivity. RIAs with tissue samples also cannot discriminate between intracellular expression and extracellular release of enkephalin. Other, more direct, approaches include collecting dialysate samples *via* microdialysis and then performing RIAs to quantify enkephalin levels in dialysate (first described in Maidment et al., 1989). Enkephalin from microdialysis samples can also be quantified *via* liquid chromatography couple with mass spectrometry (LCMS) and, while technically challenging, can distinguish between met- and leu-enkephalin (Mabrouk et al., 2011; DiFeliceantonio et al., 2012). Methods to selectively activate enkephalin expressing neurons (e.g., optogenetics or designer receptors exclusively activated by designer drugs; DREADDS) can be used to induce the release of enkephalin; however, these methods are not specific to either met- or leu-enkephalin and can also presumably induce the release of other opioid peptides (Al-Hasani et al., 2018) and/or cotransmitters, such as GABA. Therefore, while technical advancements in methodology have allowed for greater specificity in investigating enkephalin, there are still shortcomings that need to be addressed.

In the absence of direct measurements, enkephalin expression and/or levels of enkephalin can be manipulated in order to evaluate the role of enkephalin in SUDs. This has been accomplished through pharmacologically inhibiting enkephalin breakdown or by constitutive global knockout (KO) of the *PENK* gene (and recently conditional knockouts) (for review, see Charbogne et al., 2014). Studies using these tools have provided great insight into the role of the enkephalinergic system in SUDs; however, similar opioid (or non-opioid) peptides and compensatory mechanisms could distort the role of enkephalin specifically. For example, β-endorphin, which has similar affinity at MOR and DOR, may compensate for the lack of enkephalin in KO animals (Maldonado et al., 2018) or leu-enkephalin generated from *PDYN* in PENK KO animals.

Drugs that inhibit enkephalinase, such as thiorphan (Roques et al., 1980) or RB101 (Jutkiewicz, 2007; Jutkiewicz and Roques, 2012), can be used as tools to probe the enkephalinergic system in reward related behaviors by preventing the breakdown of extracellular enkephalin, increasing its activity at MORs and DORs. One limitation of this approach is that there is no way to discriminate between activity due to met- or leu-enkephalin. In addition, these enzymes may also cleave other peptides, such as cholecystokinin (Durieux et al., 1985) and substance P (Matsas et al., 1983); however, studies often perform further experiments to confirm the effects produced by enkephalinase inhibitors occur *via* the activation of opioid receptors. Importantly, β-endorphin has been shown to be a substrate of NEP and APN, but is also degraded by other enzymes (Roques et al., 2012). Many of the opioid receptor-specific behavioral effects of enkephalinase inhibitors (described below) seem to be mediated *via* enkephalins or at least by peptides binding to either MORs or DORs (Noble et al., 2008) because they are blocked by non-selective or selective opioid receptor antagonists. While these tools have been valuable for probing enkephalin peptide in reward related behaviors, there is relatively little is known about enkephalinase activity/mechanisms nor how the enzymes

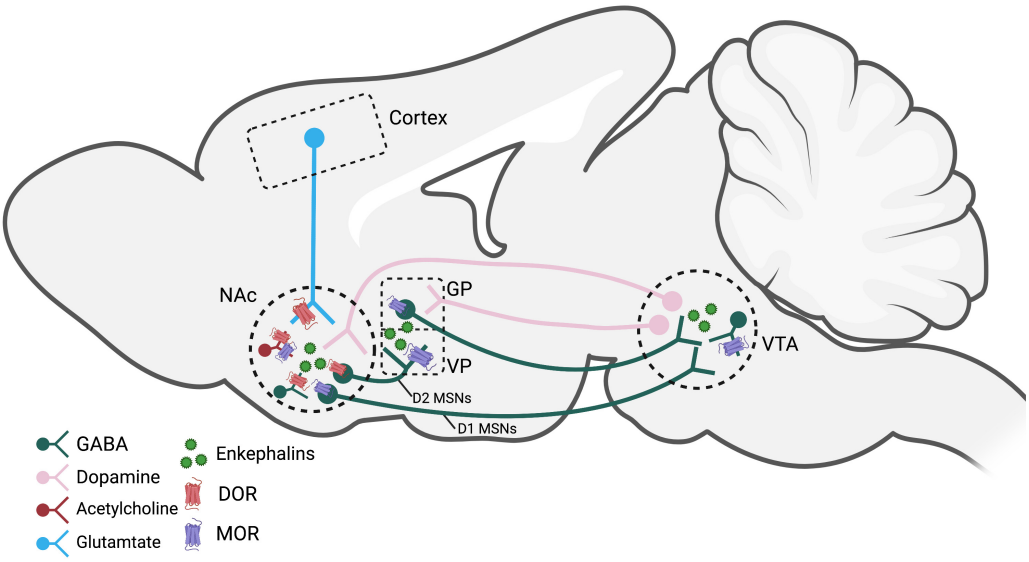

FIGURE 1
Brain regions and pathways implicated in enkephalin-mediated reward-related behaviors. Dopamine neurons in the VTA that project to the NAc are modified by MORs on GABAergic interneurons. Activation of MORs and DORs, likely by enkephalins, within the NAc modulate dopamine, GABA, glutamate, and acetylcholine release. D2 MSNs express enkephalin and project to the VP and are believed to be a crucial circuit for reinstatement behaviors. Figure created using Biorender.com. NAc, nucleus accumbens; GP, globus pallidus; VP, ventral pallidum; VTA, ventral tegmental area; MOR, mu opioid receptor; DOR, delta opioid receptor; MSNs, medium spiny neurons.

regulate synaptic enkephalin peptide levels. Recent studies have begun to investigate endogenous inhibitors of enkephalinase (Wisner et al., 2006; Tóth et al., 2012) and further investigation into the metabolism of enkephalin *in vivo* (Xu et al., 2010; Wilson et al., 2020) will be crucial for understanding the role of enkephalin in SUDs.

Indirect measurements of enkephalin also provide valuable insight into the enkephalinergic system, albeit with some deficiencies. Quantifying levels of PENK mRNA expression identifies brain regions where enkephalin is likely synthesized, but may not accurately reflect enkephalin peptide expression (intra or extracellular) nor enkephalin release. Similarly, using pharmacological methods to activate or inhibit DOR and/or MOR implicate opioid receptor signaling and requires highly selective ligands. While it is presumed that enkephalin is the endogenous ligand acting on those receptor systems, it is often not directly tested. Since all endogenous opioid peptides bind, to some degree, to all opioid receptors, peptides other than met- or leu-enkephalin may be responsible for the effects measured. Overall, while a wealth of literature has supported the notion that enkephalin modulates reward-related neurobiology and behavior, there is much still to be elucidated.

This review primarily focuses on studies investigating PENK or enkephalin peptides, as they are more closely related to the functional role of enkephalins in reward-related behaviors. Pharmacological studies investigating the effects of DOR and/or MOR activation are not the focus of this review and are thoroughly covered elsewhere (for reviews, see Shippenberg et al., 2008; Le Merrer et al., 2009; Trigo et al., 2010), but some studies are included in this review to extrapolate or corroborate the involvement of enkephalin in modulating reward-related neurotransmission and behaviors.

Effects of enkephalin on neurotransmission in reward pathways

As described above, studies have attempted to investigate the role of enkephalin in neurotransmission using PENK KO models, increasing levels of enkephalin by preventing breakdown, and activation of enkephalins' targets with exogenous ligands. By using these approaches enkephalins have been identified as neuromodulators, influencing release and extracellular levels of dopamine, GABA, glutamate, acetylcholine, and other neurotransmitters involved or implicated in reward-related circuits (for review, see Torregrossa and Kalivas, 2008).

Enkephalinergic modulation of dopamine neurotransmission

The most prevalent mechanisms underlying SUDs center around the role of dopamine in driving drug-taking and -seeking behaviors, and there is strong evidence of interactions

between enkephalinergic and dopaminergic systems. Perhaps the most obvious interaction between the two systems is that MOR activation by exogenously administered agonists, such as morphine, stimulate dopamine release in the NAc. Further, KOR activation reduces dopamine release (Escobar et al., 2020), and DOR activation may increase dopamine release to some extent (Saigusa et al., 2017) or have no effect on dopamine (Longoni et al., 1998).

To further elucidate the role of enkephalin on the dopaminergic system, studies have measured dopamine neurotransmission in PENK KO mice. Basal levels of dopamine in the NAc did not differ between PENK KO and wild-type animals (Berrendero et al., 2005), but evoked-dopamine levels appear to be altered by enkephalin and opioid receptor activation. For example, a dose of nicotine that stimulates dopamine release in wildtype mice had a blunted dopamine response in the NAc in PENK KO mice (Berrendero et al., 2005). To our knowledge, no other effects of drugs of abuse on dopamine levels in the NAc of PENK KO animals have been reported. It is possible that enkephalin promotes nicotine-stimulated dopamine release, likely *via* opioid receptor-induced inhibition of GABA release in the NAc and/or VTA.

Consistent with the study described above, opioid receptor activation also enhances psychostimulant-induced increases extracellular levels of dopamine in the NAc (see **Figure 1**). For example, increasing endogenous enkephalins by blocking hydrolysis with an enkephalinase inhibitor, thiorphan, given into the substantia nigra potentiated amphetamine-stimulated dopamine release in the striatum (Schad et al., 2002). Conversely, preventing activation of opioid receptors on inhibitory GABAergic neurons locally in the substantia nigra, VTA, or GP attenuated amphetamine-induced increases in dopamine in their projection targets, the striatum, NAc, and locally in the GP, respectively (Schad et al., 1995, 2002; Mabrouk et al., 2011). In the absence of other drugs, naloxone given locally into the GP decreased dopamine in the same brain region, suggesting that there is a tonic enkephalinergic tone in the GP which activates MORs (presumably) on GABAergic terminals to inhibit GABA release and ultimately disinhibit dopamine (Mabrouk et al., 2011).

Enkephalin binds to and activates MOR and DOR; therefore, exogenous administration of MOR and DOR agonists have been used to probe the potential involvement (albeit indirectly) of endogenous enkephalins in regulating dopamine neurotransmission. MOR agonists increase dopamine in the dorsal and ventral striatum (*via* disinhibition) by activating MORs in the VTA/substantia nigra. MORs do not seem to be located presynaptically on dopamine terminals in the NAc (Svingos et al., 1996; Britt and McGehee, 2008; Saigusa et al., 2017; but see Svingos et al., 1999), but may be present presynaptically in the VP to gate dopamine release arising from the VTA (Mitrovic and Napier, 2002; Root et al., 2015; Clark and Bracci, 2018).

While the effects of MOR activation on dopaminergic neurotransmission are fairly well-explored, the effects of DOR activation on dopamine levels are unclear. For example, the peptide DPDPE given intracerebroventricularly dose-dependently increased dopamine in the NAc of anesthetized rats, which was blocked by the DOR antagonist ICI 174,864 (Spanagel et al., 1990). Also, the small molecule DOR agonist SNC80 failed to promote dopamine efflux in rat striatal preparations directly (Bosse et al., 2008) and failed to increase dopamine levels in the NAc or caudate putamen in rats measured by microdialysis (Longoni et al., 1998). However, SNC80 did enhance amphetamine-mediated dopamine efflux in the striatum (Bosse et al., 2008) as well as amphetamine-mediated locomotor activity (Jutkiewicz et al., 2008) potentially through indirect actions with glutamatergic neurons. The effects of DOR activation on modulating dopaminergic neurotransmission is unclear (for review, see: Saigusa et al., 2017), but overall, enkephalin and opioid receptor activation seem to have some neuromodulatory effects on dopaminergic activity in the reward pathway, most likely through indirect mechanisms.

Together, these findings indicate that endogenous enkephalins in the VTA, substantia nigra, and potentially other brain regions may contribute to drug-induced increases in dopamine in the NAc. These studies indicate that opioid receptor activation enhances dopamine, likely *via* disinhibition. Extrapolating from these indirect studies of opioid receptor activation, it is plausible to think that endogenous activation of these same opioid receptors would be able to enhance dopamine reward circuitry and potentiate SUDs.

Enkephalinergic modulation of GABA neurotransmission

As described above, opioid-induced inhibition of GABAergic neurons in the VTA and substantia nigra (Galaj et al., 2020; Oliver, 2021) disinhibits dopamine neurons projecting to the NAc (Johnson and North, 1992). Thus, opioids have been shown to regulate GABA release within the reward pathway. GABA and enkephalin are thought to be cotransmitters, released from D2 MSNs projecting from the NAc to the ventral pallidum (Maneuf et al., 1994) where enkephalin likely binds to presynaptic opioid receptors (or autoreceptors) to inhibit further GABA and enkephalin release (Maneuf et al., 1994; Stanford and Cooper, 1999). Indeed, in VP slices prepared from drug naïve rats, the administration of an enkephalinase inhibitor reduced extracellular levels of GABA in the VP (Kupchik et al., 2014). Whereas naloxone given locally into the pallidum, increased GABA and also decreased dopamine in the same brain region (Mabrouk et al., 2011). At least some studies have suggested that MORs are located on GABAergic terminals in the VP and VTA, such that exogenous

activation of MORs inhibits GABA release in the VP (Kalivas et al., 2001) and in the VTA (Matsui and Williams, 2011; Matsui et al., 2014).

Additionally, activation of DORs likely influences GABAergic transmission in reward circuitry, but this has not been investigated thoroughly. Within the NAc, activation of DORs present on GABAergic terminals reduce inhibitory neurotransmission (Jiang and North, 1992; Chieng and Williams, 1998). In the VTA, postsynaptic DOR activation has been shown to augment $GABA_A$ receptor mediated inhibitory postsynaptic currents (Margolis et al., 2011). Overall, these studies suggest that enkephalins primarily act on GABAergic terminals or interneurons to inhibit GABA release in multiple brain regions.

Enkephalinergic modulation of glutamate neurotransmission

Glutamate, the primary excitatory neurotransmitter in the brain, has been shown to drive reward-related behaviors, such as sensitization and reinstatement (Scofield et al., 2016). The VTA receives glutamatergic projections from multiple brain regions (Geisler et al., 2007; Watabe-Uchida et al., 2012); however, it is unclear whether opioids influence glutamate neurotransmission in the VTA. There is evidence in other brain regions that opioids can modulate glutamate neurotransmission. For example, glutamatergic neurons project from the amygdala to the VP and release can be inhibited *via* MOR agonists (Mitrovic and Napier, 1998). VP glutamatergic neurons are preferentially innervated by D1 MSNs arising from the NAc (Heinsbroek et al., 2020), therefore there is likely opioid modulation of glutamatergic activity within the VP *via* dynorphin release, but this has not been directly tested.

Glutamate release in the NAc stems from projection neurons originating in the prefrontal cortex and enhances dopamine release in the NAc (Tzschentke and Schmidt, 2003). Glutamatergic axon terminals in the NAc express opioid receptors, specifically DOR (Winters et al., 2017; see **Figure 1**). Therefore, activation of DOR, by enkephalins, on the terminals of PFC-projecting glutamatergic neurons would be likely to decrease glutamate release in the NAc. However, DORs are not expressed exclusively on glutamatergic terminals in the NAc, highlighting the complexity of the endogenous opioid system in this brain region and how it might influence glutamatergic neurotransmission. For example, the DOR agonist, SNC80, has been shown to indirectly increase glutamate efflux in the striatum (Bosse et al., 2014). The proposed mechanism is that SNC80 activates DOR on GABAergic terminals, thereby inhibiting GABA release, which leads to local glutamate release and, subsequently, potentiation of amphetamine-induced dopamine release. An NMDA receptor antagonist, MK801, blocked the effects of SNC80 on enhancing dopamine (Bosse et al., 2014), suggesting that DOR activation can modulate the excitatory/inhibitory balance within the striatum to disinhibit dopamine release. Consistently, local administration of naltrindole, a DOR antagonist, into the caudate putamen blocked amphetamine-induced increases in glutamate, which was reversed by the DOR agonist, DPDPE (Rawls and McGinty, 2000).

Interestingly, there is some evidence that opioid receptors are also located on glia in the NAc, potentially suggesting a regulatory role of enkephalin on non-neuronal glutamate neurotransmission (Corkrum et al., 2019). Together, these studies suggest endogenous enkephalin acts as a direct or indirect neuromodulator of glutamate neurotransmission and may modulate changes in glutamate neurotransmission induced by drugs of abuse. However, the role of enkephalin has not been evaluated directly.

Enkephalinergic modulation of cholinergic neurotransmission

Cholinergic interneurons also have an important function in regulating neurotransmission in reward centers. In the NAc, cholinergic interneurons are the only source of acetylcholine and act locally to regulate efferents, particularly glutamate and dopamine (Warner-Schmidt et al., 2012). Specifically, MOR and DOR expression on cholinergic interneurons indicates that endogenous opioid peptides ligands may act as neuromodulators of acetylcholine release (Laurent et al., 2014; see **Figure 1**). Indeed, DOR and MOR activation by leu-enkephalin or DAMGO decreased acetylcholine release in the striatum (Mulder et al., 1984; Jabourian et al., 2005; Arttamangkul et al., 2021). While cholinergic neurons are also present in both the GP/VP (Chiba et al., 1995) and VTA (Rada et al., 2000; Mathon et al., 2003), it is unclear if or how endogenous opioid peptides modulate cholinergic release or signaling in these brain regions. Therefore, enkephalin may have an additional role of regulating cholinergic inhibition in brain regions within the reward pathway.

Overall, enkephalins acting at MORs or DORs modulates the transmission of multiple neurotransmitter systems enhancing the reward-related circuitry through inhibition of GABA or disinhibition of glutamate and/or dopamine. It is important to note that many of the described studies extrapolate from indirect measures of the involvement of enkephalins, because enkephalins are rarely measured directly.

Drugs alter enkephalin levels: Peptide levels and mRNA

There is also evidence that drugs of abuse may increase enkephalin release by unknown mechanisms, stimulating

MORs and DORs, further potentiating extracellular levels of glutamate and dopamine (by mechanisms described above) and thus driving reward-related behaviors. Acute and chronic administration of drugs of abuse have been shown to alter levels of enkephalin in reward brain regions, albeit with some inconsistent results across studies.

Indirect and direct dopamine receptor agonists

It has been shown that amphetamine administration increases enkephalins in multiple brain regions. Amphetamine increased met-enkephalin release in NAc and PFC (Assis et al., 2006, 2009), and in the GP (Mabrouk et al., 2011). Similarly, cocaine administration caused displacement of radioactive DAMGO at MORs in the NAc, suggesting that cocaine may stimulate endogenous opioid release, potentially enkephalins, β-endorphin, or other opioid peptides (Roth-Deri et al., 2003; Soderman and Unterwald, 2009). However, cocaine did not alter met-enkephalin in striatum or substantia nigra as measured by RIA (Sivam, 1989).

Psychostimulant administration may also alter the expression of endogenous opioid peptide mRNA, which may influence enkephalin levels and release, but there are mixed results of psychostimulant-induced changes in expression of PENK mRNA throughout reward circuitry. These may be due to differences in psychostimulant dose, time of mRNA measurement, and acute versus chronic administration. For example, psychomotor stimulants either increased, did not change, or decreased PENK mRNA in the striatum (Hurd and Herkenham, 1992; Wang and McGinty, 1996; Adams et al., 2000), decreased or did not alter PENK mRNA in the NAc (Adams et al., 2000; Turchan et al., 2002), and did not alter PENK mRNA expression in the amygdala (Turchan et al., 2002). Similar inconsistent results have been reported as a result of cocaine administration. Experimenter-administered repeated cocaine did not alter PENK mRNA in the amygdala, dorsal striatum, NAc shell or core (Mathieu-Kia and Besson, 1998; Turchan et al., 2002), but "binge" and contingent cocaine administration increased PENK mRNA in NAc, caudate putamen, PFC, and substantia nigra (Hurd and Herkenham, 1992; Spangler et al., 1997; Crespo et al., 2001; Mantsch et al., 2004; Sun et al., 2020) but not in the dorsal or ventral striatum (Hurd and Herkenham, 1992; Arroyo et al., 2000). Perhaps, these results suggest that repeated administration of psychomotor stimulants is more likely than acute drug treatment to induce changes in PENK mRNA, suggesting the involvement of long-lasting neuroadaptations as a consequence of chronic drug exposure. While few studies have investigated the effects of psychostimulants on enkephalin peptide levels or release, these limited data suggest psychostimulants may increase enkephalins in certain mesolimbic brain regions, perhaps with some differences between amphetamine and cocaine.

Opioids

Although enkephalins are an endogenous ligand for MORs, few studies have investigated the effects of exogenous MOR activation on enkephalin levels. Acute morphine (Olive et al., 1995) and heroin (Olive and Maidment, 1998) increased extracellular opioid peptides in the VP/GP thought to be enkephalin, but morphine did not alter enkephalin levels in the NAc (Olive et al., 1995). Repeated morphine was shown to either not alter (Uhl et al., 1988) or increase met-enkephalin (Nylander et al., 1995) in the striatum, NAc, and PAG (Nieto et al., 2002). Similarly, in rats with a history of heroin self-administration, MOR agonists also elevated levels of met- and leu-enkephalin in the caudal striatum and septum (Cappendijk et al., 1999). Morphine conditioning also induced an increase in enkephalin in the NAc (Nieto et al., 2002). Together, these findings suggest that exogenously administered opioids increase enkephalin in the reward pathway and may be involved in the formation of opioid-context associations.

There are few studies assessing the administration of exogenous opioids on PENK mRNA levels. Acute morphine did not alter PENK mRNA in NAc nor striatum (Turchan et al., 1997) and repeated morphine reduced PENK mRNA in NAc (Turchan et al., 1997) and striatum (Uhl et al., 1988). Morphine self-administration reduced PENK in NAc core and shell of LEW rats (Sánchez-Cardoso et al., 2007). These effects on PENK mRNA expression following chronic opioid agonist administration only evaluate enkephalin levels indirectly and are distinctly different from those found following repeated psychostimulant administration.

Together, these findings suggest that, while acute and chronic administration of MOR agonists may increase enkephalin release and peptide levels, chronic opioid administration mainly leads to a reduction in PENK mRNA expression, potentially compensating for the replacement of endogenous opioid peptides by exogenous opioid receptor ligands.

Ethanol

The effects of ethanol on enkephalin levels and PENK mRNA are highly varied across studies. Acute ethanol has been shown to increase met-enkephalin in the NAc shell and striatum, decrease enkephalin in striatum, hypothalamus, and midbrain and not alter enkephalin in VTA, amygdala, hypothalamus, midbrain, brainstem, and hippocampus (Schulz et al., 1980; Seizinger et al., 1983; Marinelli et al., 2005; Lam et al., 2008; Jarjour et al., 2009; Méndez et al., 2010). It has been hypothesized

that acute ethanol may influence enkephalin biosynthesis and release in mesolimbic areas as well (Méndez et al., 2010). Similarly, chronic ethanol increased met-enkephalin in the PAG, decreased met-enkephalin in striatum, hippocampus, brainstem, and midbrain (Schulz et al., 1980; Lindholm et al., 2000) and hypothalamus or was ineffective in altering levels in midbrain and hippocampus (Seizinger et al., 1983).

Ethanol exposure also produces varied changes in PENK mRNA levels in various brain regions. Acute ethanol treatment and voluntary consumption increased PENK mRNA in the paraventricular nucleus of thalamus, caudate putamen, amygdala, PFC, and NAc core and shell (de Gortari et al., 2000; Cowen and Lawrence, 2001; Oliva et al., 2008) and decreased PENK mRNA levels in VTA and NAc (Méndez and Morales-Mulia, 2006) in rats. Ethanol-induced changes in PENK mRNA may reflect phenotypic differences in ethanol preference, as acute ethanol increased PENK mRNA in NAc of alcohol-preferring but not alcohol-non-preferring rats (Li et al., 1998). Despite varying results of ethanol administration on enkephalin peptide and PENK mRNA levels, these studies suggest ethanol has some influence on enkephalin expression that may be brain region dependent, and further work is warranted to continue to parse apart specific effects of ethanol on the enkephalinergic system.

Nicotine

Few studies have investigated the effects of nicotine administration on endogenous enkephalin peptide levels. Acute and repeated administration of nicotine increase met-enkephalin levels in the striatum of mice as measured by immunoreactivity (Pierzchala et al., 1987; Dhatt et al., 1995; Wewers et al., 1999), and these effects were blocked by a nicotinic acetylcholine receptor antagonist (Dhatt et al., 1995). In human PET studies, nicotine smoking decreased [^{11}C] carfentanil binding in certain brain regions, such as prefrontal cortices and ventral striatum (Domino et al., 2015), further suggesting that nicotine administration increases enkephalin release in reward brain regions.

Similar to peptide levels, acute administration of nicotine in mice and rats increased PENK mRNA in the striatum and hippocampus (Dhatt et al., 1995; Houdi et al., 1998). These effects were blocked by the nicotinic acetylcholine receptor antagonist mecamylamine, but not the muscarinic antagonist atropine nor dopamine receptor antagonist haloperidol (Dhatt et al., 1995). The effects of repeated nicotine administration on PENK mRNA also vary across studies and across brain regions (Höllt and Horn, 1992; Dhatt et al., 1995; Houdi et al., 1998; Mathieu-Kia and Besson, 1998; Ugur et al., 2017). Therefore, potential compensatory adaptations in PENK mRNA following repeated nicotine may be different across reward circuitry.

Cannabinoids

Endogenous cannabinoids and their receptors (CB1) are present in many of the same brain regions as opioid receptors (Befort, 2015), indicating possible overlap and interaction between the two systems. Indeed, acute, moderate doses of THC increased enkephalin-like material in the NAc determined by RIA (Valverde et al., 2001) and increased met-enkephalin immunoreactivity in preoptic area and medial basal hypothalamus after repeated THC exposure (Patel et al., 1985).

The effects of cannabinoids on enkephalins may be greater in non-reward brain regions. Subchronic THC increased PENK mRNA levels in rat hypothalamus, PAG, and mammillary nucleus (Corchero et al., 1997; Manzanares et al., 1998), with no change in the striatum or NAc. Repeated treatment of a synthetic cannabinoid receptor agonist, CP-55,940, also increased PENK mRNA in hypothalamus and additionally the striatum and NAc (Manzanares et al., 1998). Clearly, the effects of cannabinoids on endogenous opioids and PENK mRNA are largely unknown and should be investigated further.

Other and summary

Other conditions have also been shown to change levels of enkephalin peptides. Consumption of palatable food leads to a surge of met- and leu-enkephalin in the anteromedial portion of the dorsal neostriatum, analyzed by LCMS (DiFeliceantonio et al., 2012). Optogenetic stimulation of dynorphin-expressing neurons in either ventral or dorsal NAc shell leads to increased met- and leu-enkephalin in both brain regions. This could suggest that cross-modulation of opioid peptides occurs within local circuitry in the NAc (Al-Hasani et al., 2018). Together, all of these data suggest that many drugs of abuse (and potentially non-drug reinforcers) increase enkephalin levels, which may underlie and contribute to their reinforcing effects and abuse potential by further promoting reward neurotransmission through inhibition of GABAergic signaling.

Enkephalinergic system and reward-related behaviors

The role of the endogenous enkephalinergic system has been evaluated in reward related behaviors as well as other potentially related (and co-morbid) behaviors and physiological functions, such as stress resiliency, pain, and emotion (Jutkiewicz and Roques, 2012; Henry et al., 2017; Corder et al., 2018).

For example, increasing enkephalin levels with thiorphan in the VTA (Glimcher et al., 1984) or mimicking enkephalin with a met-enkephalin peptide analog (Phillips and LePiane, 1982) given into the VTA induces conditioned place preference (CPP), in a naloxone-sensitive manner. In addition, infusions

of met-enkephalin into the NAc maintained lever pressing behavior (e.g., self-administration behavior), and this behavior was blocked by naloxone (Goeders et al., 1984). Furthermore, preventing the breakdown of endogenous enkephalins with the enkephalinase inhibitor thiorphan increased ethanol intake (Froehlich et al., 1991). These studies indicate that enkephalin may have some primary reinforcing properties and is able to activate reward circuitry.

The rewarding and reinforcing effects of various drugs of abuse are also altered by attenuating endogenous enkephalin signaling with the administration of opioid receptor antagonists or by genetic deletion of PENK. Administration of opioid receptor antagonists, which presumably block the effects of endogenous enkephalins or other opioid peptides, attenuated or blunted cocaine-induced CPP (Menkens et al., 1992), heroin self-administration (Martin et al., 2000; Tomasiewicz et al., 2012), and alcohol seeking behavior and alcohol withdrawal (Perry and McNally, 2013; Alongkronrusmee et al., 2016). Consistently, PENK KO decreased cocaine self-administration (Gutiérrez-Cuesta et al., 2014), cocaine-induced locomotor sensitization (Mongi-Bragato et al., 2016, 2021), and nicotine-induced CPP (Berrendero et al., 2005). However, PENK KO did not alter morphine (Le Merrer et al., 2011) or ethanol self-administration (Koenig and Olive, 2002; Hayward et al., 2004; Racz et al., 2008) or morphine CPP (Skoubis et al., 2005). These studies suggest enkephalinergic signaling, *via* opioid receptor activation, contributes to the rewarding effects of various drugs of abuse. This is likely due to multiple indirect mechanisms culminating in disinhibition of dopamine, either *via* disinhibiting glutamate efferents in NAc or inhibiting GABAergic interneurons in the VTA.

Interestingly, in animals trained to discriminate morphine, systemic administration of an enkephalinase inhibitor, RB 120, did not generalize to the discriminative stimulus effects of morphine and, conversely, morphine did not generalize to the discriminative stimulus effects of RB 120. Together, these data suggest that, even though enkephalins may have some rewarding properties, endogenous enkephalin and the MOR agonists may produce different subjective effects (Hutcheson et al., 2000). Therefore, targeting the endogenous enkephalinergic system for various therapeutic endpoints may lack the abuse liability of high affinity, efficacious MOR agonists. Future studies would need to investigate this further.

The endogenous enkephalinergic system may also be involved in other aspects related to the development and maintenance of SUDs. For example, MOR activation in the NAc and VP enhances hedonic impact or "liking," a distinct but related function to drug "wanting" (for reviews, see: Smith et al., 2009; Castro and Berridge, 2014). Other evidence suggests enkephalin is involved in the formation of drug-context/cue associations. Activation of MORs or DORs (specific localization unknown) by protected endogenous enkephalins in the NAc or with exogenous agonists induces reinstatement of cocaine-seeking behavior (Simmons and Self, 2009), which was blocked by a MOR antagonist given into the NAc (Simmons and Self, 2009) and VP (Tang et al., 2005) and a DOR antagonist in the NAc. Studies have also shown that cue-induced reinstatement may be a result of cocaine-induced increased enkephalinergic tone in the VP on presynaptic MORs, causing disinhibition of VP neurons projecting to VTA or other brain regions (Heinsbroek et al., 2017, 2020). These interpretations are supported by other findings demonstrating that opioid receptor blockade and MOR and DOR knockout reduced cue-induced cocaine seeking behavior and impaired morphine CPP (Burattini et al., 2008; Gutiérrez-Cuesta et al., 2014). Overall, the enkephalinergic system may act as a modulator of SUD-related behaviors by promoting drug-cue associations that enhance the rewarding effects of drugs of abuse and/or drive drug-seeking behaviors.

Conclusion

This review highlights the role of enkephalins as neuromodulators of reward-related circuitry and behaviors underlying SUDs. However, many questions still remain. As mentioned earlier, few studies directly identify and measure the specific opioid peptides involved in reward-related neurotransmission and behaviors. Therefore, in many cases, the effects are assumed to be regulated by endogenous enkephalins or other opioid peptides, such as β-endorphin. Further work identifying the specific opioid peptides and their targets (either specific or non-specific receptor targets) will provide a better understanding of the mechanisms involved in SUDs. In order to accomplish this, we must also have an improved appreciation of the sites of enkephalin synthesis, the sources of enkephalins, and the regulation of enkephalin catabolism. Finally, manipulating enkephalin directly and with brain region or cell type specificity will be crucial to measure enkephalinergic influence on reward-related behaviors.

The studies described in this review used a multitude of techniques to probe the role of enkephalin, and each technique has limitations that can influence interpretations of results. Limitations of enkephalin measurement techniques (Conway et al., 2022) are due, in part, to the complexity of the endogenous enkephalinergic system. Opioid peptides are highly homologous peptides that are rapidly degraded and bind to multiple opioid receptor types. Endogenous enkephalins are also released in smaller amounts than "classical" neurotransmitters, complicating measuring techniques. Cleavage of the opioid prepropeptides yield differential, yet overlapping, quantities of each peptide. Again, many of the studies implicating endogenous opioid release may presumably involve enkephalin due to its high prevalence in reward circuitry, yet β-endorphin cannot be ruled out as the ligand or one of the peptides involved.

Technological advancements to improve detection and quantification of endogenous opioid peptides and their regulation by enkephalinases will help our understanding of the role of enkephalins in circuitry and reward-related behaviors. Tools for measuring extracellular enkephalin specifically, such as liquid chromatography coupled with mass spectrometry analysis of *in vivo* samples (Mabrouk et al., 2011; DiFeliceantonio et al., 2012; Al-Hasani et al., 2018) and voltammetry to measure met-enkephalin (Calhoun et al., 2019) can be further applied during drug self-administration and while measuring other reward-related behaviors. Recent advancements in sensors to track dynamics of dopamine can ideally be applied to other neuromodulators like enkephalin (Patriarchi et al., 2018). Similarly, fluorescent reporters that can detect MOR activation are in development (Kroning and Wang, 2021). The ability to measure the dynamics of enkephalin degrading enzymes will also be necessary for better understanding of enkephalin regulation. Other tools such as conditional PENK knockout animal models (Gaveriaux-Ruff et al., 2011; Charbogne et al., 2014), caged-opioids, and allosteric modulators may be further implemented to study endogenous enkephalin release and function. Novel tools for more specific functional manipulations may be better for establishing causality, such as the use of CRISPR-Cas9 technology to selectively knockout enkephalin in specific cell types (Castro et al., 2021).

The studies discussed in this review provide strong evidence that the endogenous enkephalinergic system plays an important role in modulating reward circuitry and driving maladaptive behaviors to SUDs. In order to further understand the underlying mechanisms of SUDs, more research should probe the direct involvement of enkephalins and other opioid peptides in the formation, persistence, and relapse to SUDs. Furthermore, the endogenous enkephalinergic system may also be a potential target for novel therapeutics to prevent and treat SUDs and relapse.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Cellular and circuit diversity determines the impact of endogenous opioids in the descending pain modulatory pathway

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The descending pain modulatory pathway exerts important bidirectional control of nociceptive inputs to dampen and/or facilitate the perception of pain. The ventrolateral periaqueductal gray (vlPAG) integrates inputs from many regions associated with the processing of nociceptive, cognitive, and affective components of pain perception, and is a key brain area for opioid action. Opioid receptors are expressed on a subset of vlPAG neurons, as well as on both GABAergic and glutamatergic presynaptic terminals that impinge on vlPAG neurons. Microinjection of opioids into the vlPAG produces analgesia and microinjection of the opioid receptor antagonist naloxone blocks stimulation-mediated analgesia, highlighting the role of endogenous opioid release within this region in the modulation of nociception. Endogenous opioid effects within the vlPAG are complex and likely dependent on specific neuronal circuits activated by acute and chronic pain stimuli. This review is focused on the cellular heterogeneity within vlPAG circuits and highlights gaps in our understanding of endogenous opioid regulation of the descending pain modulatory circuits.

KEYWORDS

vlPAG, cellular diversity, circuit diversity, endogenous opioids, descending pain modulation

Descending pain modulation

Noxious stimuli evoke a sensory experience perceived as pain. Noxious signals initiated in the periphery are transmitted to many supraspinal structures that process the sensory, cognitive, affective, and motivational components that concurrently shape pain perception. These higher-order brain regions collectively project to the descending pain modulatory pathway, consisting of the ventrolateral column of the periaqueductal grey (vlPAG; Bandler et al., 1991; Bandler and Shipley, 1994) and the rostroventromedial medulla (RVM). RVM efferents to the dorsal horn of the spinal cord facilitate or inhibit incoming nociceptive inputs from the periphery (Basbaum and Fields, 1979; Mantyh and Peschanski, 1982; Vanegas et al., 1984b). The net output of this circuit under various acute and chronic pain conditions has been well-studied, especially the role of the RVM in bidirectional pain modulation (Basbaum and Fields, 1984; Lau and Vaughan, 2014; Heinricher and Ingram, 2020).

Bidirectional pain modulation by the RVM has been demonstrated through many studies using stimulation, pharmacology, *in vivo* electrophysiology, and various pain models. Neurons in the RVM have been characterized with respect to their role in modulating pain, identifying three distinct types of neurons: ON-, OFF-, and NEUTRAL-cells. These neuron types were initially characterized by their unique responses (*in vivo*) to acute noxious stimuli (Fields et al., 1983a; Vanegas et al., 1984b). ON-cells increase firing just prior to the tail-flick or paw withdrawal behavioral response to an acute noxious stimulus, OFF-cells pause just before the ON-cell burst and withdrawal response, and NEUTRAL-cells show no changes in firing. Some of each cell type project down to the dorsal horn (Vanegas et al., 1984b; Fields et al., 1995). Increased OFF-cell firing suppresses nociceptive reflexes (Fields and Heinricher, 1985) producing *descending inhibition of pain*, which can be seen most clearly in response to morphine or other opioids. Conversely, ON-cell firing contributes to *descending facilitation of pain*, which can be produced by many different pain models (Morgan and Fields, 1994; Porreca et al., 2002; Edelmayer et al., 2009) or pharmacological manipulations. Further, hyperalgesia and allodynia can result from either a reduction in OFF-cell firing or enhanced ON-cell firing (Martenson et al., 2009; Cleary and Heinricher, 2013). Importantly, although hyperexcited ON-cells promote descending facilitation, increased OFF-cell activation overrides the facilitation, yielding a net inhibitory output for the descending circuit (Satoh et al., 1983; Hentall et al., 1984; Fields and Heinricher, 1985; Jensen and Yaksh, 1989; Heinricher and Ingram, 2020).

In contrast to the bidirectional modulation of pain by the RVM, the upstream vlPAG has been primarily implicated in producing descending inhibition, due to the analgesic effect of both vlPAG stimulation (electrical or chemical) and locally applied opioid agonists (Reynolds, 1969; Mayer et al., 1971; Mayer and Liebeskind, 1974; Akil and Liebeskind, 1975; Soper and Melzack, 1982; Vanegas et al., 1984a; Jensen and Yaksh, 1989; Bandler et al., 1991; Bandler and Shipley, 1994; Tortorici and Morgan, 2002). vlPAG stimulation-mediated analgesia occurs predominantly via the dense projection to the RVM (Behbehani and Fields, 1979; Gebhart et al., 1983; Prieto et al., 1983), as the vlPAG sends sparse efferents directly to the dorsal horn (Basbaum and Fields, 1979). In particular, vlPAG stimulation activates an excitatory connection between the vlPAG and RVM OFF-cells (Behbehani and Fields, 1979; Basbaum and Fields, 1984; Vanegas et al., 1984a). More recent studies have reinforced the antinociceptive role of vlPAG glutamate neurons using selective, chemogenetic activation and have demonstrated that selective activation of vlPAG GABAergic neurons can produce hyperalgesia (Samineni et al., 2017a)—suggesting the capacity for the vlPAG to be an additional locus of bidirectional pain modulation. Interestingly in opposition to the role vlPAG glutamate neurons play in descending inhibition, a recent study has identified a subpopulation of dynorphin-expressing v/lPAG glutamate neurons, which when chemogenetically activated facilitate nociception (Nguyen et al., 2022). The key question remains whether distinct vlPAG populations, such as RVM-projecting glutamatergic or GABAergic neurons or more specific subpopulations within these groups, are activated by acute noxious stimuli or in persistent and chronic pain conditions mirroring these different experimental manipulations that produce descending inhibition or facilitation.

The vlPAG is a key site of opioid-induced analgesia mediated by mu-opioid receptors (MOR; Heinricher and Morgan, 1999). Activation of postsynaptic MORs, expressed on a subpopulation of vlPAG neurons, produces a hyperpolarizing current; whereas, activation of MORs in presynaptic terminals within the vlPAG inhibits neurotransmitter release. Pre- and postsynaptic MORs coupled to different signaling pathways work in concert to promote descending inhibition. However, other forms of global inhibition of the vlPAG (e.g., muscimol or baclofen) result in descending facilitation of pain, not the inhibition (analgesia) produced by opioid infusion. These contradictory findings were explained by a circuit mechanism referred to as the opioid-mediated *disinhibition of pain hypothesis* (Basbaum and Fields, 1984). This mechanism hypothesizes that opioids inhibit GABA release onto vlPAG neurons, either through selective postsynaptic MOR expression on inhibitory interneurons or at the level of the presynaptic GABAergic afferent terminals, disinhibiting excitatory RVM-projecting neurons that promote descending inhibition of pain. In subsequent sections, we consider critical studies that identify additional complexity in MOR expression, signaling, and regulation, that provide many loci for pain-mediated alterations to influence opioid-mediated pain modulation.

The descending pain modulatory circuit also exhibits sexual dimorphism (Fullerton et al., 2018). Females have ∼33% more RVM-projecting neurons than males, however, persistent inflammation activates significantly more RVM-projecting neurons in males than females (Loyd and Murphy, 2006, 2014). In addition, the antinociceptive potency of bicuculline, a $GABA_A$ receptor antagonist, injected into the vlPAG is greater in male rats compared to females (Bobeck et al., 2009), indicating differences in GABA tone within the vlPAG between males and females (Tonsfeldt et al., 2016). This same study showed that the antinociceptive potency of kainic acid, which activates glutamate receptors, is the same for males and females. This indicates that direct activation of the vlPAG activates a sufficient number of RVM OFF-cells needed to suppress nociception, which has been estimated to require relatively few neurons (<100; Hentall et al., 1984). Furthermore, RVM-projecting vlPAG neurons are more strongly disinhibited by systemic morphine in male rats compared to females (Loyd et al., 2007), emphasizing the need to continue to improve our understanding of how descending modulation of pain varies between males and females.

To date, studies have focused on defining the net output of the vlPAG and RVM in response to nociceptive stimuli and different pain states. However, many features of cellular- and circuit-based diversity suggest additional layers of complexity relevant to increasing our understanding of the role of the vlPAG in descending pain modulation in uninjured and pain states. Specifically, features including cortical and subcortical afferent inputs, efferent targets, neurotransmitter content, receptor and channel expression, morphology, intrinsic membrane properties, and responses to stimuli, can be used to discern between subpopulations of vlPAG neurons (Hamilton, 1973; Heinricher et al., 1987; Barbaresi and Manfrini, 1988; Chieng and Christie, 1994a; Park et al., 2010; Heinricher and Ingram, 2020; McPherson et al., 2021). The vlPAG is involved in many behavioral circuits associated with survival (i.e., threat, fear, pain) as well as vital autonomic functions like breathing, feeding, and respiration (Bandler et al., 2000; George et al., 2019; Silva and McNaughton, 2019). Thus, it is a prime brain area for using genetically encoded circuit-mapping tools to understand how specific PAG afferents participate in different behaviors. The broad categorization of excitatory and inhibitory neurons defined by these genetic methods is a useful starting point, however, does not account for the diversity that further distinguishes subpopulations of vlPAG neurons. In addition, methods dependent on gene expression (such as neurotransmitter content or MOR expression) assume that large populations defined by one key descriptive feature activate or inactivate in unison in response to stimuli, whereas studies are increasingly showing that this interpretation does not hold (Vaaga et al., 2020; McPherson et al., 2021). This review is focused on what is known about different aspects of heterogeneity within the vlPAG in relation to the descending pain modulatory system.

Opioids in the descending pain modulatory pathway

Endogenous opioids

Stimulation of the vlPAG produces analgesia in humans and antinociception in rats that is blocked by the MOR antagonist naloxone (Adams, 1976; Akil et al., 1976; Hosobuchi et al., 1977; Behbehani and Fields, 1979; Barbaro, 1988; Bach and Yaksh, 1995), providing evidence for the release of endogenous opioids in the vlPAG (Bagley and Ingram, 2020). Stimulation of the PAG produces an increase in the release of met-enkephalin (ME; Bach and Yaksh, 1995), a full MOR agonist, that is typically below detection limits under basal conditions (Del Rio et al., 1983). It is not clear where the ME originates as a subset of neurons distributed throughout the vlPAG express enkephalin and enkephalin-containing afferent terminals from other brain areas (Moss et al., 1983; Williams and Dockray, 1983). Enkephalin-containing vlPAG neurons send projections to the amygdala and the nucleus accumbens (Li et al., 1990a,b) but they may also send local collaterals. Both of these areas send inputs to vlPAG indicating multiple reciprocal circuits exist between the vlPAG and supraspinal brain areas where endogenous opioids may influence descending modulation. This is further supported by studies showing activation of enkephalin-expressing inhibitory interneurons in the central nucleus of the amygdala (CeA) increases Fos expression in non-serotonergic vlPAG neurons, inducing analgesia (Poulin et al., 2008; Paretkar and Dimitrov, 2019).

The vlPAG also receives β-endorphin-containing fibers from the arcuate nucleus of the hypothalamus (Finley et al., 1981; Ibata et al., 1985; Sim and Joseph, 1991), with confirmed β-endorphin release in the vlPAG following stimulation of the arcuate nucleus (Bach and Yaksh, 1995). The hypothalamus sends endomorphin-2 containing projections to the PAG (Chen et al., 2008) and high levels of endomorphin-2 are observed within the PAG (Martin-Schild et al., 1999). β-endorphin is a full agonist while endomorphin-2 is a partial agonist of the MOR (Narita et al., 2000) suggesting that these two agonists will activate MORs differently. In addition to endogenous MOR agonists, electrical stimulation of the CeA increases levels of the kappa-opioid receptor (KOR) agonist dynorphin A in the lateral PAG (Nakamura et al., 2013), however, dynorphin microinjection within the PAG is not analgesic (Fang et al., 1989). A recent study discovered a subpopulation of dynorphin-expressing vlPAG glutamate neurons that can facilitate nociception through KOR signaling within the RVM (Nguyen et al., 2022).

As one would anticipate, the release of endogenous opioid peptides in response to pain states and exogenous opioids varies significantly. Substance P induces ME release in the PAG that is correlated with antinociception (Del Rio et al., 1983; Rosén et al., 2004). The CFA-induced inflammatory pain model increases neuropeptide release (neurotensin increased 133% and ME increased 353%), with differential time courses for recovery (Williams et al., 1995). After 7 d of inflammation, neurotensin returns to baseline but ME remains elevated. Further, the enhancement in ME release in the vlPAG with inflammation is seen uniformly across the rostral-caudal axis and is maintained between 4 h, 4 d, and 14 d post-CFA in male rats (Hurley and Hammond, 2001). Other opioid peptides are also increased with other pain models. Formalin-induced inflammation increases the release of β-endorphin and endomorphin-2 within the PAG (Sun et al., 2001; Nakamura et al., 2013) and β-endorphin is released within the vlPAG during stress-induced analgesia (Külling et al., 1989). Interestingly, endogenous ME release is increased ∼50% by systemic morphine injection (Williams et al., 1995). Similarly, antinociception produced by DAMGO injections into the basolateral amygdala (BLA) is occluded by blocking endogenous MOR activation with MOR antagonists in the

vlPAG (Tershner and Helmstetter, 2000), demonstrating synergy between exogenous and endogenous opioid effects. The extent of the endogenous opioid release and efficacy during naïve and pain states with and without exogenous opioid use are key points of an ongoing investigation.

Importantly, extensive work is still required to understand endogenous opioid peptides in descending modulation in females, as most of the early studies were conducted in male rats. This is of particular importance given the sex differences observed in pain states both in animal models and the clinical population (Fullerton et al., 2018; Shansky and Murphy, 2021). Similarly, these studies are largely carried out in adult rats, overlooking the possible differences in the development of the descending pain modulatory circuit. One prime example of this is that activation of opioid receptors in the PAG produces opposite effects in young rats compared to adults (Kwok et al., 2014). This is a significant area of research considering 37% of children in the clinical population experience chronic pain (King et al., 2011). Our understanding of the mechanisms by which endogenous opioids produce analgesia during pain states is further complicated by the role of endogenous opioids in other circumstances (i.e., stress-induced analgesia; Ferdousi and Finn, 2018).

Endogenous opioids must be released with the spatial and temporal precision necessary to activate the circuit without directly inhibiting excitatory PAG efferents that target RVM OFF-cells involved in descending pain inhibition. Furthermore, how the release and efficacy of endogenous opioids are impacted by acute or ongoing pain states is not understood. Complementary to release, it is crucial to understand the specificity of opioid receptor expression and signaling across diverse neuron populations and cellular compartments with distinct mechanisms of action. Next, we consider many important studies that shape our understanding of MOR action in the vlPAG.

MOR expression and signaling

The PAG contains a high density of MOR expressing neurons (Mansour et al., 1986; Kalyuzhny et al., 1996; Gutstein et al., 1998; Commons et al., 1999, 2000; Wang and Wessendorf, 2002). As previously discussed, the disinhibition of pain hypothesis provides a possible circuit mechanism for opioid-mediated analgesia at the level of the vlPAG. Specifically, this mechanism proposes selective MOR expression on the cell bodies of vlPAG inhibitory interneurons within the vlPAG (Basbaum and Fields, 1984) and was later updated to include expression on GABAergic presynaptic terminals within the vlPAG (Chieng and Christie, 1994b; Lau and Vaughan, 2014).

MORs are $G_{i/o}$-coupled G protein-coupled receptors (GPCRs) expressed on postsynaptic cell bodies within the vlPAG. Agonist-bound MORs initiate a cascade of many signal transduction processes, including the activation of G protein-coupled inwardly-rectifying potassium channels (GIRKs) by $\beta\gamma$-subunits of activated $G_{i/o}$ G proteins (Logothetis et al., 1987) that produces a K^+ efflux and subsequent hyperpolarization of the neuron, inhibiting firing (North et al., 1987). MOR agonists exhibit functional selectivity differences in $G_{i/o}$ recruitment in MOR-GIRK signaling. In particular, maximal GIRK currents induced by DAMGO and fentanyl require G_o G proteins, compared to ME, which requires G_i (McPherson et al., 2018).

MORs are also expressed on presynaptic terminals within the vlPAG where they inhibit the release of the neurotransmitter (Chieng and Christie, 1994b; Vaughan et al., 1997). In GABAergic terminals, MORs couple to voltage-gated potassium channels through the phospholipase $A_2 \rightarrow$ arachidonic acid $\rightarrow$ 12-lipoxygenase cascade (Vaughan et al., 1997). This signaling pathway is not necessary for MOR inhibition of glutamate release in the vlPAG and is distinct from that used by other presynaptic GPCRs that inhibit GABA release (i.e., $GABA_B$; Vaughan et al., 1997; Bouchet and Ingram, 2020). Agonist-specific functional selectivity in the recruitment of G_i or G_o G proteins also occurs during the inhibition of presynaptic GABA release. Specifically, in order to achieve maximal efficacy for inhibiting spontaneous GABA release DAMGO requires G_o, fentanyl requires both G_o and G_i, and ME sufficiently inhibits release with either (Bouchet et al., 2021). Comparatively, for maximal inhibition of evoked GABA release, DAMGO requires both G_o and G_i, and fentanyl and ME require G_i.

Postsynaptic MOR expression has been found on ∼30%–60% of vlPAG neurons where MOR agonist application produces a GIRK current response reversible by a MOR antagonist (Chieng and Christie, 1994a; McPherson et al., 2018). Many studies have concluded that this subset of MOR-expressing neurons is GABAergic interneurons that tonically inhibit glutamatergic projection neurons (Yaksh et al., 1976; Basbaum and Fields, 1984; Reichling et al., 1988; Park et al., 2010; Lau and Vaughan, 2014). This selective neuron-type expression has been challenged by several studies showing evidence of postsynaptic MORs on neurons with varied neurotransmitter content, intrinsic firing properties, and morphology (Chieng and Christie, 1994a; Osborne et al., 1996; Commons et al., 2000; Morgan et al., 2008; Zhang et al., 2020; McPherson et al., 2021). Furthermore, MOR activation has been shown to directly inhibit a subset (∼14%) of RVM-projecting vlPAG neurons (Osborne et al., 1996). However, this does not rule out the possibility that MORs can be expressed on GABAergic projection neurons that send local collaterals within the vlPAG. Thus, it is clear that the actions of opioids in vlPAG are more complex than their ability to disinhibit excitatory RVM-projecting vlPAG neurons.

The analgesic effect of morphine microinjected into the PAG is reversed by muscimol also microinjected into the PAG (Moreau and Fields, 1986), underscoring the overall role of opioids in alleviating inhibitory tone to produce analgesia.

As a result of both pre- and postsynaptic mechanisms, MOR activation disinhibits excitatory RVM-projecting vlPAG neurons (Lau et al., 2020), which can activate downstream nociception-inhibiting OFF-cells within the RVM (Fields et al., 1983b; Basbaum and Fields, 1984; Cheng et al., 1986). The non-selective excitatory amino acid (EAA) receptor antagonist kynurenate in the RVM abolishes systemic opioid-mediated activation of OFF-cells and antinociception (Heinricher et al., 1999), confirming the antinociceptive role of glutamate release from afferents within the RVM. However, both GABAergic and non-GABAergic vlPAG neurons have also been found to project to ON- and OFF-cells within the RVM, with varied MOR expression on their cell bodies and axon terminals within the RVM (Commons et al., 2000; Zhang et al., 2020). Parallel descending circuits, both excitatory and inhibitory vlPAG afferents within the RVM, have been discussed to encompass these findings (Lau and Vaughan, 2014).

Although MORs are most effective in inhibiting presynaptic GABA release in the vlPAG, they also inhibit release to a lesser extent from glutamatergic afferents (Lau et al., 2020). This suggests that in the presence of opioids, there is a net excitatory effect (increased E/I balance). Additionally, the EC_{50} for DAMGO-mediated inhibition of presynaptic release is roughly four times lower than that for postsynaptic K^+ current (Pennock and Hentges, 2011). This creates the possibility for a MOR-expressing neuron to be either disinhibited by a low dose of opioids (removing inhibitory afferent tone) or inhibited by a higher dose (triggering a hyperpolarizing GIRK-mediated K^+ current). Interestingly, there is functional selectivity between opioid agonists for pre- vs. postsynaptic signaling. To achieve maximal antinociceptive efficacy morphine requires presynaptic MOR activation and fentanyl requires postsynaptic MOR activation (Morgan et al., 2020). Overall, the activation of presynaptic MORs alone sufficiently produces analgesia. These findings present interesting questions about how smaller concentrations of targeted endogenous opioid release may alter vlPAG neuron activity differently than larger concentrations of globally delivered exogenous opioids. These compartment-specific differences in opioid potency also demonstrate the ability for opioids to have many different effects on vlPAG neurons and the subsequent signaling they trigger at efferent targets depending on E/I balance and postsynaptic MOR expression.

Interestingly, KOR, and not DOR, activation inhibits evoked inhibitory synaptic release from afferent terminals comparably to MOR activation (Lau et al., 2020). Despite this overlap in presynaptic function, vlPAG KOR activation does not produce analgesia in rats (Bodnar et al., 1988; Smith et al., 1988; Fang et al., 1989; Ossipov et al., 1995). Optogenetic studies examining KOR and MOR sensitivity of specific afferents may be able to solve this contradictory observation. It is likely that KORs are expressed on different afferent terminals from brain areas that do not have a strong role in opioid analgesia, further reinforcing the importance of identifying whether inhibiting selective vlPAG afferent inputs are necessary to produce analgesia, how these inputs are altered by pain states, and whether these alterations impact the ability for endogenous opioids to sufficiently dampen their signal.

Pain-state-mediated alterations to these parallel circuits, such as the E/I balance onto RVM-projecting vlPAG neurons or vlPAG afferent inputs onto specific neuron types in the RVM, have yet to be defined but seem likely due to known changes in opioid efficacy in these regions during pain states. Persistent inflammation (24 h) prior to systemic morphine administration significantly increases the analgesic response compared to uninjured animals (Eidson and Murphy, 2013). A study completed in male rats showed greater analgesic efficacy by DAMGO locally infused downstream in the RVM 14 d after CFA-induced inflammatory pain (Hurley and Hammond, 2001). The attenuation of morphine tolerance by persistent peripheral inflammation aligns with clinical literature, where chronic pain patients do not readily demonstrate opioid tolerance (Collett, 1998; Dworkin et al., 2005). Altogether, the effect of opioids within the vlPAG is much more complex than selective postsynaptic MOR expression inhibiting GABA interneurons. The next critical questions include whether the E/I balance is distributed uniquely across distinct subpopulations of vlPAG neurons, how opioid modulation of neuronal activity is impacted by alterations induced by pain states (i.e., altered intrinsic activity neuronal activity or presynaptic inputs), and how this influences vlPAG efferent engagement with functionally distinct ON- and OFF-cells within the RVM. In the next section, we consider different MOR signaling regulation mechanisms that reveal additional compartmental specificity in MOR signaling.

Regulation of MOR signaling

Multiple mechanisms exist to regulate ongoing MOR signaling. Continuous MOR activation triggers the phosphorylation of the intracellular C-terminal tail of the receptor by several different protein kinases, including protein kinase A, protein kinase C (PKC), and G protein receptor kinases (Williams et al., 2013). Phosphorylation of the C-terminus triggers desensitization and recruitment of β-arrestin (βarr), resulting in the internalization of the receptor. MOR signaling is recovered around 60 m following maximal desensitization, indicating the time course for receptor recycling back to the membrane. Postsynaptic MOR-mediated GIRK currents within the vlPAG are relatively small, however, they do desensitize during prolonged MOR agonist exposure, and this desensitization is even greater in morphine-tolerant rats (Ingram et al., 2008). Desensitized MOR-GIRK signaling, enhanced by morphine tolerance, reduces the ability for opioids to hyperpolarize vlPAG neurons, suppressing their firing rates.

Although postsynaptic MORs in the vlPAG desensitize, the inhibition of GABA release by presynaptic MORs within the vlPAG does not desensitize during prolonged exposure to an agonist in drug naïve or chronic morphine treated rats (Fyfe et al., 2010). Additional evidence from the arcuate nucleus of the hypothalamus confirms presynaptic MORs, as well as other presynaptic GPCRs, are resistant to desensitization (Pennock et al., 2012). Interestingly, presynaptic MORs have been shown to undergo internalization in the continued presence of ligand, but are quickly replaced by lateral diffusion along the axon surface (Jullié et al., 2020). Thus, despite the dynamic movement of MORs in the presynaptic compartment, signaling is maintained.

In addition to βarr-mediated desensitization, activated G proteins that bind and activate effector targets are also regulated by the regulator of G protein signaling (RGS) proteins. RGS proteins bind to active α-subunits driving GTP-hydrolysis to GDP, boosting the affinity between the α- and βγ-subunits resulting in the reformation of the inactive heterotrimer. Many RGS proteins are involved in the regulation of MORs, including RGS4 (Garzón et al., 2005a; Roman et al., 2007; Leontiadis et al., 2009; Santhappan et al., 2015), RGS9-2 (Psifogeorgou et al., 2007; Papachatzaki et al., 2011; Gaspari et al., 2017), RGS19 (Wang and Traynor, 2013), and RGSz (Garzón et al., 2005b; Gaspari et al., 2018; Sakloth et al., 2019). Within the vlPAG, a mouse model with RGS-insensitive G proteins exhibits increased opioid-mediated inhibition of presynaptic GABA release and increased morphine antinociception (Lamberts et al., 2011). These findings support the idea that RGS proteins negatively modulate MOR inhibition of evoked GABA release (eIPSCs), influencing supraspinal nociception. Antagonizing hydrolysis by RGS4 in the vlPAG enhances morphine-mediated analgesia, but not fentanyl, which may be a function of their different signaling pathways (Morgan et al., 2020).

In contrast, RGS proteins positively modulate postsynaptic MOR-mediated GIRK activation in the vlPAG (McPherson et al., 2018). RGS proteins playing a facilitatory role in MOR-GIRK signaling is counterintuitive, as RGS proteins inactivate G proteins which activate GIRK channels. However, a "kinetic scaffolding" model outlines the necessity of rapid turnover of G proteins to replenish the inactive G protein substrate pool for quick re-activation by the receptor (Clark et al., 2003; Zhong et al., 2003). The proximity of substrates and binding partners, here MORs and GIRKs, allows for expedient activation→ channel gating → inactivation. As a result, when the RGS binding is disrupted in the RGS-insensitive mouse model, the efficiency in coupling is lost and the substrate pool turnover is hindered, reducing the overall K^+ conductance through the GIRK channel. Thus, this model suggests that RGS proteins serve as key components in receptor and effector coupling, enhancing the efficiency of the signal transduction pathway. Distinct actions of RGS proteins and agonist-specific G protein recruitment, in pre- and postsynaptic MOR signaling provide another avenue for compartment-specific MOR signaling that can affect the analgesic circuit. Future studies on how acute and persistent pain states may influence RGS actions in pre- and postsynaptic MOR signaling will further our understanding of how RGS-mediated positive and negative modulation of compartment-specific MOR signaling within the vlPAG influence pain states. Furthermore, the duration of opioid (i.e., morphine) exposure impacts the association between MORs and specific RGS proteins in the PAG (Garzón et al., 2005a), highlighting one mechanism by which treating pain states with exogenous opioids can influence MOR regulation.

Sustained MOR activation can also produce heterologous desensitization at adjacent receptors that use the same intracellular signaling components (Leff et al., 2020; Adhikary et al., 2022). As a result, these mechanisms associated with MOR desensitization could be adapting the signaling of other receptors, which then influence tolerance and withdrawal. Additional receptors within the vlPAG have been hypothesized to contribute to the analgesic tolerance of opioids, such as the nociceptin receptor (NOP), which is another GPCR that is densely expressed within the vlPAG with similar homology to MOR/DOR/KOR but low affinity for opioid agonists and antagonists (Anton et al., 1996). Activation of NOP blocks analgesia and NOP antagonists microinjected into the vlPAG stop the development and expression of analgesic tolerance to systemic morphine administration (Parenti and Scoto, 2010; Scoto et al., 2010). Ongoing activation of NOP produces PKC-mediated heterologous desensitization of MORs in cultured cells (Mandyam et al., 2002), providing an example of how cross-talk between these receptor signaling systems can influence analgesic efficacy.

Cellular diversity

Many methods have been used to characterize different neuronal populations within brain regions. Tools that utilize genetic approaches to selectively alter neuronal activation, such as optogenetics or DREADDs, have reinforced our understanding of how the activation of excitatory and inhibitory vlPAG neurons influences the net output of the descending pain modulatory pathway. However, these studies do not address the question of which vlPAG neurons are recruited during acute and persistent pain states to influence ongoing nociception. Additional features such as intrinsic firing and membrane properties, receptor and channel expression, endogenous opioid peptide production, afferent inputs, and efferent targets can collectively define vlPAG neurons engaged by acute nociceptive stimuli, ongoing pain states, and endogenous or exogenous opioids. Other cell types within the vlPAG, such as microglia, also play important and extensive roles in the pain response and analgesia (Loyd and Murphy, 2006; Fullerton et al., 2018; Averitt et al., 2019).

Neurotransmitter content

Early work using GAD-immunoreactivity, labeled a subset of ∼33% of vlPAG cell bodies (Barbaresi and Manfrini, 1988; Reichling and Basbaum, 1990). The GABAergic subpopulation combined with the identification of a direct, excitatory connection between the vlPAG and the RVM that contributes to stimulation-mediated analgesia (Behbehani and Fields, 1979), leads to the proposed circuit where inhibitory neurons in the vlPAG serve as an interneuron population that control the intensity of the output signal to the RVM (Basbaum and Fields, 1984). To determine the effect of GABA on vlPAG output, GABA receptor antagonists were locally infused into the vlPAG increasing vlPAG firing and the firing of downstream RVM OFF-cells, producing analgesia (Moreau and Fields, 1986; Behbehani et al., 1990; Knight et al., 2002). These studies do not determine the source of GABA, which can come from GABAergic interneurons or GABAergic afferents originating from many different brain regions. Direct evidence for GABAergic interneurons within the vlPAG has not been provided to date.

Selective activation of vlPAG GABA neurons using DREADDs produces hyperalgesia and confirms the pronociceptive role of vlPAG GABA neurons independent of GABA afferents from other regions (Samineni et al., 2017a). However, this experiment does not rule out that this behavioral outcome could be the result of GABAergic neurons that project to the RVM that directly inhibit spontaneous OFF-cell firing (Heinricher et al., 1991). Histological studies confirm that GABAergic afferents in the RVM coming from the vlPAG come in contact with both OFF- and ON-cell populations (Morgan et al., 2008). Additionally, DREADD-mediated activation of GABAergic neurons does not address whether acute or ongoing nociceptive stimuli activate the same neurons within the vlPAG, demonstrating the physiological relevance of the impact of selectively activating this population or if neuronal activation is more heterogeneous, and if so, what resulting output that produces. Additionally, it is important to identify whether there are other circuit consequences of increased activity of vlPAG GABAergic neurons, such as increasing GABA release onto RVM OFF-cells, which would also produce hyperalgesia.

The proinflammatory cytokine Tumor Necrosis Factor-α (TNF-α) has been recently shown to selectively activate GABAergic neurons within the vlPAG (Pati and Kash, 2021), suggesting a possible mechanism by which a pain state can produce the targeted activation of vlPAG GABA neurons. TNF-α is one of many proinflammatory cytokines released by activated microglia, which are activated by inflammatory pain states (Fullerton et al., 2018). Interestingly, the enhanced activity of vlPAG GABA neurons by TNF-α did not increase GABAergic synaptic inputs onto neighboring vlPAG dopamine (DA) neurons—suggesting that if these GABAergic neurons send local collaterals within the vlPAG, they do not target DA neurons. Altogether, it is possible that pain states activate microglia, which release TNF-α, activating GABA neurons to enhance local GABA tone—resulting in descending facilitation through a specific subpopulation of vlPAG neurons. However, this possible mechanism would need to be confirmed in a pain model to implicate selective activation of vlPAG GABA neurons in altered pain modulation during pain states.

Selective activation of glutamatergic neurons in the vlPAG with DREADDs promotes analgesia (Samineni et al., 2017a). This reinforces the conclusion from many early studies that stimulation-mediated analgesia is driven by the activation of glutamatergic neurons (Reynolds, 1969; Mayer et al., 1971; Mayer and Liebeskind, 1974; Akil and Liebeskind, 1975; Behbehani and Fields, 1979; Soper and Melzack, 1982; Jensen and Yaksh, 1989). However, selective activation of glutamatergic vlPAG neurons also enhances anxiety (Taylor et al., 2019), one of the many off-target effects precluding this stimulation target as a therapeutic option for clinical pain management. Deep brain stimulation targeting the vlPAG has been applied therapeutically for treatment-resistant hypertension (Patel et al., 2011; O'Callaghan et al., 2014), emphasizing the many subcircuits that utilize this region and the importance of understanding whether specific stimuli engage different neuronal subpopulations within the vlPAG. Furthermore, a recent study using single nucleus RNA-sequencing and Multiplexed Error-Robust Fluorescence *in situ* Hybridization (MERFISH) identified over 100 excitatory and inhibitory neuronal populations (Vaughn et al., 2022). In addition to unique transcriptional profiles, these neurons were found to be spatially distributed uniquely along the rostral-caudal axis, and multiple populations were activated in unison by different instinctive behaviors (i.e., mating, aggression, etc.)—underscoring the complexity in subpopulations of excitatory and inhibitory vlPAG neurons.

An increasing number of studies are providing evidence that glutamate neurons do not represent a functionally homogeneous population of vlPAG neurons. One example is a Chx10-expressing subpopulation of glutamate neurons that are specifically involved in mediating freezing behaviors (Vaaga et al., 2020). Activation of another subpopulation vlPAG glutamate neurons that express dynorphin produces dynorphin-mediated facilitation of nociception through signaling at terminals in the RVM (Nguyen et al., 2022). Findings such as these emphasize the caution we should take when using neurotransmitter content as the only genetic marker in behavioral studies. Although it is interesting to know that the net output of activating all glutamate neurons is analgesia, important questions remain. First, how many vlPAG glutamate neurons are necessary to produce analgesia? Second, do glutamate neurons that promote descending inhibition overlap with glutamate populations involved in other outputs (i.e., freezing)? Lastly, are there markers for subpopulations of glutamate neurons that are activated by nociceptive stimuli or necessary for producing analgesia that can be harnessed to

develop targeted drug delivery methods? Together, the answers to these questions will equip us with the information needed to develop therapeutic manipulations that produce the smallest intervention possible that drives descending inhibition from the level of the vlPAG.

Additional neuron populations within the vlPAG with different neurotransmitter content engage with descending modulation differently. Most notably, DA neurons have been implicated in the broader supraspinal pain circuitry and analgesia (Hökfelt et al., 1976; Meyer et al., 2009; Taylor et al., 2019; Yu et al., 2021) despite not projecting directly to the RVM (Suckow et al., 2013). Interestingly, these DA neurons co-release both DA and glutamate at terminals in the bed nucleus of the stria terminalis (BNST; Li et al., 2016). Selective activation of these vlPAG DA neurons produces antinociception in male rats (Yu et al., 2021). Serotonergic neurons that are densely populated in the dorsal raphe and extend diffusely up into the most ventral portion of the vlPAG (Crawford et al., 2010), have also been implicated in opioid-mediated analgesia (Samanin et al., 1970).

Altering the activity of vlPAG neuron populations with distinct neurotransmitter content can influence pain modulation, however, this alone does not answer important questions: (1) are these molecularly defined subpopulations selectively engaged by pain states, mirroring these activation/inhibition studies in a physiological condition; (2) how does this change over the course of acute, persistent, and chronic stages; and (3) how do endogenous and exogenous opioids influence how these neurons participate in descending circuitry in naïve and pain states?

Receptor or channel expression

In addition to neurotransmitter content, the expression of receptors and channels amongst vlPAG neurons can differentiate distinct populations and potentially define any selective, population-specific engagement by pain states or opioids (Chieng and Christie, 1994a; Park et al., 2010; Liao et al., 2011; Du et al., 2013; Lau and Vaughan, 2014; McDermott et al., 2019). Although MORs mediate morphine antinociception (Matthes et al., 1996) all three opioid receptors (MOR, DOR and KOR) are expressed in vlPAG. DOR and KOR are densely expressed within the vlPAG, both on cell bodies (including RVM-projecting neurons) and on afferent terminals (Mansour et al., 1986; Kalyuzhny et al., 1996; Gutstein et al., 1998; Kalyuzhny and Wessendorf, 1998; Wang and Wessendorf, 2002). Neither DOR nor KOR activation elicits GIRK currents from vlPAG neurons in rats (Chieng and Christie, 1994a), although both activate GIRK currents in mouse vlPAG (Vaughan et al., 2003). Interestingly, DOR activation alone does not produce analgesia but potentiates MOR-mediated analgesia (Rossi et al., 1994). This effect is observed when MOR agonist DAMGO is microinjected into the vlPAG or RVM and DOR agonist deltorphin is microinjected into the other region and not when they are microinjected into the same region—suggesting synergy is occurring at the circuit and not cellular level.

Several studies have tried to use MOR as a marker for a specific functional subpopulation of vlPAG neurons. One interesting possibility identified in mice showed that MOR expressing, tonic firing GABAergic neurons also expressed T-type calcium channel, indicated by low-threshold spiking (LTS; Park et al., 2010). Using MOR-mediated GIRK currents, they observed that T-type channel expressing GABAergic neurons were opioid-sensitive (five neurons) and the remaining GABAergic (four neurons) and phasic firing, non-GABAergic neurons were opioid-insensitive. However, in a larger data set in the vlPAG of rats, LTS was not a predictor of opioid sensitivity (McPherson et al., 2021). Furthermore, LTS was observed in phasic firing neuronal populations in rats in addition to the tonic firing populations that exclusively had LTS in the mouse study. Together these discrepancies suggest either T-type channels are more broadly expressed in rats than in mice or the mouse data set did not capture a large enough sample to observe phasic firing, non-GABAergic, opioid-sensitive neurons.

Other receptors expressed in the vlPAG can also modulate the effects of opioids. For example, activating NOP with the endogenous ligand nociception/orphanin FQ reduces the analgesic efficacy of endogenous opioids and systemic morphine (Mogil et al., 1996). Conversely, NOP antagonists potentiate DAMGO efficacy, whether the animal is pretreated or given the antagonist after DAMGO administration (all microinjected within the vlPAG; Scoto et al., 2007). NOP activation also appears to be contributing to the development and expression of allodynia in acute inflammatory pain and chronic neuropathic pain (Scoto et al., 2009), making it a potential therapeutic target to both modulate opioid efficacy and nociceptive thresholds in the absence of opioid use.

Additionally, recent studies found that GPR171, a recently deorphanized GPCR, is expressed on GABAergic vlPAG neurons where it regulates opioid-mediated antinociception (McDermott et al., 2019). GPR171 agonists enhance morphine efficacy while antagonists do the opposite, with the most substantial effect seen with the supraspinal antinociceptive test (hotplate). The GPR171 agonist MS15203 administered daily after injury alleviated thermal hypersensitivity (after CFA) and allodynia (after neuropathic pain) in males only (Ram et al., 2021). Of note, the neuropathic pain model reduced PAG GPR171 expression in male mice only, which was recovered by the agonist treatment.

The DA and opioid receptor systems provide examples of signaling interactions that influence pain modulation at the level of the vlPAG. Activation of vlPAG DA receptors directly with agonist (-) apomorphine or indirectly with D-amphetamine, produces robust antinociception via the descending circuit with the RVM, and is attenuated by D_2 receptor blockade (Flores et al., 2006; Meyer et al., 2009; Ferrari et al., 2021). In

addition to DA-mediated antinociception, blocking either D_1 or D_2 DA receptors inhibits opioid-mediated antinociception in a dose-dependent manner (Flores et al., 2004; Meyer et al., 2009; Tobaldini et al., 2018). These results are consistent with previous findings that show a significant reduction in the antinociceptive effect of systemic opioids (specifically, heroin and morphine) after selectively ablating DA neurons within the vlPAG (Flores et al., 2004). Mechanistically, activation of D_2 receptors induces GIRK currents (Pillai et al., 1998; Marcott et al., 2014) and dopamine applied on slices *in vitro* reduces presynaptic GABA release (Meyer et al., 2009). Interestingly, unlike the antinociceptive tolerance observed with repeated opioid administration, the DA-receptor system sensitizes to repeat (-) apomorphine administration, producing increased antinociception, making the furthered understanding of these mechanisms of particular relevance for the development of novel therapeutics (Schoo et al., 2018).

Overall, defining the specific combinations of receptor and channel expression in combination with other features of cellular heterogeneity (neurotransmitter content, intrinsic properties, and specific circuitry) will increase our understanding of neuron types within the vlPAG. Compiling these features into comprehensive vlPAG neuron profiles may provide interesting insight into how pain states alter these neurons, the descending modulatory circuit, and the efficacy of drugs targeting these receptor-channel complexes.

Intrinsic firing properties

Characterizing intrinsic membrane and firing properties is a common approach to defining neuronal heterogeneity (Prescott and De Koninck, 2002; Sedlacek et al., 2007; Van Aerde and Feldmeyer, 2015; Pradier et al., 2019) and determining these properties in naïve animals allows for the evaluation of alterations induced by persistent inflammation (Li and Sheets, 2018; Adke et al., 2021; McPherson et al., 2021). Neuronal firing properties and response to noxious stimuli have been used to define important, functionally distinct neurons within the RVM (Fields et al., 1983a; Vanegas et al., 1984b). These landmark papers that characterized responses of distinct neuron types to noxious stimuli (ON-, OFF-, and NEUTRAL-cells) have served as a useful framework for subsequent findings.

ON- and OFF-cells respond differently to opioids, application of EAAs, and blocking inhibitory inputs. First, RVM ON-cells selectively express MORs and as a result, iontophoretic application of morphine inhibits ON-cell firing without affecting OFF-cell firing (Heinricher et al., 1992). ON- and OFF-cells respond differently to excitatory and inhibitory afferent input. Iontophoretic application of a glutamate receptor antagonist reduces the ON-cell burst triggered by the noxious stimulus and ON-cell spontaneous firing and does not alter OFF-cell firing (Heinricher and Roychowdhury, 1997; Heinricher et al., 1999). Conversely, iontophoretic application of the GABA antagonist bicuculline eliminates the OFF-cell pause triggered by the noxious stimulus but does not change ON-cell firing (Heinricher et al., 1991). Together these studies suggest that enhanced glutamate release within the RVM can increase ON-cell firing while keeping the OFF-cells unaltered and enhanced GABA release within the RVM can reduce OFF-cell firing without impacting ON-cell firing. This highlights the importance of identifying which vlPAG neurons are activated by pain states, how they alter afferent inputs in the RVM, and where endogenous or exogenous opioids intervene in the circuit.

In addition to determining how specific synaptic inputs can affect RVM ON- and OFF-cells, studies have examined how these cells respond to noxious stimuli during different pain stages. Upon CFA injection, both ON- and OFF-cell spontaneous activity are enhanced but spontaneous firing for both neuron types returns to baseline after a couple of hours; however, mechanical thresholds are reduced into the innocuous range (Cleary et al., 2008). Furthermore, blocking excitatory afferent inputs within the RVM prior to chronic constriction injury results in slower and diminished development of mechanical allodynia, correlating with a reduction in the hyperexcitability of spinal neurons (Sanoja et al., 2008). Combined with what is known about the effect of afferent inputs that impinge onto distinct neuron types in the naïve condition, this suggests that glutamatergic inputs onto ON-cells are important for the development of hyperalgesia, calling into question how excitatory and inhibitory projections from the vlPAG contribute to these changes.

In vivo recordings from the vlPAG have also identified neurons that respond to nociceptive stimuli (Heinricher et al., 1987; Samineni et al., 2017b), finding ON-, OFF-, and NEUTRAL-cells. Neuropathic pain induced by paclitaxel, a commonly used chemotherapy drug, enhances spontaneous firing and lowers the response thresholds in vlPAG ON-cells and OFF- and NEUTRAL-cells in response to noxious and previously innocuous stimuli (Samineni et al., 2017b). These studies provided evidence that pain states selectively activate subpopulations of neurons within the vlPAG and that the acute firing response to a noxious stimulus can be used to distinguish distinct vlPAG neuron populations.

An *ex vivo* survey of nearly 400 neurons using *in vitro* whole-cell patch-clamp experiments identified four distinct neuron types based on their intrinsic firing properties: Tonic (35%), Phasic (46%), Onset (10%), and Random (9%; McPherson et al., 2021). Tonic neurons (35%) fired continuously in response to depolarizing current steps compared to Phasic neurons (46%) which reached depolarization block in the more strongly depolarizing steps. These neuron types allowed the same study to identify that persistent CFA-induced inflammation (5–7 d) selectively enhances the spontaneous firing rate of Phasic neurons. Identifying activation of specific subtypes of vlPAG neurons prompts many interesting follow-up studies, including

examining intrinsic changes in receptors and/or channels or adaptations in afferent inputs. A study evaluating GABAergic neurons in a genetically defined mouse model observed that firing patterns in mice largely correlated with neurotransmitter content, with 31/33 GABAergic neurons having a tonic firing pattern with the other 2/33 showing a phasic pattern (Park et al., 2010). If this correlation observed in mice is upheld in rats, enhanced spontaneous activity of Phasic neurons after persistent inflammation may be producing the glutamate afferent input onto RVM ON-cells that contributes to allodynia. These interpretations are made even more interesting if neurons with distinct firing patterns have unique afferent inputs, that could for example contribute to enhanced Phasic firing after persistent inflammation or unique efferent targets that implicate the enhanced Phasic firing in altering signaling within different circuits.

In addition to providing a useful framework to identify mechanisms of targeted neuronal activation after different stimuli, firing patterns provide insight into how neurons may encode noxious stimuli. For example, a tonic firing neuron can entrain stimuli of varying intensities, whereas a phasic neuron can only do so at low-intensity ranges. At the higher depolarizing intensities a Phasic neuron becomes a coincidence detector, similar to the Onset neuron (Prescott and De Koninck, 2002). This can change whether presynaptic release from these neurons onto their downstream targets is ongoing (Tonic) or transient (Phasic). Recently published work has discovered opposing functional outputs produced by activating the same GABAergic neuron population with different channelrhodopsin-2 variants that have distinct off-kinetics (Baleisyte et al., 2022). The two variants produce two different firing patterns with the same optogenetic stimulation paradigm; the faster variant has identical action potentials with each stimulation, whereas the slower variant leads to significant attenuation of the action potential peak over repeated stimulation—demonstrating the importance of combining firing properties with neurotransmitter content to more completely understand the implication of neuron populations within a circuit.

Circuit diversity

Afferent inputs

The vlPAG receives inputs from many cortical and subcortical regions associated with nociceptive, cognitive, and affective components of pain. Ascending nociceptive inputs to the vlPAG come through the spinothalamic, spinoparabrachial, and spinomesocenphalic tracts, with some inputs coming directly from the spinal cord to the vlPAG (Menétrey et al., 1982; Yezierski and Mendez, 1991). The spinomesencephalic tract provides direct inputs to the PAG, however, these inputs have been linked to nociception and analgesia, as well as aversive behaviors (Willis and Westlund, 1997). Additional ascending nociceptive inputs come from the parabrachial complex (Gauriau and Bernard, 2002), which receives inputs from the superficial and deep dorsal horn (Roeder et al., 2016). Forebrain regions including the medial prefrontal, agranular insular, and anterior cingulate cortices, amygdala, BNST, and hypothalamus send the most significant supraspinal inputs to the PAG (Shipley et al., 1991; An et al., 1998; Floyd et al., 2000; Hao et al., 2019; Silva and McNaughton, 2019). In addition to anatomical studies showing connections between these regions and the vlPAG, studies using lesions and pharmacological manipulations have provided evidence that these regions participate in pain circuitry (Donahue et al., 2001; Ikeda et al., 2007; Starr et al., 2009; Bliss et al., 2016; Mills et al., 2018). For example, antinociception induced by morphine injected into the basolateral and medial nuclei of the amygdala is interrupted by lesioning the vlPAG (Helmstetter et al., 1998; McGaraughty et al., 2004)—emphasizing the importance of the vlPAG as an integration site for cortical inputs involved in pain modulation and opioid-mediated analgesia.

Supraspinal inputs are both excitatory and inhibitory so vlPAG neuronal activity is dictated by the E/I balance onto an individual neuron. Opioid-mediated disinhibition of pain is one example where it is presumed that glutamatergic PAG output neurons are biased towards a more inhibited state by GABAergic afferent inputs. In one study, glutamatergic inputs from the medial (fastigial) cerebellar nuclei synapse onto 20% of Chx10-expressing glutamatergic neurons, 21% of GABAergic ($GAD2^+$) neurons, and 70% of DA neurons within the vlPAG (Vaaga et al., 2020), clearly demonstrating that afferent inputs are not universally distributed within the vlPAG. These results highlight the importance of identifying specific afferent inputs that are activated by either pain or opioids that could be useful in defining subpopulations of vlPAG neurons.

Persistent inflammation induced with CFA enhances GABA tone in the vlPAG of female rats (Tonsfeldt et al., 2016). In addition to changes in GABA release by pain states, the glutamatergic release was decreased in the vlPAG 3 and 10 d after spinal nerve ligation (Ho et al., 2013). Although different pain models were used in these studies, the results suggest possible changes in the balance of excitatory and inhibitory inputs (E/I balance). Altered afferent release from either excitatory or inhibitory terminals can influence firing rates, and the changes to firing induced by opioid-mediated inhibition of presynaptic release, thus yielding altered engagement with downstream targets like the RVM. For example, if the afferent inputs onto a neuron are excitatory-dominant in the naïve condition, opioids will remove the excitatory drive, resulting in inhibition of firing. However, if the known enhanced GABA tone after persistent pain shifts the E/I balance onto this same neuron to becoming inhibitory-dominant, opioids will now activate firing. This shift in how opioids can alter vlPAG neuronal firing can have a

significant effect when considering how any specific neuron engages with ON- or OFF-cells within the RVM.

Efferent targets

Projection target is another important feature that can increase our understanding of how distinct types of vlPAG neurons engage with downstream targets and how that connection is altered by persistent inflammation or opioid action. The vlPAG contributes to the overall output of the descending pain modulatory pathway at the level of the dorsal horn of the spinal cord through its connection with the RVM (Behbehani and Fields, 1979; Gebhart et al., 1983; Prieto et al., 1983). The RVM-projecting population contains both GABAergic and non-GABAergic neurons (Commons et al., 2000; Morgan et al., 2008). In the mouse, both tonic firing (7/12) and phasic firing (5/12) neurons project to the RVM with comparable density; however, low-threshold spiking, MOR-expressing GABAergic tonic firing neurons did not project to the RVM (Park et al., 2010). Lau et al. (2020) found that RVM-projecting vlPAG neurons lacking MOR expression are disinhibited by DAMGO application compared to non-RVM-projecting neurons which are inhibited (n = 9), however, other findings show that RVM-projecting vlPAG neurons can express MORs (Commons et al., 2000). A subset of dynorphin-releasing glutamatergic vlPAG neurons (∼32%) project to the RVM, making up ∼10% of the RVM-projecting vlPAG neurons (Nguyen et al., 2022). Altering the activity of this particular subpopulation of excitatory neurons can impact responses to cold, thermal, itch, and nociception.

Recent studies have also shown that vlPAG projections to regions other than the RVM can be implicated in antinociception, expanding the definition of subpopulations involved in descending pain modulation beyond RVM-projecting neurons. One example is DA neurons that project to the BNST (Hasue and Shammah-Lagnado, 2002; Yu et al., 2021), which interestingly has reciprocal connections with the vlPAG via GABAergic efferents (Hao et al., 2019). Despite not projecting to the RVM (Suckow et al., 2013), these DA neurons have been implicated in the broader supraspinal pain circuitry and analgesia (Meyer et al., 2009; Taylor et al., 2019; Yu et al., 2021). Another is the connection between the central medial nucleus of the thalamus, which when lesioned temporarily alleviates mechanical hyperalgesia in a neuropathic pain model (Sun et al., 2020). Additional examples of reciprocal connections between the vlPAG and other brain regions, such as the amygdala (Ottersen, 1981; Hasue and Shammah-Lagnado, 2002; Oka et al., 2008; Sun et al., 2019), show pain-induced alterations (Li and Sheets, 2018). Multi-region circuits, such as that between the vlPAG, central medial thalamic nucleus, and the BLA are activated by neuropathic pain (Sun et al., 2020), which is known to project back to the vlPAG via neurons with distinct intrinsic membrane properties within the central medial and lateral nuclei of the amygdala (Rizvi et al., 1991; Li and Sheets, 2018). These reciprocal connections could account for the polysynaptic responses that lead to latent changes in RVM neuronal firing in response to vlPAG stimulation (Odeh et al., 2003).

The vlPAG has many other efferent targets that are associated with other behaviors. GABAergic projections to the VTA have been implicated in freezing behaviors (Laurent et al., 2020). Single-unit recordings in awake behaving animals have linked vlPAG cellular activity to threat probability evaluation (Wright et al., 2019). The subpopulation of neurons in mice involved in freezing with distinct connectivity, molecular markers (Chx10 and glutamate), and electrophysiological features (Vaaga et al., 2020). An entire field of work has implicated this region in the acquisition, expression, and extinction of fear, anxiety, or defensive response (Borszcz et al., 1989; Fanselow, 1991; De Oca et al., 1998; McDannald, 2010; Wright and McDannald, 2019; Wright et al., 2019). It is important to understand whether the circuits associated with behaviors or physiological states other than pain overlap with the vlPAG neurons that are specifically engaged in pain modulation. This could shed light on possible circuit mechanisms for comorbidities observed with chronic pain or other conditions that increase an individual's susceptibility to developing pain conditions.

Conclusion

The heterogeneity of the vlPAG calls for understanding neuronal subpopulations that comprise pain circuits with a greater resolution than the field currently uses. Combining multiple features, such as neurotransmitter content, receptor/channel expression, intrinsic firing properties, afferents inputs, efferent targets, etc., will create the opportunity to identify novel targets that interfere with pain processing, especially in chronic pain states. As new innovative approaches are developed, we can address key questions that remain in the field regarding the spatial and temporal specificity of endogenous opioid release within the descending pain modulatory pathway.

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Conflict of interest

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Dynorphin/kappa opioid receptor system regulation on amygdaloid circuitry: Implications for neuropsychiatric disorders

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Amygdaloid circuits are involved in a variety of emotional and motivation-related behaviors and are impacted by stress. The amygdala expresses several neuromodulatory systems, including opioid peptides and their receptors. The Dynorphin (Dyn)/kappa opioid receptor (KOR) system has been implicated in the processing of emotional and stress-related information and is expressed in brain areas involved in stress and motivation. Dysregulation of the Dyn/KOR system has also been implicated in various neuropsychiatric disorders. However, there is limited information about the role of the Dyn/KOR system in regulating amygdala circuitry. Here, we review the literature on the (1) basic anatomy of the amygdala, (2) functional regulation of synaptic transmission by the Dyn/KOR system, (3) anatomical architecture and function of the Dyn/KOR system in the amygdala, (4) regulation of amygdala-dependent behaviors by the Dyn/KOR system, and (5) future directions for the field. Future work investigating how the Dyn/KOR system shapes a wide range of amygdala-related behaviors will be required to increase our understanding of underlying circuitry modulation by the Dyn/KOR system. We anticipate that continued focus on the amygdala Dyn/KOR system will also elucidate novel ways to target the Dyn/KOR system to treat neuropsychiatric disorders.

KEYWORDS

basolateral amygdala, central nucleus of amygdala, dynorphin, kappa-opioid receptor (KOR), G-protein coupled receptors, stress, anxiety, addiction

Dynorphin/kappa opioid receptor signaling overview

Translational/clinical significance of dynorphin/kappa opioid receptor signaling

The neuropeptide dynorphin (Dyn) and its cognate receptor, the kappa opioid receptor (KOR), have been implicated in maladaptive behaviors associated with several psychiatric disorders. The Dyn/KOR system constitutes a class of opioidergic signaling that is in large part distinct from other opioid systems such as the mu-or delta-opioid receptor system. In humans, stimulation of KORs drives anxiety, dysphoria, and psychotomimesis (Pfeiffer et al., 1986). These behavioral effects have been observed with various KOR agonists, including the naturally occurring KOR agonist Salvinorin A (Tejeda and Bonci, 2019). Salvinorin A is the primary psychoactive compound in *Salvia divinorum,* a hallucinogenic plant. KOR antagonists have been investigated in the clinic primarily as potential treatments for mood and substance use disorders (Carlezon and Krystal, 2016; Fava et al., 2020) but see Jacobson et al. (2020). Selective antagonism of KORs in humans alleviates symptoms of anhedonia in transdiagnostic studies (Pizzagalli et al., 2020), with a corresponding rescue of activation of the ventral striatum in anticipation of reward delivery (Krystal et al., 2020). Similarly, in rodents, KOR activation with endogenous Dyn or exogenous agonists promotes anxiety-like behavior, aversion, and anhedonia, impairs social interactions, and drives deficits in active coping in response to stressors [reviewed in Bruchas et al. (2010), Wee and Koob (2010), Tejeda et al. (2012), Crowley and Kash (2015), Karkhanis and Al-Hasani (2020)]. It has been speculated that over short periods, Dyn-mediated agonism of KOR may act as an acute punisher to reduce the seeking for other drugs and reinforcers (Freeman et al., 2014; Butelman and Kreek, 2015; Heinsbroek et al., 2018), but over the long term, it may act as a negative reinforcer of such behaviors (Bruchas et al., 2010; Wee and Koob, 2010; Walker et al., 2012; Chartoff et al., 2016; Escobar et al., 2020). Consistent with effects produced by synthetic KOR agonists, the recreational drug salvinorin A promotes similar behavioral effects and blocks the reinforcing effects of other drugs, most notably psychostimulants (Gonzalez et al., 2006; dos Santos et al., 2014; Brito-da-Costa et al., 2021). Salvinorin A, unlike other hallucinogens, does not bind the 5-HT2A receptor (Sheffler and Roth, 2003). Ketamine, a recreational and rapid-acting antidepressant, has also been used as a means to model certain domains of schizophrenia and other dissociative disorders in animal models (Moghaddam and Jackson, 2003; Frohlich and Van Horn, 2014; Beck et al., 2020; Schmack et al., 2021). Ketamine antagonizes NMDA receptors, as well as KORs (Nemeth et al., 2010; Bonaventura et al., 2021). However, it is currently unclear whether direct actions of ketamine on KOR mediates any of the behavioral effects produced by ketamine. The Dyn/KOR system interacts with other stress-related neuropeptide systems, including corticotropin-releasing factor (CRF), which is enriched in neuronal circuits that control affect and motivation (e.g., the central nucleus of the amygdala). Dyn/KOR interactions with CRF contribute to dysregulation of innate and learned fear responses relevant to anxiety-like behavior and affect.

The effects of Dyn/KOR agonism by exogenous or endogenous agonists in animal models result in affective, motivational, and cognitive phenotypes relevant to psychiatric disorders including PTSD, depression, schizophrenia, and substance use disorder. Indeed, such disorders have been associated with alterations in Dyn/KOR expression or function [see Hang et al. (2015), Bruchas et al. (2010), Jacobson et al. (2020) for reviews on this topic]. In psychotic disorders, such as schizophrenia, altered Dyn/KOR signaling may be one factor that contributes to the dysfunction of dopaminergic transmission in mesolimbic and mesocortical circuitry, which mediate various features of positive and negative symptoms, and cognitive deficits [see Tejeda et al. (2012), Clark and Abi-Dargham (2019)]. The Dyn/KOR system may also be involved in substance use disorder, potentially contributing to the development of pro-addictive behaviors during stressful experiences [see Bruchas et al. (2010)]. In mice, stress is also thought to promote alcohol-and drug-seeking behaviors through Dyn/KOR interactions with the CRF system [see Bruchas et al. (2010), Wee and Koob (2010), Walker et al. (2012), Anderson and Becker (2017), Karkhanis and Al-Hasani (2020)]. Together, this highlights a role for Dyn/KOR activation during aversive and stressful experiences and underscores its potential to contribute to psychiatric dysfunction in humans.

The Dyn/KOR system regulates stress-related and goal-directed behaviors *via* actions in circuits that subserve the aforementioned behaviors. Early studies identified that the Dyn/KOR system is embedded in the substantia nigra and the ventral tegmental area, nucleus accumbens (NAcc), amygdala, hypothalamus, paraventricular thalamus (PVT), hippocampus, and septum, and caudate/putamen (Chavkin et al., 1985; Fallon et al., 1985; Slater and Cross, 1986; Mansour et al., 1987; Rattan et al., 1992; DePaoli et al., 1994; Jamensky and Gianoulakis, 1997; Chou et al., 2001). These regions are part of an interconnected limbic network that control various facets of learning/memory, goal-directed behavior, stress, arousal, attention, and energy homeostasis. The Dyn/KOR system is also found in multiple cortical areas including the auditory cortex (Ramsdell and Meador-Woodruff, 1993), the somatosensory cortex (Loh et al., 2017), periamygdaloid cortex (Anderson et al., 2013), and the parietal cortex (DePaoli et al., 1994). Prodynorphin-expressing neurons have also been identified in mouse and human brainstem (Agostinelli et al., 2021), as well as the lateral parabrachial nucleus (Chiang et al., 2020; Norris et al., 2021).

In the vein of translational considerations, there have also been reports of sex differences in the Dyn/KOR system. A PET imaging study detected increased KOR-selective tracer in healthy human males relative to healthy females (Vijay et al., 2016). Multiple single nucleotide polymorphisms (SNPs) in the prodynorphin gene have also been associated with differential susceptibility to development of opioid dependence, with the overall risk of each SNP often differing between sexes and across ethnic populations (Chartoff and Mavrikaki, 2015). The Dyn/KOR system may also be regulated through hormonal signaling, either through direct or indirect regulation of transcription factor binding or other signaling pathways (Chartoff and Mavrikaki, 2015). For example, in the mouse spinal cord, KORs heterodimerize with MORs in a sex-dependent manner, which is regulated by estrogen signaling (Chakrabarti et al., 2010; Liu et al., 2011). The extent to which KOR/MOR heterodimerization occurs in the human brain is still largely unknown, however, thus highlighting the need for additional studies on sex-dependent regulation of this system.

Mechanisms of circuit neuromodulation by dynorphin and kappa opioid receptor

Ultrastructural evidence shows KOR immunoreactivity within dendritic spines and axon terminals. These results provide an anatomical substrate by which KOR activation regulates the presynaptic release and postsynaptic neuron activity (Drake et al., 1996; Svingos et al., 1999; Svingos and Colago, 2002), to ultimately impact the activity of limbic circuits. KOR is a G-protein coupled receptor (GPCR), coupled to inhibitory $G_{i/o}$ proteins to decrease the membrane excitability *via* activation of G-protein gated inwardly rectifying potassium channels (Kir3 family). The activation of GIRK causes cellular hyperpolarization and inhibits neural activity (Torrecilla et al., 2002; Margolis et al., 2003; Ford et al., 2007; Chen et al., 2015). Besides Kir3 activation, KOR activation inhibits Ca^{2+} currents mediated by P/Q-type, N-type, and L-type channels to reduce calcium conductance and/or interfere with presynaptic release machinery downstream of Ca^{2+} entry (Grudt and Williams, 1993; Castillo et al., 1996; Simmons and Chavkin, 1996; Rusin et al., 1997; Hjelmstad and Fields, 2003; Iremonger and Bains, 2009; Tejeda et al., 2017). The impact of Dyn/KOR signaling may be complex depending on how this system is integrated into circuits. For example, Dyn/KOR signaling inhibits glutamate release in the NAcc from specific excitatory inputs, in addition to acting on local inhibitory connections from KOR-expressing accumbal medium-sized spiny neurons. The direct inhibitory effects of KOR signaling on presynaptic inputs filters glutamate release from incoming KOR-sensitive inputs and any influence those inputs may have on post-synaptic activity. Conversely, KOR acting on local circuit collaterals disinhibits other MSNs and facilitates the integration of incoming excitatory input from KOR-lacking afferent inputs (Tejeda et al., 2017). In summary, the available evidence indicates that the KORs are localized on axon terminals as well as on neuronal cell bodies to modulate the activity of the presynaptic compartment or the neuronal activity acting on the postsynaptic cell. A deep understanding of how KORs are embedded within circuits (e.g., on presynaptic vs. post-synaptic compartments, excitatory vs. inhibitory cells, etc.) is essential to deconstruct how the Dyn/KOR system regulates affect and motivation *via* its actions in limbic structures, including the amygdala.

Amygdala overview

Functional KORs and Dyn peptides have been described in the amygdala, an area which, in humans and other mammals, is critical for cognitive and emotional processing, learning and memory (LeDoux, 2000; Phelps, 2006). Amygdala dysfunction has been implicated in mediating symptomology in a host of psychiatric conditions. Often described as a hub for learning and memory and regulating affective states, the amygdala, like the Dyn/KOR system, is recruited during motivationally-charged experiences, including those associated with physiological or psychological stress (Roozendaal et al., 2009; Janak and Tye, 2015). It is also a region associated with pronounced changes following exposure to stress or stress-related hormones (McEwen, 2007; Roozendaal et al., 2009) and Dyn/KOR signaling within amygdala circuitry may be a key player in this process. Humans bearing the gene polymorphism (T) allele of prodynorphin at rs1997794 show impaired fear extinction and significant decreases in functional connectivity between the amygdala and PFC (Bilkei-Gorzo et al., 2012). As such, the Dyn/KOR system within the amygdala may serve as an interface through which stressors and noxious signals modulate key behavioral and affective states.

Importantly, key populations of excitatory and inhibitory neurons alike have been reported to be largely similar across humans and mice. One study performed a single-nucleus expression profiling of human amygdala and compared these results with a previous profiling study on mouse amygdala, finding that just 10.4% of detected genes were human-specific (Tran et al., 2021). A separate study found that sexual dimorphism in gene expression is largely conserved across human and mice (Lin et al., 2011). Additional controlled studies are needed to further characterize the peptidergic cell types of the human amygdala and contrast them with other species, especially given that the conventional notion of "cell type" has become harder to define in the era of transcriptomics (Yuste et al., 2020). Similarly, investigations on how Dyn/KOR expression, signaling, and/or modulation of amygdala circuit function changes across development are warranted. Research in this area may elucidate how neurodevelopmental challenges, such as early life stress, may contribute to dysfunction of limbic circuits and/or the function of the Dyn/KOR system in

promoting maladaptive behaviors in adulthood in patients with psychiatric disorders.

Functions of the amygdala in behavior

Amygdala circuits control multiple domains of behavior and contribute to cognitive, affective, and social processing. Situated between the cortex and deeper brain regions, connectivity of the amygdala suggests it may serve to integrate incoming sensory streams with state-and experience-dependent information to guide behavior (Sah et al., 2003; Pape and Pare, 2010; Janak and Tye, 2015). The amygdala has been studied extensively as a brain region critical for associative learning for stimuli of both positive and negative valence [see LeDoux (2007), Calu et al. (2010), Pape and Pare (2010), Janak and Tye (2015), Namburi et al. (2015), Kim et al. (2016), O'Neill et al. (2018), Kong and Zweifel (2021)]. Furthermore, the role of the amygdala extends beyond acquisition, as it is also critical for the extinction of associative memories, similarly processing conditioned stimuli of both positive and negative valence (Maren and Quirk, 2004; Tye et al., 2010; Zhang et al., 2020; Whittle et al., 2021). Cognitive flexibility and decision-making are also impacted by amygdala function (Keefer et al., 2021). A recent study also showed the amygdala processes cue contingencies and motivational states to help select behavioral responses under a range of environmental and internal state demands (Courtin et al., 2022). In addition to learning and memory, the amygdala regulates facets of affective behavior (Gallagher and Chiba, 1996). Amygdala dysfunction has been linked to major depressive disorder (MDD) in humans (Nestler et al., 2002), and in mice may also play a role in governing anhedonia-like phenotypes (Ramirez S. et al., 2015). Similarly, because anxiety and depression often co-occur (Britton et al., 2011; Tiller, 2013), it is possible that dysregulated amygdala activity can contribute to both of these conditions (He et al., 2019; Espinoza Oyarce et al., 2020).

Alterations in amygdala activity have been linked to post-traumatic stress disorder (PTSD) as well as substance use disorders in humans (Grillon et al., 1996; Sharp, 2017; Morey et al., 2020; Zhang W.H. et al., 2021; Alexandra Kredlow et al., 2022). Following its role in learning, memory, and affect, especially in the context of psychiatric conditions such as PTSD, the amygdala is also susceptible to stress and itself regulates components of stress processing (Zhang W.H. et al., 2021). As previously mentioned, stress produces lasting changes in amygdala circuitry and connectivity (McEwen, 2007; Roozendaal et al., 2009; Zhang et al., 2019), and the amygdala contains neural populations that express and release CRF (Gray and Bingaman, 1996; George and Koob, 2010; Koob et al., 2014; Zorrilla et al., 2014; Marcinkiewcz et al., 2016). CRF in turn activates other stress-associated neuromodulatory systems in the amygdala, namely cell populations expressing norepinephrine (NE) and dynorphin (George and Koob, 2010; Knoll and Carlezon, 2010; Koob et al., 2014). Together, these studies suggest that changes in stress-related signaling perturb amygdala function and may promote behavioral reactions in response to stressors or gate active behaviors that promote avoidance or escape from stressors and threats.

Overall, the precise mechanisms through which the amygdala governs diverse behaviors are complex. Recently there have been significant advances in understanding the cell types and amygdala microcircuits and long-range interactions that permit amygdala circuitry to control cognitive, emotional, and social behaviors. Studies such as these are necessary to resolve questions on how or why the amygdala specifies affective states such as anxiety and depression as well as cognitive tasks such as learning and extinction.

Amygdala subregions and circuitry

The amygdala comprises several subregions which vary in their connectivity, cellular subtypes, and function. This includes the central nucleus of the amygdala, basal, and lateral nuclei of the amygdala, and the intercalated cell masses (ITCs). The bulk of this review will focus primarily on the basolateral amygdala (BLA) and central amygdala (CeA), which are two of the most characterized regions of the amygdala.

Most hypotheses of amygdala function contend that the BLA integrates the temporal structure of sensory and state information before passing it to other regions, such as the downstream CeA, through glutamatergic projection neurons. The excitatory inputs from BLA target both the lateral and medial compartments of the CeA, with innervation of the lateral compartment coming primarily from the LA and innervation of the medial compartment from the BA. The lateral nucleus of the CeA (CeL) also targets the medial nucleus of the CeA (CeM), but reciprocal projections from CeM to CeL have not been observed. Importantly, the CeL is involved in fear acquisition, while the CeM is critical for fear expression and extinction (Ciocchi et al., 2010; Haubensak et al., 2010). A third subregion of the CeA is the capsular region, although this area is less well-studied (**Figure 1**).

Recent studies have refined the model of BLA-CeA processing by investigating the effects of this pathway on appetitive behaviors. Kim et al. found that genetically distinct populations of BLA neurons target specific populations of CeA neurons that are responsible for appetitive or aversive behaviors (Kim et al., 2017).

Basolateral amygdala inputs and outputs

The regions supplying the densest afferents to the BLA include the thalamus, ventral hippocampus, dorsal raphe

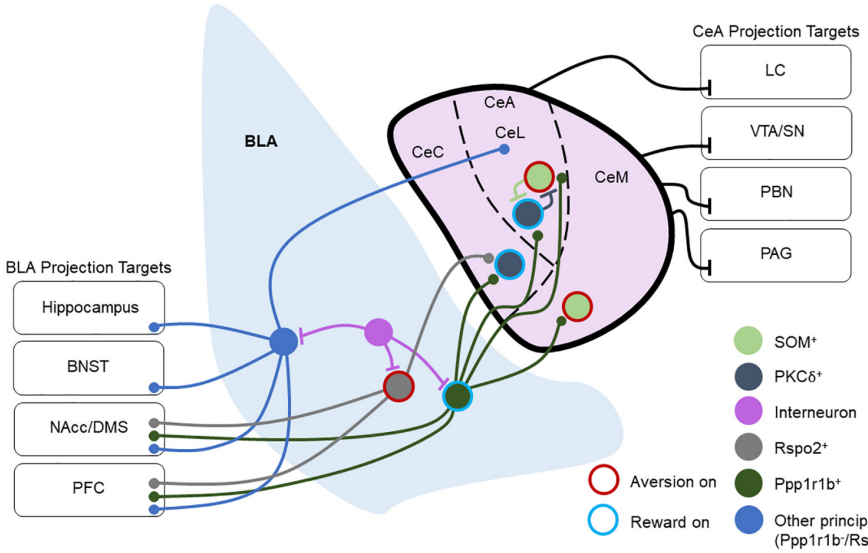

FIGURE 1
Overview of basolateral amygdala (BLA) and central amygdala (CeA) connectivity and projection targets. This depicts a layout of multiple BLA and CeA cell types and their connectivity schemes in local and long-range circuitry. Cells are labeled as "Aversion on" or "Reward on" that has been tested experimentally. Ppp1r1b^{+} BLA neurons target PKCδ^{+} and SOM^{+} neurons throughout the CeA, and they also project to the NAcc and PFC. Rspo2^{+} BLA neurons target PKCδ^{+} neurons in the CeC and send projections to the NAcc and PFC. Other BLA principal neurons project to the CeA as well as to several other brain regions including the hippocampus, BNST, NAcc, DMS, and PFC. Within the CeL, SOM^{+}, and PKCδ^{+} populations reciprocally inhibit one another. CeM neurons also send long-range inhibitory projections to the VTA, SN, PBN, and PAG, while the CeL sends long-range inhibitory projections to the LC. Through these projections, the CeA regulates arousal, attention, movement, and defensive behaviors.

nucleus, posterior intralaminar nucleus, medial geniculate nucleus, ventral tegmental area, nucleus accumbens, and cortical areas including the prefrontal cortical areas and other sensory cortical regions (Steinbusch, 1981; Bocchio et al., 2016; Fu et al., 2020; Hintiryan et al., 2021; Morikawa et al., 2021). Each of these input pathways relays different forms of state, motivational, or sensory information to the BLA. Synaptic integration of these converging pathways into the BLA is regulated by neuromodulators, including monoamines and neuropeptides, *via* target receptors in the BLA and presynaptic terminals from afferent inputs.

Outputs of the BLA include the CeA, BNST, lateral hypothalamus (LH), nucleus accumbens, ventral tegmental area, and the BLA's reciprocal connections with the mPFC and ventral hippocampus (Pape and Pare, 2010; Janak and Tye, 2015; Hintiryan et al., 2021; Murray and Fellows, 2022). Some of these BLA projection targets also provide reciprocal inputs. This implies that while the BLA exerts unidirectional control on specific targets, other targets may exert influence on BLA activity as well, but the degree to which the BLA is capable of regulating aspects of its activity through these feedback loops has remained elusive thus far (**Figure 1**). Future research is needed to understand more about how these feedback circuits are wired through specific cell types and how various feedback mechanisms orchestrate amygdala activity to control behavioral responses.

Recent studies have suggested that the projection targets of the BLA may offer insight into the roles of various projecting populations that originate in the BLA. For example, BLA neurons that project to the NAcc are critical for reward and avoidance learning (Ambroggi et al., 2008; Jones et al., 2010; Pascoli et al., 2011; Stuber et al., 2011; Britt et al., 2012; Namburi et al., 2015; Ramirez F. et al., 2015; Ramirez S. et al., 2015; Zhang X. et al., 2021), while those that project to the CeA are critical for fear (Jimenez and Maren, 2009; Namburi et al., 2015; Kim et al., 2016). However, differential valence processing may exist even within the same pathway as reward and aversion activated neurons differentially engage CeA circuits (Kim et al., 2017) and control different compartments of ventral striatal circuitry (Zhang X. et al., 2021; **Figure 1**). Further, the encoding of motivationally-relevant behaviors by the BLA may also be influenced by anterior-posterior gradients in reward and aversion neurons in the BLA and their outputs (Kim et al., 2016; Beyeler et al., 2018). Recently, the ITCs of the amygdala have been shown to inhibit BLA output neurons to the prelimbic and infralimbic prefrontal cortex, which are involved in fear acquisition and extinction, respectively (Hagihara et al., 2021). This not only indicates that the BLA encodes valence but also suggests that an understanding of the efferent and topographical organization of the BLA may help to resolve its involvement in various behaviors, offering a greater lens into how the BLA may be engaged in both appetitive and aversive tasks.

Central amygdala inputs and outputs

Unlike the BLA, the CeA receives few cortical inputs apart from the insular cortex (Kargl et al., 2020), with virtually non-existent outputs of the CeA to cortical areas. The CeA receives afferent inputs from various limbic regions including the BLA, thalamus, BNST, and the ITCs (Royer et al., 1999; Bienkowski and Rinaman, 2013; Kim et al., 2017). Most output pathways of the CeA arise from the CeM and send inhibitory projections to various brainstem regions as well as the hypothalamus, periaqueductal gray (PAG), substantia nigra/ventral tegmental area, BNST, PVT, and parabrachial nucleus (PBN) (Pape and Pare, 2010; Penzo et al., 2014; Gilpin et al., 2015; Douglass et al., 2017; Ahrens et al., 2018; Baumgartner et al., 2021; Borrego et al., 2022). These outputs are capable of rapidly modulating defensive behaviors in response to threats, approach behavior, and reward-related responses. Work has also demonstrated that CeA projection neurons are involved in appetitive behaviors. GABAergic serotonin receptor 2a (Htr2a)-expressing CeA neurons modulate food consumption in mice (Douglass et al., 2017), while CRF-expressing CeA neurons are involved in the motivation to consume rewards and their activation enhances the recruitment of brain areas involved in motivation and reward (Calu et al., 2010; Steinberg et al., 2020; Warlow and Berridge, 2021; **Figure 1**).

Subpopulations of basolateral amygdala cell types

The BLA and CeA structures are quite different in terms of their cellular composition. The BLA bears features largely similar to cortical structures (Carlsen and Heimer, 1988). The BLA primarily contains cortical neuron types, with glutamatergic cells comprising approximately 80% of the total neurons in the BLA and GABAergic interneurons making up the residual 20% (Pape and Pare, 2010). Genetic profiling techniques have revealed that multiple subpopulations of BLA principal neurons differentially contribute to valence encoding. For example, subpopulations of principal neurons expressing Rspo2 and Fezf2 have been shown to contribute to aversive behaviors, while neurons expressing Ppp1r1b promote appetitive behaviors (Kim et al., 2016; Rovira-Esteban et al., 2019; Zhang X. et al., 2021). Anatomical arrangement from dorsoventral/anteroposterior in BLA may in part contribute to the encoding of positive or negative valence (Kim et al., 2016; Beyeler et al., 2018; **Figure 1**).

The GABAergic population of BLA neurons consists of many of the same interneuron subtypes found in cortical regions, with distinct subpopulations of interneurons positive for the calcium-binding proteins parvalbumin, calbindin, and calretinin, along with neuropeptides including vasoactive intestinal peptide (VIP), somatostatin (SOM), cholecystokinin (CCK), among others. These various interneuron subtypes each play a distinct role in the modulation of BLA excitability and synaptic integration. As peptides diffuse over larger areas than amino acid neurotransmitters (Nassel, 2009), the peptidergic interneurons of the BLA are thought to provide modulatory inputs to local interneurons and principal neurons alike. Many studies have examined the involvement of BLA peptidergic neurons in behavior, although very few of these have probed the roles of the various peptides themselves in these functions (Mascagni and McDonald, 2003; Krabbe et al., 2019).

Kappa opioid receptors (KORs) are expressed in BLA neurons, providing a means for Dyn inputs to the BLA to regulate BLA microcircuit function (**Figure 2**). However, the role of the Dyn/KOR system within BLA microcircuits that control input-output transformations remains largely unresolved. As such, the precise BLA circuits and cell types through which this system regulates behavior must be clarified in greater detail.

Central amygdala cell types

The cell types and circuits of the CeA bear more resemblance to the inhibitory neurons of striatal circuits, in contrast to primarily excitatory cell types seen in the BLA. Most CeA neurons express GAD65 or GAD67, with very few expressing vGlut1 or vGlut2 (Poulin et al., 2008). Like the striatum, the CeA is embryonically derived from the lateral ganglionic eminence (Swanson and Petrovich, 1998). The CeA and striatum are also characterized by similar expression patterns of cell fate markers (Medina et al., 2011) [see Aerts and Seuntjens (2021) for a recent review on amygdala development].

Neurons in the lateral region of the CeA (CeL) are often categorized by expression, or lack thereof, of the delta isoform of protein kinase C (PKCδ) (Haubensak et al., 2010). PKCδ$^+$ neurons in the CeL also tend to express oxytocin receptors and are inhibited during fear states. Furthermore, this population inhibits PKCδ$^-$ neurons in the CeL, although PKCδ$^+$ and PKCδ$^-$ neurons alike project to the CeM. The peptide SOM is also expressed in the CeA, primarily in the CeL (Li et al., 2013; Kim et al., 2017). Here it is important to note that the properties and functions of these CeA SOM cells differ from the SOM cells found in the BLA. Furthermore, the CeL SOM and PKCδ$^+$ populations are distinct, with very little overlap (Li et al., 2013; Kim et al., 2017; Wilson et al., 2019). Owing to the differential functions of these populations, CeA SOM and PKCδ$^+$ neurons have a role in the consolidation of differential threat memories in CeL, inhibition of SOM or PKCδ$^+$ interneurons impaired the time the animals freeze to a threat and safety responses (Wilson et al., 2019; Shrestha et al., 2020; **Figure 1**).

Central amygdala (CeA) GABAergic neurons co-express mRNA of several peptides including SOM, enkephalin, CRF,

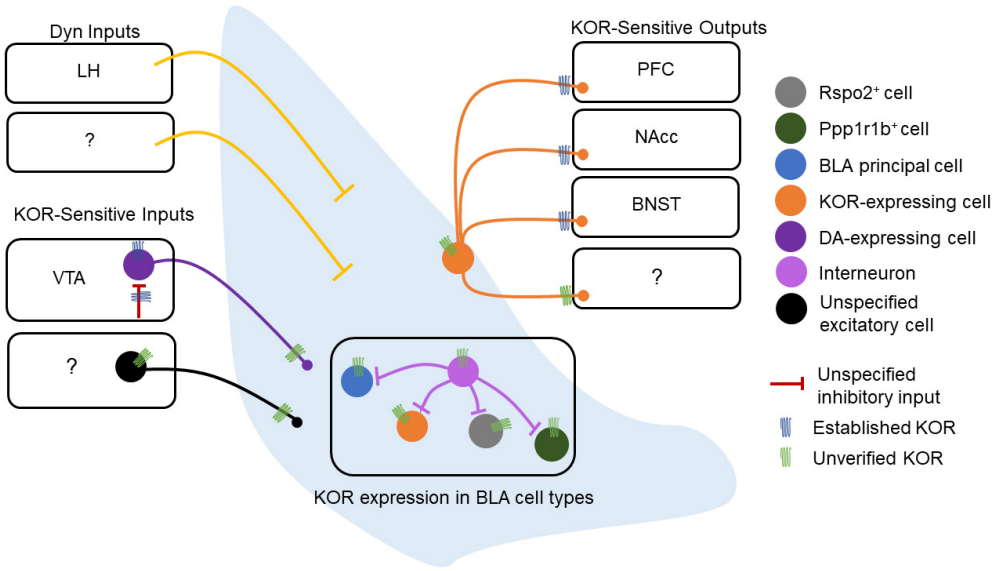

FIGURE 2
Dynorphin/kappa opioid receptor expression in basolateral amygdala (BLA) circuitry. Schematic of the experimentally-established components of the BLA Dyn/KOR system [e.g., verified kappa opioid receptors (KORs)] as well as some outstanding questions (e.g., unverified KORs). The lateral hypothalamus (LH) is the only structure currently reported to send Dyn inputs to the BLA, while other potential sources of Dyn to the BLA remain to be discovered. Other inputs to the BLA are regulated by the Dyn/KOR system as well. The VTA expresses KORs at DAergic cell bodies, and these same cell bodies are regulated by an inhibitory input that expresses KOR presynaptically, suggesting that the Dyn/KOR system may modulate the release of other neuromodulators such as DA into the BLA. These VTA inputs, as well as other potential inputs to the BLA, may also express KORs presynaptically. The BLA does not contain Dyn neurons, and the cell type-specific expression of KOR in BLA neurons is also poorly understood. Similarly, whether local BLA interneurons express KOR, and, if so, whether KOR^+ and KOR^- interneurons differentially regulate different populations of BLA neurons is unknown. KOR^+ BLA principal neurons target the PFC, NAcc, and BNST, other projection targets of KOR-expressing neurons remain to be discovered. Likewise, whether KOR is present at somatodendritic compartments or regulates local collaterals within the BLA is not known.

neurotensin, and tachykinin (Day et al., 1999). Dyn-expressing neurons are located primarily in the CeL and CeM subregions of the CeA (Kim et al., 2017). Populations of CeA neurons impact the function of the HPA axis (Buller et al., 2001). In mice, the BLA-CeA circuit regulates anxiety-like behavior (Tye et al., 2011), and the knockdown of CRF in CeA neurons reduces anxiety-like behavior (Pomrenze et al., 2019; Ventura-Silva et al., 2020). As such, it is thought that chronic stress produces changes that remodel amygdala circuitry, which can negatively impact performance on cognitive tasks (Roozendaal et al., 2009; Cacciaglia et al., 2017; de Quervain et al., 2017). Conditioned fear responses driven by CeM neurons, presumably $PKC\delta^+$ neurons, disinhibit CeL output neurons for fear acquisition (Ciocchi et al., 2010; **Figure 1**).

Dynorphin/kappa opioid receptor in amygdala circuits

Basolateral amygdala

Dynorphin/kappa opioid receptor signaling may shape synaptic transmission in BLA circuits. KOR mRNA expression and protein immunoreactivity in the BLA has been described in several studies since the 1990s (DePaoli et al., 1994; Knoll et al., 2011; Van't Veer et al., 2013; Tejeda et al., 2017; Maiya et al., 2021), while Dyn-expressing neurons are largely absent in the BLA. In humans, prodynorphin mRNA expression is observed in the amygdalohippocampal and accessory basal nuclei, and this expression is reduced in patients with major depressive disorder or bipolar disorder (Hurd, 2002). Pdyn mRNA in the BLA is generally not observed in mice but has been reported in lateral ITCs (Gomes et al., 2020). The KOR agonist U50 reduces excitatory synaptic transmission (as assessed by field EPSPs) in the BLA and blocks high frequency stimulation-induced long-term potentiation of excitatory synapses (Huge et al., 2009). However, in another study examining glutamatergic transmission onto BLA pyramidal neurons with spontaneous excitatory postsynaptic currents using whole-cell slice electrophysiology, the KOR agonist U69,593 was without effect (Przybysz et al., 2017). These inconsistent findings may result from differential sampling of excitatory synapses as the former study examined fEPSPs evoked by LA stimulation, while the latter study was agnostic to the source of excitatory synapses. In contrast, GABAergic sIPSC frequency, but not amplitude, was increased by KOR agonists (U69 and Dyn) in the BLA of adolescent, but not adult, rat

brain slices (Przybysz et al., 2017). The U69 effect on sIPSC frequency was blocked by the KOR antagonist nor-BNI and TTX bath application, which indicates that this effect was KOR-dependent and is consistent with a mechanism that acts on action potential-dependent inhibitory transmission and/or indirect circuit-level mechanisms. These results suggest that KORs may act to limit synaptic transmission within BLA circuits. However, from these studies, it is unclear whether KORs act on BLA principal neurons, interneurons, or KOR-expressing afferent inputs to the BLA. KOR in BLA neurons also regulates their outputs to downstream targets (Tejeda et al., 2013, 2015; Crowley et al., 2016). KORs are expressed in BLA terminals innervating the NAcc where they preferentially inhibit glutamate release onto D1 vs. D2 MSNs (Tejeda et al., 2017). KORs also inhibit the release of glutamate from BLA terminals in the mPFC and the BNST (Tejeda et al., 2015; Crowley et al., 2016; **Figure 2**). These studies suggest that Dyn released within BLA target regions, such as the NAcc, mPFC, and BNST, may modulate glutamatergic inputs from BLA neurons, decoupling BLA terminal control of target cells without inhibiting the BLA projection neuron at the soma. KORs preferentially inhibit inputs to NAcc D1 versus D2 MSNs (Tejeda et al., 2017), raising the possibility that either KORs are trafficked to specific BLA terminals based on their postsynaptic targets and/or that KOR-containing and KOR-lacking BLA projecting neurons differentially innervate D1 and D2 MSNs. It is unclear whether KOR regulation of BLA outputs onto molecularly-or projection-defined targets in other BLA terminal brain regions differs, such as the BNST and mPFC (**Figure 2**). Together, these studies demonstrate that the Dyn/KOR system can regulate inhibition and excitation onto BLA neurons and their outputs to target structures.

Central amygdala

In contrast to the BLA, neurons of the CeA express Dyn. *Pdyn* and *Oprk1* mRNA expressing cells are primarily non-overlapping populations in the CeA, with a smaller subset of cells expressing both *Pdyn* and *Oprk1* mRNA (Bloodgood et al., 2021). Dyn is also co-expressed with other neuropeptides within the CeA. Nearly all Dyn-expressing neurons in the CeL co-express proenkephalin (Penk), SOM, and Tac2 mRNA (Kim et al., 2017), and approximately 80% of Dyn-expressing CeL neurons co-express SOM peptide (Jungling et al., 2015). About 35% of Penk-expressing neurons in CeL co-express dynorphin (Kim et al., 2017). In the CeM, nearly all Dyn-expressing neurons co-express the dopamine receptor Drd1a, and nearly all Tac1 neurons co-express Dyn (Kim et al., 2017), consistent with Dyn expression patterns observed in striatal cells expressing Tac1 and Drd1a. Furthermore, a subset of GABAergic CeL neurons co-expresses CRF and prodynorphin (Marchant et al., 2007; Kim et al., 2017; Sanford et al., 2017; **Figure 3**). Collectively, these results suggest that Dyn-expression is embedded in CeA circuits in a sub-region and cell-specific manner. Given that different molecularly defined CeA cells and sub-regions play fundamentally different roles in motivationally-charged behaviors then Dyn in distinct cell types and sub-regions is hypothesized to differentially control behavior and circuit function. Like we mentioned in the prior section, it will be important for human postmortem studies to investigate the distribution of the Dyn/KOR system among populations of CeA neurons and contrast these with the established circuitry of mice.

Dynorphin acting within the CeA regulates inhibitory synaptic transmission. KOR activation inhibits GABA release onto CeA cells *via* a presynaptic site of action (Kang-Park et al., 2013, 2015; Gilpin et al., 2014; Hein et al., 2021). These results suggest that Dyn may decrease inhibition of CeA cells and contribute to the disinhibition of CeA circuits. Approximately half of CeA neurons characterized by strong spike accommodation and lack of an after-depolarization potential (ADP) are hyperpolarized by the KOR agonist U69,593, while cells lacking spike accommodation and with ADPs were insensitive to U69,593 but directly hyperpolarized by met-enkephalin (Zhu and Pan, 2004). These results demonstrate that Dyn/KOR signaling may also directly hyperpolarize subsets of cells in the CeA. Given that intrinsic firing properties differ between CeA cell types (Zhu and Pan, 2004; Haubensak et al., 2010; Wilson et al., 2019; Adke et al., 2021), these results suggest that Dyn may decrease the excitability of subsets of CeA cells *via* actions at somatodendritic KORs. Further, CeA cells form local collaterals within CeA circuits (Cassell et al., 1999), and as such, it is possible that pools of KOR-sensitive inhibitory synapses may arise from within the CeA. KORs inhibit glutamatergic transmission of electrically evoked glutamatergic transmission in the BLA to subsets of CeL neurons (Kissiwaa et al., 2020), but fails to modify PBN to CeL synapses (Kissiwaa et al., 2020; Hein et al., 2021; **Figure 3**). These results suggest that Dyn released from CeA neurons may regulate local circuit inhibition and incoming afferent inputs in a pathway-specific manner.

Central amygdala Dyn neurons target several regions involved in reward-, fear-, and stress-related behaviors. The CeA sends a Dynergic projection to the locus coeruleus (LC), and about 42% of those projection neurons co-express CRF and Dyn (Reyes et al., 2011). Dyn-expressing CeA neurons are modulated by LC neurons, specifically those expressing NE (Kravets et al., 2015). Furthermore, approximately 30% of these NE-expressing terminals from LC target CeA neurons that co-express CRF and Dyn. Dynergic CeL neurons project to the LC, and approximately 50% of the CeL inputs to the LC express both Dyn and SOM (Jungling et al., 2015). Additionally, less than 3% of the PKCδ^{-} expressing CeL neurons that project to peri-LC are positive for Dyn, suggesting that PKCδ and Dyn populations are largely non-overlapping (Jungling et al., 2015). In addition to the LC, Dyn CeA neurons also project to the PBN.

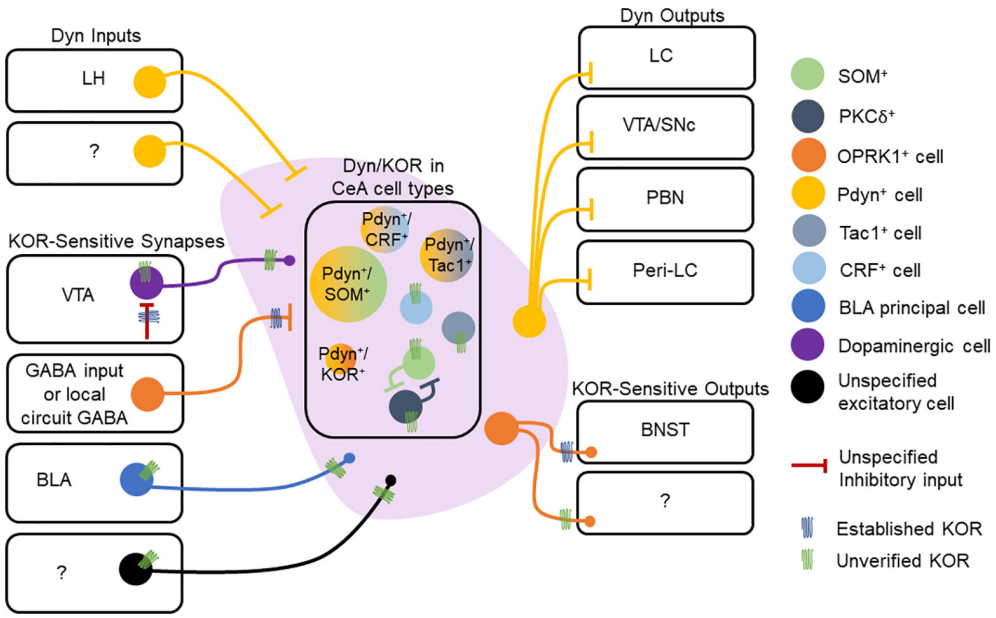

FIGURE 3
Dynorphin/kappa opioid receptor expression in central amygdala (CeA) circuitry. Dyn is expressed in multiple cell types within the CeA. Extrinsic Dyn inputs to the CeA arise from lateral hypothalamus (LH), but other sources remain unknown. Presynaptic KOR regulation of inhibitory synapses onto CeA neurons has documented, but the source of KOR + GABA neurons is not known. Other inputs to the CeA may also express KOR, including those arising from the VTA, basolateral amygdala (BLA), and other uncharacterized regions. Within the CeA, KOR expression within specific cell types is not understood. KORs may directly hyperpolarize or inhibit collaterals established by CeA neurons. CeA projections to the BNST are inhibited by presynaptic KORs. KOR expression at other CeA neuron outputs has not been demonstrated. CeA Dyn neurons are known to project to multiple regions including the LC, VTA/SNc, PBN, and Peri-LC. The molecular profiles of Dyn neurons may also vary across the CeA's medial and lateral subdivisions, which we are omitted in the figure.

Approximately 20% of retrograde fluorogold-labeled neurons in CeA that project to the PBN are Dyn^{+}, and another 15% co-express Dyn and SOM (Raver et al., 2020), suggesting that co-expression of Dyn and SOM may differ between CeA outputs (**Figure 3**). The CeA pathway is inhibited in mouse models of chronic pain, and stimulation of this pathway blocks pain-related behavioral phenotypes, providing a potential circuit-based mechanism by which CeA Dyn neurons may influence nociceptive and affective behaviors driven by pain states. PDyn- and SOM-expressing neurons in the CeA also project to the substantia nigra (Steinberg et al., 2020), thus targeting a key brain region involved in appetitive behaviors and motivational drive.

External inputs of Dyn to the CeA were documented by Zardetto-Smith et al. (1988) who reported the presence of Dyn-expressing neurons in the LH and perifornical nucleus that project to the CeA of rats. Orexinergic neurons from the LH also innervate the CeA, and about 95% of orexinergic neurons express dynorphin (Peyron et al., 1998; Chou et al., 2001). KORs also regulate CeA projections *via* inhibition of presynaptic GABA release in downstream targets. For example, application of KOR agonists reduced GABAergic transmission onto BNST neurons from CeA afferents (Li et al., 2012). Dyn released from BNST neurons activates presynaptic KORs located on CeA afferents to inhibit GABAergic transmission to the BNST and opposes the facilitatory effect of endogenously released neurotensin from BNST neurons on CeA GABA inputs (Normandeau et al., 2018). These results suggest that Dyn released from BNST neurons may act as a retrograde signal to limit KOR-sensitive inputs. Dynergic tone on inhibitory synapses in the BNST, which may potentially arise from CeA SOM-expressing CeA neurons, is enhanced in stressed mice and mice lacking ErbB4 in SOM-positive neurons (Ahrens et al., 2018; **Figure 3**). Collectively, these results suggest that Dyn/KOR is integrated within CeA circuits and regulates CeA local circuits and outputs. Taken together, robust Dyn/KOR expression and regulation of amygdala circuit function position this system to regulate amygdala-dependent control of motivationally charged behaviors and experience-dependent appetitive and aversive learning.

Stress and amygdala dynorphin/kappa opioid receptor signaling

The stress response plays a key role in the ability of organisms to adapt following exposure to threatening stimuli or experiences (LeDoux, 2000). CRF plays a significant role

in the integration of endocrine and behavioral responses to stress (Vale et al., 1981). CRF is released as a neurotransmitter from neurons in the CeA and BNST and is secreted as a neurohormone from PVT neurons to induce the secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland. ACTH enters the bloodstream to access the adrenal gland cortex, where it stimulates the secretion of glucocorticoids, initiating the systemic stress response (Dedic et al., 2018). Importantly, glucocorticoids can regulate gene expression by binding glucocorticoid receptors (GR) (Meijsing, 2015). Dyn cells in the central amygdaloid nucleus contain GR immunoreactivity (Cintra et al., 1991), suggesting that glucocorticoids may regulate Dyn expression in CeA as they do in other brain regions like the hippocampus (Thai et al., 1992). Furthermore, early life stress may increase GR binding to the second intron of OPRK1, the gene that encodes KOR expression, to influence gene expression (Lutz et al., 2018). These studies suggest that stressor-driven fluctuations in CRF and glucocorticoids may modulate the Dyn/KOR system in the amygdala to regulate behavior. Since stress modalities and durations are diverse, different stress-related signaling molecules and defensive behaviors may be mobilized to promote resilience or susceptibility. Therefore, future work further dissecting how the Dyn/KOR system is engaged or regulates different forms of stress is imperative for furthering our understanding.

Basolateral amygdala

Psychological stress increases Dyn/KOR activity in the BLA. Dyn/KOR activation is necessary for a variety of stress-related and aversive behaviors. Interestingly, acute forced swim stress and CRF both increase phosphorylation of KOR (a putative index of KOR activity) in the BLA, but not in the CeA (Land et al., 2008). Dyn expression is also increased in the BLA of male mice following 14 days of social defeat stress (Zan et al., 2022). Moreover, systemic KOR antagonists abolish the increase of phosphorylation of ERK after social defeat stress in the BLA, suggesting that KOR signaling is potentially upstream of ERK induction. KOR agonist decreases GABAergic transmission in most BLA neurons from stressed adolescent male mice relative to unstressed controls (Varlinskaya et al., 2020), suggesting that stress may impact KOR regulation of inhibitory transmission. Increased BLA KOR phosphorylation induced by CRF injection is similarly blocked by KOR antagonism (Bruchas et al., 2009; Knoll et al., 2011). BLA KORs also interact with stress-related signaling pathways to drive the expression of stress-induced nicotine drug reinstatement (Nygard et al., 2016). Together, these findings highlight that activation of the BLA Dyn/KOR system during stress may impact the function of the BLA in regulating stress-associated behavioral responses. Given the central role of stress in fear and anxiety, it is also important to cover the role of the BLA Dyn/KOR system in these states.

Fear-related behaviors in response to threats are, by definition, driven by aversion, and many signaling pathways associated with stress are often similarly engaged during fear states. As such, the Dyn/KOR system in the BLA is well-suited to contribute to the formation and/or maintenance of fear memory. In rats, KOR mRNA in the BLA increases with conditioning with fear-potentiated startle (FPS) and decreases with extinction of FPS (Knoll et al., 2011). Microinjection of the KOR antagonist JDTic into the BLA of rats reduces fear expression. In Dyn KO mice, levels of c-fos protein in the BLA are decreased relative to WT controls in response to fear extinction but not during fear conditioning to auditory cue and footshock pairings (Bilkei-Gorzo et al., 2012). These studies imply not only that fear expression is associated with changes in Dyn/KOR signaling in the BLA, but also that the Dyn/KOR system mediates fear behavior by acting within the BLA.

Similarly, activation of the BLA Dyn/KOR system during stress may serve to regulate depressive-or anhedonia-like states that persist after exposure to a stressor. BLA activity, as indexed by c-fos, is reduced in Dyn KO mice relative to controls following exposure to the anxiogenic zero maze (Bilkei-Gorzo et al., 2008), suggesting that loss of Dyn/KOR signaling reduces BLA neuronal activation. However, it is important to note that this study did not provide measurements of BLA c-fos from naive animals not exposed to the zero maze. Microinjection of KOR antagonists in the BLA increases the time in the interaction zone after social defeat stress and prevents the development of depressive-like behaviors induced by chronic social defeat stress (Zan et al., 2022). Intra-BLA administration of a KOR antagonist produces anxiolytic-like effects in the elevated plus maze in rats (Knoll et al., 2011). Consistent with the hypothesis that BLA KOR is involved in anxiety-related behavior, deletion of KOR from the BLA of adult male mice results in more time in the open arms of the elevated plus maze, suggesting that loss of BLA KOR function may confer an anxiolytic effect (Crowley et al., 2016). Microinjections of the KOR antagonist nor-BNI into the BLA reduce anxiety-like behavior following acute stress exposure or CRF administration in mice (Bruchas et al., 2009). Together, these results demonstrate that Dyn/KOR signaling within the BLA may be engaged to promote innate anxiety-like behavior and learned fear.

Central amygdala

The CeA Dyn/KOR system has also been studied in the context of stress responsivity. Forced swim stress increases dynorphin expression in the CeA of mice following stress exposure (Chung et al., 2014). Another study showed that unescapable tail shock increased DynA (1–8) immunoreactivity in the anterior portion of the lateral CeA (Gouty et al., 2021). In CeA, CRF, a neuropeptide that is a critical mediator of the stress response, is co-expressed with Dyn (Reyes et al., 2008; Kravets et al., 2015) and Dyn KO mice have been shown to

have reduced expression of CRF in CeA (Wittmann et al., 2009). Activation of KORs in the CeA with U69,593 drives aversion and anxiety-like behavior, and this effect can be blocked by optogenetic inhibition of CeA CRF neurons. KORs disinhibit CeA CRF neurons *via* inhibition of presynaptic GABA release and feed forward inhibition driven by the PBN onto CeA CRF neurons (Hein et al., 2021). These results suggest that CeA Dyn/KOR signaling may modulate CRF neuron activity and CRF peptidergic transmission, which would influence defensive responses to stressors and anxiety-related behavior under the control of CeA CRF neurons (Fadok et al., 2017; Sanford et al., 2017).

Loss of KORs in the CeA, as well as loss of Dyn inputs to the CeA, increases anxiety-like behavior and promotes fear generalization wherein defensive freezing is observed in response to both threat-predictive cues and neutral cues alike (Baird et al., 2021), suggesting that Dyn released from CeA neurons may limit excessive passive defensive behaviors to non-threatening experiences and environmental cues. In ErbB4 gene deficiency mice, the Dynergic activity of SOM-expressing CeA inputs to BNST also promotes anxiety-like behavior *via* a disinhibitory effect (Ahrens et al., 2018). Likewise, Dyn knockdown in CRF-expressing CeA neurons reduces anxiety-like behavior as evidenced by increased exploration of open arms in the EPM as well as increased time in the center of the OFT (Pomrenze et al., 2019). Fear conditioning and fear retrieval increase pCREB in Dyn-expressing CeL neurons, relative to both naive home cage mice as well as mice that received unpaired CS/US delivery (Jungling et al., 2015), suggesting that CeA Dyn neurons may not be solely engaged by threats, but rather cues that predict threats.

Regulation of drug and alcohol seeking behavior by amygdala dynorphin/kappa opioid receptor neurons

The basolateral amygdala dynorphin/kappa opioid receptor system modulates drug-seeking behavior

The BLA Dyn/KOR system has also been studied in the context of alcohol and nicotine seeking behavior, where it has been implicated in playing a role as a negative reinforcer that promotes drug-seeking behavior aimed at curbing drug withdrawal-induced and stress-induced negative affect and/or anhedonia which maintain drug-seeking behavior (Koob et al., 2014). Male constitutive Dyn-KO mice have a higher preference for alcohol consumption relative to WT controls, and footshock increases alcohol consumption in WT mice but not Dyn-KO mice (Racz et al., 2013). Furthermore, mild footshock following chronic alcohol exposure increases BLA c-fos levels in WT mice but reduces BLA c-fos in Dyn-KO mice (Racz et al., 2013). A more recent study identified the KOR-encoding gene OPRK1 as a target of the transcriptional regulator LMO4 (Maiya et al., 2021). The authors also found that U50,488-mediated increases in alcohol consumption are attenuated in LMO4-shRNA^{BLA} mice and that local infusion of the KOR antagonist nor-BNI in the BLA reduces alcohol consumption in mice. The role of Dyn/KOR signaling in mediating the reinforcing properties of other drugs has not been thoroughly investigated. KOR antagonism during reinstatement of stress-induced nicotine conditioned place preference (CPP) reduces c-fos expression in the BLA and deletion of KOR from BLA neurons blocks reinstatement of stress-induced nicotine CPP (Nygard et al., 2016). The finding that nor-BNI blocks stress-induced reinstatement effects on BLA c-fos immunoreactivity suggests that the function of KORs in the BLA is more complex than simply blocking the inhibitory actions of KOR signaling. Similar effects of KOR antagonism on stress-induced nicotine-seeking behavior were observed in a separate study, although the specific amygdala subregion studied was not delineated (Smith et al., 2012). DREADD-mediated activation of G_{ai} signaling, an inhibitory signaling pathway that is known to be activated by KORs, in BLA PNs is also sufficient to drive this reinstatement effect (Nygard et al., 2016). These studies demonstrate that Dyn/KOR signaling specifically in the BLA regulates negative reinforcement processes that drive drug- and alcohol-seeking behaviors.

The central amygdala dynorphin/kappa opioid receptor system modulates the consumption of alcohol, psychostimulants, and opioids

Repeated alcohol exposure produces maladaptive behavioral effects which in part are hypothesized to be mediated by Dyn/KOR signaling in the CeA (Pohorecky et al., 1989; Dar, 1998; Matsuzawa et al., 1999; Walker and Kissler, 2013; Kissler et al., 2014; Anderson et al., 2018). Alcohol consumption increases the expression of both Dyn and KOR in the amygdala, including the CeA and BLA (D'Addario et al., 2013). In a rat model of alcohol dependency induced by ethanol vapor exposure, dependent rats displayed increased Dyn immunoreactivity and functional KOR coupling to G-proteins in the amygdala (Kissler et al., 2014). However, binge alcohol drinking in the drinking in the dark paradigm did not result in changes in the expression of Pdyn and Oprk1 mRNA in the CeA of mice (Bloodgood et al., 2021). An *in vivo* microdialysis study also reported that extracellular Dyn peptide levels in the CeA are increased following high doses of ethanol associated with intoxication (Lam et al., 2008). These

studies highlight that Dyn expression and release may be recruited by intoxicating or dependence-producing ethanol exposure. In slices, KOR activation by dynorphin impairs ethanol-induced increases in IPSP amplitude in the CeA, while KOR antagonism with nor-BNI increases CeA IPSPs (Gilpin et al., 2014), suggesting that alcohol increases the activity of KOR-mediated inhibitory synapses. Alcohol drinking drives sex-specific effects on the excitability of Pdyn-expressing neurons in the CeA without impacting excitatory synaptic drive onto these neurons (Bloodgood et al., 2021). Together, these studies indicate that ethanol consumption promotes Dyn/KOR signaling in the CeA. Consistent with the hypothesis that enhanced CeA Dyn promotes negative reinforcement of alcohol seeking behavior, inhibition of the Dyn/KOR signaling pathway in the CeA reduces alcohol consumption. Deletion of *Pdyn* decreases ethanol drinking in both male and female mice, while CeA *Oprk1* ablation in reduces alcohol-seeking in males but not females (Bloodgood et al., 2021). Administration of the KOR antagonist nor-BNI in the CeA reduces ethanol self-administration across multiple models of alcohol consumption, including a drinking in the dark model and in alcohol-dependent rats (Kissler et al., 2014; Kissler and Walker, 2016; Anderson et al., 2019). Intra-CeA nor-BNI also reduces ethanol self-administration during acute withdrawal and protracted abstinence, suggesting mitigated psychological sensitivity to withdrawal symptoms, although this surprisingly did not affect physiological measures of alcohol withdrawal (Kissler and Walker, 2016). Finally, alcohol consumption increases Pdyn-mRNA expression in CeA, and inhibition of PDyn-expressing neurons in the CeA or KOR antagonism in the CeA or bed nucleus of the stria terminalis, also reduces alcohol consumption (Anderson et al., 2019; Haun et al., 2022). These behavioral studies underscore a causal role for Dyn/KOR signaling in mediating negative reinforcement underlying compulsive alcohol seeking behavior.

Dynorphin/kappa opioid receptor activity in the CeA is also associated with psychostimulant and opioid seeking behavior. One study found that the KOR antagonist nor-BNI and KOR agonist U50,488 reduced and increased, respectively, GABAergic neurotransmission in the CeA of rats with long access (6 h) to cocaine (Kallupi et al., 2013). Interestingly, in controls, CeA GABAergic transmission was inhibited by KOR activation, suggesting that long access to cocaine inverts KOR regulation of CeA inhibitory synaptic transmission. In this study, CeA KOR antagonism blocked cocaine sensitization, indicating that Dyn/KOR signaling in the CeA may promote incentive salience with repeat cocaine exposure, as well as decreasing anxiety-like behavior during cocaine withdrawal. One study used the chemical stressor yohimbine to drive stress-induced reinstatement of heroin seeking in rats. Here, dynorphin precursor mRNA levels were enhanced in the CeA, but not BLA or medial amygdala, of yohimbine-treated mice, suggesting that CeA Dyn may promote stress-induced heroin-seeking behavior (Zhou et al., 2013). Collectively, these studies demonstrate that Dyn/KOR signaling within the CeA is engaged by various misused substances and this subsequently regulates drug-seeking behavior and ensuing maladaptive behaviors.

Regulation of pain by amygdala dynorphin/kappa opioid receptor neurons

It is hypothesized that Dyn/KOR signaling in the CeA may mediate aspects of pain processing. In a spinal nerve ligation (SNL) model of pain, nor-BNI in the right CeA blocked conditioned place preference (CPP) driven by gabapentin (an FDA approved treatment for neuropathic pain), suggesting that CeA KOR signaling is necessary for pain-induced negative affect (Navratilova et al., 2019). Further, intra-CeA KOR antagonism blocks anxiety-like behavior and ultasonic vocalizations in a rat functional pain model wherein morphine priming sensitizes stress-induced pain-like and affective behavior (Yakhnitsa et al., 2022). Moreover, increased CeA Dyn signaling may shape pain-related behavior as intra-CeA KOR antagonism blocks defensive behaviors in response to noxious stimuli using Randall Selitto to measure the paw withdrawal threshold (Phelps et al., 2019) or sensitivity to capsaicin left forepaw injection in a functional pain model involving morphine priming in rats exposed to a bright light stimulus (Nation et al., 2018). Furthermore, electrically evoked IPSCs onto CeA neurons are only potentiated by nor-BNI in SNL rats but not sham controls (Navratilova et al., 2019). Moreover, PBN-evoked polysynaptic inhibition or electrically-evoked IPSCs are potentiated by nor-BNI in the functional pain model described above (Yakhnitsa et al., 2022). These studies suggest that heightened Dyn signaling may be contributing to CeA neuron disinhibition in pain states. Disinhibition of CeA neurons by increased Dyn tone facilitates synaptically-evoked spiking in SNL rats, suggesting that Dyn may influence input-output transformations within CeA circuits (Navratilova et al., 2019). Administration of complete Freund's adjuvant (CFA) in mice increases G-protein stimulation in the CeA with the KOR agonist ICI 199,441 relative to saline-injected control mice (Narita et al., 2006), and Dyn content in the CeA of a functional pain model (Nation et al., 2018). Furthermore, intra-CeA KOR activation with U69,593 potentiates responsivity of amygdala and spinal cord neurons in response to noxious stimuli, an effect that is reversed by optogenetic silencing of CeA CRF neurons (Ji and Neugebauer, 2020). These findings suggest that Dyn disinhibition of CeA CRF neurons is a critical component underlying the effects of Dyn on CeA circuits and control of behavior. Recently, CeA SOM and PKCδ neurons have been shown to be differentially involved in pain regulation, with PKCδ and SOM cells promoting and

inhibiting nociceptive responses, respectively (Wilson et al., 2019). Given that CeA SOM neurons robustly respond to threats [reviewed above and in Yu et al. (2016)], it is possible that pain, a strong threat that promotes maladaptive passive defensive behaviors, robustly recruits SOM neurons co-expressing Dyn. Pain-induced recruitment of CeA Dyn neurons would disinhibit incoming afferent inputs to the CeA that process noxious stimuli, including the PBN. The aforementioned hypothesis would address the discrepancy in the field wherein CeA SOM neurons have been widely implicated in mediating fear-related freezing (fear ON cells) and paradoxically inhibit nociceptive responses in animal models of chronic pain (pain OFF cells). However, further work is needed to understand how Dyn may be interacting with distinct cell populations, such as PKCδ, SOM, and CRF neurons, to orchestrate maladaptive behavior induced by CeA dysfunction driven by pain, stress, and/or misused substances.

Cracking the shell on the almond: A circuit-based framework for amygdala dynorphin/kappa opioid receptor control of behavior

Here we posit that the Dyn/KOR system is poised to orchestrate coordinated waves of inhibition or disinhibition by targeting specific cell types and afferent inputs to amygdala circuits *via* the circuit-specific actions of amygdala Dyn on KOR-expressing neurons. As KOR is a G_i-coupled receptor, which regulates presynaptic neurotransmitter release or intrinsic excitability, activation of KOR by Dyn will produce fundamentally different outcomes depending on which amygdala cell type and/or afferent input expresses KORs. An obvious major distinction is that the BLA and CeA consists primarily of glutamatergic and GABAergic projection neurons that also collateralize within local circuits. Thus, KOR signaling in excitatory BLA neurons vs. CeA neurons would produce distinct outcomes since divergent circuit motifs would be engaged by the Dyn/KOR system, including decreased excitatory drive or disinhibition, respectively. Within the BLA, for example, activation of KORs on principal neurons would suppress transmission of those neurons to downstream targets such as the CeA. Activation of KORs on GABAergic CeA neurons, meanwhile, would serve to disinhibit downstream targets of those neurons and in efferent regions (**Figure 2**). Since molecularly- and projection-defined cells in the BLA and CeA in large part account for various aspects of threat and reward processing, future work is needed to further resolve the specificity of KOR expression within amygdala cell types. It is currently unclear whether KOR is widely expressed in different sub-classes of molecularly-defined neurons such as RSPO2, PPP1R1B, and Fezf2 (Kim et al., 2016; Zhang X. et al., 2021; **Figure 2**). KORs are on various presynaptic terminals of BLA efferents where they inhibit glutamate release in areas including the NAcc, PFC, and BNST, but whether KORs are ubiquitously expressed across all BLA outputs remains to be resolved. Further, it remains unclear whether KORs in the BLA are poised to control microcircuitry within BLA circuits or solely BLA outputs to downstream targets *via* presynaptic inhibition (**Figure 2**). KORs in the BLA may be expressed solely on excitatory neurons, or potentially in any of the plethora of interneuron populations within the BLA (**Figure 2**). Further, KORs may regulate excitatory synapses from KOR-expressing BLA principal neurons to other BLA cell types and interneuron populations. Dyn release and subsequent KOR signaling within BLA circuits may depress recurrent excitatory connections between BLA principal neurons or engaged inhibitory circuit motifs by limiting interneuron recruitment or outputs, depending on how KORs are embedded. This information will be critical for understanding how the Dyn/KOR system shapes activity dynamics of BLA projection neurons. Lastly, Dyn expression is spare or absent in the BLA, which raises the question of what specific sources of Dyn for the BLA may be or whether all sources of BLA Dyn confer the same effects on BLA circuit physiology and behavior. A recent study reported expression of Pdyn mRNA in the lateral ITCs raising the interesting possibility that this may be a source of Dyn to BLA circuits (Gomes et al., 2020).

Within the CeA, Dyn expression is primarily concentrated in SOM neurons, but is also expressed in other SOM-negative cell types (**Figure 3**). Whether these subpopulations of Dyn-expressing neurons are differentially integrated within CeA local circuits and innervate downstream brain regions is not known. Moreover, CeA Dyn neurons may release Dyn locally to regulate local CeA microcircuits that directly or indirectly impacted by KOR signaling. Determining the specific expression profile of KORs in molecularly-defined cell types and CeA projections would be significantly advance our understanding of how the Dyn/KOR system regulates CeA control of behavior (**Figure 3**). Dyn release from SOM neurons may regulate GABA release from KOR-expressing terminals from defined cell types within the CeA microcircuit, which has been hypothesized to be critical for regulating different aspects of threat-related behaviors (Moscarello and Penzo, 2022). For example, $PKC\delta^+$ neurons in the CeA may inhibit SOM^+ neurons *via* GABA release to diminish freezing behavior in response to a threat, and Dyn release from SOM neurons may limit $PKC\delta^+$-mediated lateral inhibition if these cells express KORs. Therefore, further research into KOR expression on specific cell types will be critical for our understanding of how Dyn signaling regulates activity among amygdala targets. Through specific functional effects on cellular physiology (e.g., regulation of synaptic transmission and excitability), Dyn signaling in specific cell types expressing KOR may forge inter-cellular communication within amygdala circuitry. Such a mechanism, when considered

at the population scale, may help determine the selection and deselection of specific subpopulations to form neuronal ensembles within the amygdala whose signal is able to stand out above the noise of the network (**Figure 3**). This, of course, remains to be tested with rigorous functional studies that examine the activation patterns of Dyn-or KOR-expressing neurons and how their activity influences inputs and outputs of the amygdala during behavior.

The amygdala Dyn/KOR system may also interact with other neuromodulatory systems that influence amygdala dependent behavior. Dyn neurons in the CeA target other brain regions rich in specific neuromodulators. For example, CeA Dyn neurons project to the VTA and substantia nigra, two known dopaminergic hubs (Fallon et al., 1985; Steinberg et al., 2020; **Figure 3**). Dyn decreases excitability BLA-projecting VTA DA neurons (Ford et al., 2007; Margolis et al., 2008; Baimel et al., 2017) in addition to inhibiting GABAergic transmission onto these cells (Ford et al., 2007; **Figure 2**). However, whether the Dyn supplied to the VTA that influence nigro- and meso-amygdaloid neurons arises from the CeA or some other Dynergic region remains to be determined. An alternative, but not mutually exclusive possibility, is that dopaminergic terminals in the amygdala express presynaptic KORs similar to what is observed in mesolimbic and mesocortical dopaminergic pathways (Chefer et al., 2013; Tejeda et al., 2013; **Figure 2**). If KORs regulate presynaptic DA terminals in amygdala circuitry it would provide a mechanism for Dyn inputs to the BLA or CeA Dyn-expressing neurons to regulate incoming DA inputs which are critical for aversive and reward learning. Amygdala Dyn/KOR signaling may also influence other monoamines. CeA Dyn neurons project to the norepinephrine-rich LC (Kravets et al., 2015), which in turn influences amygdala circuitry and distributed networks (**Figure 3**). However, how the Dyn/KOR system regulates the norepinephrine system during various behaviors is unknown. In CeA, Dyn expression highly overlaps with SOM, another neuropeptide. Although Dyn and SOM are often expressed in the same CeA neurons (Jungling et al., 2015), much remains unknown about the implications of this co-expression. It is unclear what the behavioral effects of SOM neuropeptide transmission is on affective and motivated behaviors. Further, it is unknown whether Dyn and SOM are differentially released during behavior, and, if so, whether they exert complementary or antagonistic effects on amygdala microcircuitry. It is possible that differences in expression patterns of KOR and the SOM receptor on distinct CeA cell types may confer another layer of complexity by which these two peptides engage or disengage discrete microcircuits. Together, these results highlight the importance of understanding the way the Dyn/KOR system may interact with other neuromodulators in amygdala circuits to influence behavior.

Future efforts should be aimed at investigating how the compartmentalization of the Dyn/KOR system within the amygdala shapes different aspects of amygdala-dependent behavior. Though tremendous progress has been made in understanding the role of amygdala circuitry in controlling associative learning, primarily threat conditioning, over the last couple of decades it has become increasingly clear that the amygdala as a whole regulates many nuanced facets of emotion, goal-directed behavior, and motivation. Through the selection of distinct cell types and circuit motifs, the Dyn/KOR system may aid in mediating specific behavioral outcomes, including associative learning. The Pearce-Hall learning model posits that attention and salience are also critical factors underpinning associative learning, and a growing body of work underscores the role of the amygdala in regulating attentional processing (Roesch et al., 2012). Furthermore, given the restriction of Dyn expression to largely PKCδ^- neurons, which are thought to be engaged during fear (Ciocchi et al., 2010; Haubensak et al., 2010), the Dyn/KOR system in the amygdala may regulate valence processing. Therefore, understanding whether the amygdala Dyn/KOR system may be regulating appetitive associative and instrumental behavior is needed to further understand how cells expressing Dyn and/or KOR may be involved in learning processes. Because dopamine in the amygdala is critical for appropriate associative learning (Jo et al., 2018; Lutas et al., 2019). Therefore, in addition to the direct actions of Dyn/KOR signaling on amygdala neurons, the potential for Dyn to inhibit KOR-expressing dopaminergic neurons that project to the amygdala could constitute a mechanism that regulates learning. As models of BLA and CeA circuit function are refined and projection-and molecularly-defined neurons are characterized in terms of how their activity is explained by different learning models, we will be able to place activity of amygdala Dyn neurons or KOR-expressing cells in the context of neural correlates that adhere to different leaning models, such as the Pearce-Hall learning model (Roesch et al., 2012). Moreover, manipulating Dyn/KOR signaling (e.g., pharmacologically, genetically, etc.,) and monitoring the activity of amygdala circuitry broadly in the context of molecular markers and/or connectivity will be essential for determining the role this system plays in learning and cognitive processes by shaping amygdala circuit dynamics. Activation of the Dyn/KOR system by stressors, in conjunction with its ability to itself drive aspects of the stress response, raises the possibility that the Dyn/KOR system may be an important neuromodulator at the interface between an organism's internal state and external events and stimuli essential for guiding behavior. The amygdala receives significant inputs from structures that incorporate interoception, environmental features, flexible behavior, and action selection, including various prefrontal cortical and neuromodulatory factors such as dopamine to ultimately influence overall behavioral states (Grundemann et al., 2019; Courtin et al., 2022). To summarize, several lines of research are needed in order to piece together how the Dyn/KOR system in the amygdala functions to shape the selection/deselection

of distinct cell types, microcircuits, and pathways couple with internal states to regulate complex innate and learned behaviors.

Conclusion

In conclusion, here we provide an overview of the literature on the amygdala Dyn/KOR system (**Figures 2**, **3**). Despite the considerable evidence that implicates the Dyn/KOR system in the amygdala complex in promoting threat reactivity, chronic pain, and negative reinforcement in models of alcohol and substance use disorder, there is still a major gap in our understanding of the Dyn/KOR system in the amygdaloid nuclei. We identify unknowns and provide a framework that places the function of the Dyn/KOR system in the context of the recent advancements in identifying the role of specific cell types and incoming and outgoing pathways of the amygdaloid complex (**Figures 2**, **3**). This model will also provide general principles that are shared or distinct across neuropeptide signaling in amygdala circuits and the brain. A better understanding of this system will be invaluable in identifying how the Dyn/KOR systems regulate information processing in amygdala circuits and behaviors related to motivation. Additionally, uncovering novel potential targets and translational work will help elucidate new treatments for neuropsychiatric disorders and provide potential mechanisms for targets currently in clinical trials.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Endogenous opioid systems alterations in pain and opioid use disorder

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Decades of research advances have established a central role for endogenous opioid systems in regulating reward processing, mood, motivation, learning and memory, gastrointestinal function, and pain relief. Endogenous opioid systems are present ubiquitously throughout the central and peripheral nervous system. They are composed of four families, namely the μ (MOPR), κ (KOPR), δ (DOPR), and nociceptin/orphanin FQ (NOPR) opioid receptors systems. These receptors signal through the action of their endogenous opioid peptides β-endorphins, dynorphins, enkephalins, and nociceptins, respectfully, to maintain homeostasis under normal physiological states. Due to their prominent role in pain regulation, exogenous opioids—primarily targeting the MOPR, have been historically used in medicine as analgesics, but their ability to produce euphoric effects also present high risks for abuse. The ability of pain and opioid use to perturb endogenous opioid system function, particularly within the central nervous system, may increase the likelihood of developing opioid use disorder (OUD). Today, the opioid crisis represents a major social, economic, and public health concern. In this review, we summarize the current state of the literature on the function, expression, pharmacology, and regulation of endogenous opioid systems in pain. Additionally, we discuss the adaptations in the endogenous opioid systems upon use of exogenous opioids which contribute to the development of OUD. Finally, we describe the intricate relationship between pain, endogenous opioid systems, and the proclivity for opioid misuse, as well as potential advances in generating safer and more efficient pain therapies.

KEYWORDS

opioids, pain, addicition, opioid use and abuse, opioid use disorder (OUD), reward, endogenous opioids, opioid receptors

Introduction

The intersection between pain and opioid use presents a major dilemma for public health. Efforts to curb the burden of the ongoing opioid crisis continue to be challenged by the need to provide adequate relief for pain patients and at the same time lessen the negative impact of opioid misuse. Pain is extremely prevalent with over half of US adults reporting pain symptoms within the past 3 months (Lucas et al., 2021). Similarly, detriments of opioid abuse are evident in the annual increases in opioid overdose deaths, with the most recent provisional estimates exceeding 80,000 in 2021 (Ahmad et al., 2022). Although the prevalence of problematic opioid use in pain patients is difficult to pin-point for a myriad of reasons (Ballantyne, 2015; Voon et al., 2017), estimates derived from a number of metanalyses suggest rates of problematic prescription opioid use may occur in >80% of pain patients (Minozzi et al., 2013; Ballantyne, 2015; Chou et al., 2015; Vowles et al., 2015; Voon et al., 2017). Collectively, pain and opioid use pose tremendous societal costs, with pain-related health care and lost productivity exceeding $635 billion and opioid abuse-related health care, criminal justice, lost productivity, reduced quality of life, and life lost due to overdose exceeding $1.03 trillion annually (Institute of Medicine Committee on Advancing Pain Research, 2011; Gaskin and Richard, 2012; Florence et al., 2021). Linking the putative relationship between pain and maladaptive opioid use, is the endogenous opioid system, a primary biological substrate of pain and opioid reward. In the present review, we examine how pain and concurrent opioid use may disrupt endogenous opioid system function leading to alterations in reward signaling pathways and ultimately, higher risk for negative outcomes associated with opioid use.

Problematic opioid use in the context of pain

In 2019, the National Survey on Drug Abuse reported that almost all (>96%) instances of opioid misuse, or use deviating from physicians' instructions, was restricted to prescription opioid pain medications (Center for Behavioral Health Statistics and Quality, 2019). This same report indicated that among those that misused prescription opioids, the most common reason for misuse was to relieve physical pain (65%). Based on this evidence and the lack of therapeutic alternatives to prescription opioids suggests that the US is undertreating pain or undermining an overlapping and vulnerable population. The former could have likely been fueled by pain management initiatives in the 1990s that recognized pain as a fifth vital sign (Morone and Weiner, 2013; Meisenberg et al., 2018). This notion encouraged physicians to prioritize pain reduction through the liberalization of opioid prescriptions (Compton and Volkow, 2006; U.S. Department of Health and Human Services, 2019) which led to the initial wave of prescription overdose deaths (Rudd et al., 2016). This was addressed by several opioid diversion and mitigation strategies, including revisions to opioid prescribing practices in 2016 by the Center of Disease Control (CDC) that limit the number of opioid prescriptions (Lappin, 2016; Volkow and McLellan, 2016). Although these efforts appeared to bring prescription overdoses to a plateau, synthetic opioid overdoses (both illicit and prescribed) increased at alarming rates (CDC WONDER, 2018). It is difficult to pin down whether the continued rise in opioid overdoses was driven by the unmet needs of pain patients, growth in illicit markets, or a combination of both. Despite additional government-backed initiatives intended to curb opioid use and facilitate research for pain management alternatives (U.S. Department of Health and Human Services, 2019), the prevalence of chronic pain and opioid overdose deaths continue to rise each year (Goldstick et al., 2021; Zajacova et al., 2021; Ahmad et al., 2022), and have even been amplified by the COVID-19 pandemic (Fallon et al., 2021; Manchikanti et al., 2021; Soares et al., 2021). The National Institute of Health's (NIH) most recent endeavor, the HEAL initiative (Helping to End Addiction Long-term), recognized the need to address the opioid crisis through improvements to pain management (Wandner et al., 2022). As such, our ability to curtail opioid abuse and improve the treatment of pain relies heavily on our capacity to understand the neurobiological mechanisms underlying pain and opioid systems.

According to the International Association for the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (Raja et al., 2020). The intersection between these two dimensions of pain—sensation and emotion—present a substantial problem for chronic pain patients on long-term opioid therapy which can play a synergistic role in perpetuating pain, mood disruptions, and problematic opioid use. The occurrence of mood disorders can predict not only opioid misuse liability (NIDA, 2008; Davis et al., 2017; Center for Behavioral Health Statistics and Quality, 2019; Jones and McCance-Katz, 2019; Smit et al., 2020) but also, susceptibility to pain conditions (Viana et al., 2018; Rizvi et al., 2021). Likewise, patients with opioid use disorder (OUD), a chronic and relapsing disorder characterized by persistent and compulsive drug-seeking behavior despite negative outcomes, frequently report comorbidities of chronic pain (up to 65%) and mood disorders (up to 82%) (Davis et al., 2017; Hser et al., 2017; Peciña et al., 2018; Jones and McCance-Katz, 2019; Higgins et al., 2020; Latif et al., 2021). It is therefore not surprising that chronic pain patients are 2–3 times more likely to meet diagnostic criteria for an anxiety, mood, and mental disorders (Pereira et al., 2017) and are at higher risk (>50%) for developing opioid or substance use disorder (Højsted and Sjøgren, 2007; Morasco et al., 2011).

Collectively, the co-occurrence of pain, mood disruptions, and problematic opioid use can have additive effects on the severity and risk for the other. The extensive overlap of these conditions alludes to a common underlying mechanism; and while each of these conditions are associated with dysfunction across multiple biological systems, one potential source of shared functional disruption lies within the endogenous opioid system (Jarcho et al., 2012; Witkin et al., 2014; Peciña et al., 2018; Jones and McCance-Katz, 2019; Toubia and Khalife, 2019).

The endogenous opioid system

Both pain and exogenous opioids can disrupt function of the endogenous opioid system (Roeckel et al., 2016) and similarly, alterations in endogenous opioid activity can predict variations in pain thresholds, opioid-induced analgesia, and the proclivity for opioid misuse and abuse (Corder et al., 2018; Jassar et al., 2019; Llorca-Torralba et al., 2019a; Massaly and Morón, 2019; Bodnar, 2021). The endogenous opioid system plays an important role in analgesia, but it is also critically involved in autonomic regulation, immunological responses, gastrointestinal function, learning and memory, and many other functions (Bodnar, 2021). As such, the endogenous opioid system is crucial for maintaining homeostasis and alterations in its activity are largely state dependent (Darcq and Kieffer, 2018; Valentino and Volkow, 2018). This system is also highly integrated with other biological systems involved in stress regulation, mood, and reward such as the endocannabinoid, serotonin, oxytocin, vasopressin, and dopamine (DA) systems and the hypothalamic adrenal pituitary axis (Leknes and Tracey, 2008; Toubia and Khalife, 2019; Emery and Akil, 2020; Koob, 2020; Bodnar, 2021; Mohammadkhani and Borgland, 2022). Implicitly, the extensive crosstalk between these contributes to the highly adaptive nature of the opioid system and its ability to acutely respond to noxious stimuli. However, chronic perturbations to opioid systems can leave the system vulnerable to dysfunction and have debilitating consequences (Stoeber et al., 2018). Here, we focus on the impact of pain and opioids on function of the endogenous opioid system and reward pathways and examine their putative role in provoking maladaptive patterns of opioid use and OUD.

Opioids

Opioids are natural, synthetic, or semi-synthetic chemicals acting on opioid receptors to produce analgesia among other peripheral effects (Zöllner and Stein, 2007). Opium is a dried milky exudate obtained from the unripe seed pods of the opium poppy, *papaver somniferum* (Brownstein, 1993). Among the dozens of alkaloids found in opium, the pharmacologically relevant constituents include morphine (10–15%), codeine (1–3%, noscapine (4–8%), papaverine (1–3%), and thebaine (1–2%) (Zöllner and Stein, 2007). The antiquity of opium for medicinal use was documented as early as ∼2100 BCE in Sumerian medical tablets (Duarte, 2005). The unrivaled ability of opium to relieve pain was recognized in texts for millennia, but the therapeutic application of opioids was transformed when a young German apothecary's assistant, F.W.A. Sterürner, isolated crystalline morphine (1803–1817), naming it after Greek god of sleep and dreams (Krishnamurti and Rao, 2016). The subsequent invention of the hypodermic syringe needle in the 1850s facilitated the use of morphine for surgical procedures, pain relief, and as an adjunct to general anesthetics (Brownstein, 1993). Since then, the broad application of various opioid analgesics has facilitated a greater understanding of the opioid system and the clinical utility of opioids for pain management.

The existence of opioid receptors was first proposed in the 1950s (Beckett and Casy, 1954), but it was not until the 1970s that different bioassays began to identify stereospecific binding sites for opioids in the brain (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973; Martin et al., 1976; Lord et al., 1977). These studies revealed that exogenous opioid ligands produce their narcotic effects through actions at different opioid receptors which led to the discovery that endogenous opioid-like peptides can produce similar effects through their activity at the same peptide receptors (Cox et al., 1976; Hans et al., 1977; Olson et al., 1979). The first evidence of distinct opioid receptor types was determined by detailing the actions of several analgesic drugs. As such, the first two opioid receptor types were named after the prototypic drugs used in these studies to distinguish them, mu (μ) for morphine and kappa (κ) for ketocyclazocine (Martin et al., 1976). Pharmacological analysis revealed a third opioid receptor type in the mouse vas deferens that exhibited a pharmacological profile markedly different from those previously identified (μ and κ) and was accordingly, named delta (δ) to signify this difference (Lord et al., 1977). The heterogeneity of these receptor types was later confirmed when distinct mRNAs for each receptor type were cloned and characterized (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Yasuda et al., 1993). Together, the μ, κ, and δ opioid receptors (MOPR, KOPR, DOPR, respectively) are considered the classical opioid peptide receptors based on their structural homology and sensitivity to the non-selective opioid receptor antagonist, naloxone (Dietis et al., 2011). A fourth opioid receptor-like (OPRL1) gene was later revealed to encode a receptor with a primary structure analogous to previously identified opioid receptors and yet, it lacked sensitivity to traditional opioid ligands (Bunzow et al., 1994; Mollereau et al., 1994). As such, OPRL1 remained an 'orphan' receptor until two independent groups isolated its endogenous ligand, nociception (Meunier et al., 1995) or orphanin FQ (Reinscheid et al., 1995) (N/OFQ), for which the OPRL1 or N/OFQ opioid receptor is referred to here on as NOPR. While MOPR, KOPR, DOPR,

and NOPR comprise the four major opioid receptor systems due to homology in structure and function, NOPR is often excluded from "classical" opioid receptor types based on its lack of sensitivity to naloxone or prototypical opioid ligands.

Four major opioid peptide families are commonly associated with complimentary opioid receptor systems for which they exhibit preferential activity: β-endorphins (MOPR), dynorphins (KOPR), enkephalins (DOPR), and N/OFQ (NOPR) (Hughes et al., 1977; Nakanishi et al., 1979; Horikawa et al., 1983; Pathan and Williams, 2012; Shenoy and Lui, 2018). However, despite greater selectivity of these endogenous ligands and their respective receptors, the activity of both endogenous and exogenous opioids at distinct receptor types is rarely exclusive to one family and can often activate multiple receptor types to varying degrees (Stein, 2016). Opioid receptors are seven-transmembrane G protein-coupled receptors (GPCR) that generally couple to inhibitory G proteins, thereby reducing signal transduction and neurotransmission by engaging several second- and third-messenger systems and regulating ion channel activity (Zöllner and Stein, 2007; Al-Hasani and Bruchas, 2011; Toll et al., 2016; Corder et al., 2018). Different opioids can also engage biased signaling pathways to preferentially activate GPCR-dependent signaling or β-arrestin-dependent signaling, which can produce analgesia or unwanted side-effects, respectively (Ballantyne and Chavkin, 2020). Allosteric binding sites on opioid receptors, distinct from orthostatic sites or the ligand binding pocket, can also modulate opioid receptor function through activation by various other neurotransmitters and neuropeptides (Kathmann et al., 2006; Burford et al., 2015; Remesic et al., 2017; Livingston and Traynor, 2018). For example, cannabidiol (CBD), an exogenous cannabinoid ligand, can act as a negative allosteric modulator at MOPR or DOPR in rat cerebral cortices to reduce their function (Kathmann et al., 2006). Positive allosteric modulators for MOPR have also been sought after as they may reduce some of the unwanted side-effects attributed to traditional opioid medications or facilitate the activity of endogenous opioids (Burford et al., 2015). Adding another layer of complexity to opioid receptor signaling is the fact that different opioid receptors can associate with each other to form heteromers (e.g., MOPR-DOPR, DOPR-KOPR, KOPR-MOPR). For example, DOPR antagonism of DOPR-MOPR heteromers can act to enhance MOPR agonist-mediated analgesia (Gomes et al., 2004). The complexity of opioid receptor signaling mechanisms shed light on the multiple means by which opioid system function can be disrupted.

Opioid receptors are among the most widely expressed receptors in the central and peripheral nervous systems, although the composition and distribution of different opioid receptor types varies across regions (Corder et al., 2018). In the periphery, opioid receptors expressed in the lungs, heart, kidney, small intestine, and pancreas, can modulate organ function, inflammation, as well as multiple homeostatic processes (Peng et al., 2012). Opioid receptors can also be found in neuroendocrine (adrenals, pituitary), immune (leukocytes), and ectodermal cells, where they can modulate nociception and inflammation (Zöllner and Stein, 2007; Stein, 2013). In the context of pain, opioid receptors are ideally situated among, and connected with, somatosensory neurons of dorsal root ganglion (DRG) and second-order neurons of the dorsal horn of the spinal cord where they transmit ascending nociceptive signals to cortical areas through the spinothalamic, spinoreticular, and spinoparabrachial pathways (Basbaum and Fields, 1984; Marchand, 2008; DosSantos et al., 2017; Ringkamp et al., 2018). Local release of endogenous opioids or acute application of exogenous opioids at injury sites can suppress DRG activity to reduce nociceptive signaling and pain perception (Dickenson et al., 1990; Stein et al., 2003; Spahn et al., 2017; Corder et al., 2018; Massaly et al., 2020). Similarly, top-down regulation by opioid receptor systems within the periaqueductal gray (PAG) and rostral ventral medulla (RVM) can exert descending modulatory control over nociceptive signal transduction (Marchand, 2008; Ringkamp et al., 2018). The level of top-down control over anti-nociceptive responses can also be influenced by opioid receptor systems in other brain regions involved in cognition, affect, sensation, and motivation (Corder et al., 2018; Bannister and Dickenson, 2020; Dickenson et al., 2020). As such, the central and peripheral presence of opioid systems yields the ability of opioid receptors to functionally modulate reward-aversion networks through ascending and descending modes of control, and therefore, play a substantial role in aversive pain states, reward from pain relief, and hedonic balance (Darcq and Kieffer, 2018).

Proper functioning of the endogenous opioid system is essential for survival mechanisms involved in reward- and aversion-based learning and behavior. When the integrity of this system becomes compromised, the ability to integrate opioid reward- and pain aversion-related information will also become impaired. Among the many debilitating consequences associated with compromised opioid system function, is the risk of OUD. After repeated drug exposure, reward-processing centers can undergo neuroadaptations that leave affected individuals with enhanced incentive salience and habit formation, impulsivity, stress reactivity, and negative affect in the absence of drug; thereby producing overall disruptions in motivation (Koob and Volkow, 2010). As a result, maladaptive drug use is perpetuated through cycles of binge/intoxication, withdrawal/negative affect, and preoccupation/craving (Koob and Volkow, 2010). OUD and other substance use disorders are linked with adaptations to the opioid system (Darcq and Kieffer, 2018) because of its central role in reward processing (le Merrer et al., 2009). Therefore, the ability of the opioid system to regulate both pain states and the actions of opioid drugs may exacerbate the risk for the development of OUD in pain patients on long-term opioid therapies. Here, we focus on adaptations

within mesolimbic reward pathways and the putative synergistic effects of pain and opioid use in driving opioid misuse liability.

μ Opioid receptor

The role of MOPR in mediating opioid-dependent analgesia and reward provides support for the abundance of research on this opioid receptor family. The analgesic effects of MOPR activity are attributed to their hyperpolarizing effects and suppression of neuronal activity. This is regulated by $G_{\alpha i}$-mediated inhibition of cAMP production (Raffa et al., 1994), activation of G protein-coupled inwardly rectifying potassium (GIRK) channels (Ikeda et al., 2000), $G_{\beta\gamma}$ -mediated inhibition of L-type calcium channels (Bourinet et al., 1996), and inhibition of voltage-dependent calcium channels (VDCC) (Saegusa et al., 2000). Alternatively, β-arrestins can modulate MOPR signaling by decoupling the receptor from G proteins and facilitating receptor internalization (Siuda et al., 2017; Cong et al., 2021). β-arrestins can also engage multiple intracellular signaling cascades independent of G proteins (Macey et al., 2006) and biased signaling mechanisms through β-arrestins or G proteins often produce distinct effects (discussed below).

Endogenous MOPR agonists, like β-endorphins, can be locally released at injury sites to provide acute pain relief through their signaling at the MOPR (Hassan et al., 1993; Truong et al., 2003; Stein et al., 2009). Similarly, acute administration of exogenous MOPR agonists, like morphine, can provide both pain relief and reinforcement. Evidence from positron emission tomography (PET) studies in humans demonstrate that acutely painful stimuli increase MOPR activity in multiple brain regions, including those implicated in nociception and reward processing, such as the PAG and the nucleus accumbens (NAc), respectively (Zubieta et al., 2001, 2002; Bencherif et al., 2002). Relative to pain-free conditions, acute pain enhances MOPR activity while its activity is decreased under conditions of chronic pain. In animal models of neuropathic pain, MOPR expression is downregulated in the spinal cord, DRG, and several cortical regions in the days and weeks following injury (Porreca et al., 1998; Zhang et al., 1998; Rashid et al., 2004; Pol et al., 2006; Thompson et al., 2018). Similarly, patients with chronic lower back pain exhibit lower circulating levels of β-endorphin (Bruehl et al., 2012, 2014, 2017, 2013; Rhodin et al., 2013), while deficits in MOPR binding potential have been linked with multiple pain conditions including fibromyalgia, chronic migraine, trigeminal neuropathic pain, and chronic lower back pain (Harris et al., 2007; DosSantos et al., 2012; Hagelberg et al., 2012; Martikainen et al., 2013; Schrepf et al., 2016; Jassar et al., 2019; Toubia and Khalife, 2019). Therefore, the function of the MOPR system can differ depending on the persistence of pain conditions, losing efficacy over time.

Importantly, MOPR activity can contribute to both sensational and emotional aspects of pain. In healthy controls, baseline MOPR binding can predict pain thresholds, such that lower MOPR binding in multiple cortical regions is associated with higher pain sensitivity (Zubieta et al., 2001, 2002; Hagelberg et al., 2012). Moreover, MOPR binding is negatively correlated with affective pain ratings (Zubieta et al., 2001, 2002), adding further support to the idea that MOPR activity can modulate sensory and affective components of pain. In patients with various chronic pain conditions, the ability of MOPR binding to predict pain sensitivity is similar. For example, patients with trigeminal neuropathic pain exhibit reduced MOPR binding in the NAc which is negatively correlated with pain ratings (DosSantos et al., 2012). Consistent with this relationship, reduced MOPR binding in the prefrontal cortex is associated with migraine severity (DaSilva et al., 2014). Similar results have been recapitulated in rodent models of neuropathic pain. Months after spared nerve injury, rats show reduced MOPR availability and expression in the insula, caudate putamen, and motor cortices, and these levels are correlated with deficits in sucrose preference, a measure of anhedonia (Thompson et al., 2018). Together, these findings indicate that chronic pain disrupts MOPR function to negatively regulate sensory and affective components of pain.

The MOPR system is also influenced by acute or chronic exposure to exogenous opioids. In patients undergoing surgery under general anesthesia, plasma β-endorphin levels are increased, and this effect is inhibited by administration of fentanyl, a potent MOPR agonist (Dubois et al., 1982; Cork et al., 1985). Fentanyl administration also induces MOPR phosphorylation in the striatum of mice at sites involved in receptor desensitization and internalization (Macey et al., 2006), suggesting that acute opioid exposure can have rapid effects on receptor desensitization and tolerance. In contrast, MOPR antagonism increases β-endorphin levels (Hargreaves et al., 1986), adding further support to the idea that endogenous β-endorphin release is regulated by MOPR activity. Chronic opioid exposure can have detrimental effects on endogenous opioid production and MOPR system function. For example, chronic morphine treatment reduces expression levels of the β-endorphin precursor protein, proopiomelanocortin (POMC), in rats (Bronstein et al., 1990; Wardlaw et al., 1996; Przewlocki, 2004), and reduces MOPR density in β-endorphin-expressing neurons of the hypothalamus (site of synthesis) in guinea pigs (Zhang et al., 1996). As such, chronic exposure to exogenous MOPR agonists reduce MOPR system function by reducing endogenous production of MOPR agonists (β-endorphins) and overall MOPR availability. Chronic opioid exposure can also alter function of remaining MOPR by producing a switch in MOPR G-protein coupling from Gi/o to Gs, leading to activation of adenylyl cyclase rather than inhibition (Wang et al., 2005). MOPR activation and subsequent phosphorylation by GPCR kinases can also lead to the recruitment of β-arrestins,

which—in conjunction with many other effectors—leads to MOPR receptor desensitization and internalization (Koch and Höllt, 2008; Roeckel et al., 2016; Corder et al., 2017; Derouiche et al., 2020; Massaly et al., 2021). MOPR phosphorylation at sites involved in receptor desensitization and internalization are observed in mice seven days after partial sciatic nerve ligation, a manipulation that produces tolerance to both the analgesic and conditioned reinforcing properties of morphine (Petraschka et al., 2007). Together, these disruptions to endogenous opioid production and MOPR function in response to chronic opioid exposure can lead to long-term plasticity underlying the development of opioid-induced hyperalgesia, analgesic tolerance, and negative affect, contributing to problematic opioid use.

The ability of pain and exogenous opioids to modify MOPR system function can lead to alterations within the mesolimbic reward pathway that may "prime" the system to be more vulnerable to the abuse of opioids, alcohol, and other substances of abuse (Contet et al., 2004). Opioid activity at MOPR produces rewarding effects by hyperpolarizing GABAergic inputs onto ventral tegmental area (VTA) DA neurons, thereby disinhibiting DA release (Elman and Borsook, 2016; Mitsi and Zachariou, 2016; Stoeber et al., 2018). Local infusion of MOPR agonists in the VTA is sufficient to produce reinforcing behaviors and conditioned reward-seeking behavior (Devine and Wise, 1994). Additionally, VTA MOPR function is necessary for opioid-dependent reward (Cui et al., 2014). Based on the ability of opioids to provide both positive reinforcement and pain relief, it seems evident that pain-induced alterations on MOPR signaling within mesolimbic circuits may facilitate tendencies toward opioid abuse (Koob, 2020). A large body of evidence indicates that pain augments opioid reward thresholds by disrupting DA transmission within the mesolimbic system (Hipólito et al., 2015; Martikainen et al., 2015; Taylor et al., 2016; Selley et al., 2020; Ren et al., 2021). This is regulated at least partly by deficits in MOPR system function (Markovic et al., 2021). Preclinical studies have shown that inflammatory and nerve injury pain reduces MOPR agonist efficiency at silencing VTA GABAergic transmission (Hipólito et al., 2015; Taylor et al., 2015), thus decreasing the ability of MOPR agonists to disinhibit VTA DA neurons (Ozaki et al., 2004, 2003, 2002; Hipólito et al., 2015) and evoke DA release in the nucleus accumbens (NAc) (Niikura et al., 2010; Hipólito et al., 2015; Taylor et al., 2015). These pain-induced deficits in mesolimbic function significantly dampen the rewarding properties of MOPR agonists. For example, rats with sciatic nerve ligation exhibit reduced placed preference induced by intra-VTA administration of the MOPR agonist, DAMGO, or systemic administration of morphine—an effect paralleled by attenuated MOPR binding in the VTA (Niikura et al., 2008). Consistent with this idea, chronic pain patients at low risk for opioid misuse exhibit less pain-induced activation of MOPR in the NAc, and this effect is associated with fewer mood disturbances and negative affect (Ballester et al., 2022). Taken together, MOPR signaling is a primary mechanism by which opioids yield high potential for abuse. As such, the MOPR system has received interest as therapeutic target for the treatment of chronic pain and OUD since the 1960s. Methadone, a long-acting MOPR agonist, has been used as a substitution therapy for chronic pain patients with long-term opioid therapy and maintenance treatment for patients with OUD (Kreek, 1973, 1991, 2000; Ferrari et al., 2004; Axelrod and Reville, 2007; Shi et al., 2008; Mattick et al., 2009; Kreek et al., 2010). The unique pharmacokinetic profile of methadone (slow onset, slow offset) yields a useful strategy to target the MOPR system while reducing the potential for opioid abuse, but the efficacy of these treatments is often limited by inter-individual variability, resources, and appropriate implementation (Dole and Nyswander, 1976; Ward et al., 2009; Kreek et al., 2010). As such, recent approaches have examined allosteric modulators of MOPR and biased signaling mechanisms as a means of offsetting the negative side effects of opioid pain medications (Manglik et al., 2016). A better understanding of how different pain conditions alter MOPR function with consideration of the interplay with ongoing opioid use will aid the development of future pharmacotherapeutic targeting strategies.

κ Opioid receptor

In contrast to the rewarding effects exerted by MOPR activity, the KOPR system is often attributed to dysphoria, anhedonia, and aversion (Spanagel et al., 1992; Darcq and Kieffer, 2018; Liu et al., 2019; Massaly et al., 2019; Cahill et al., 2022b). The opioid peptide, dynorphin, and its activity at KOPR have been implicated in negative affect, pain, analgesia, stress, and addiction (Bruchas et al., 2009; Darcq and Kieffer, 2018). A large body of evidence demonstrates that pain increases dynorphin mRNA expression and peptide production in the spinal cord of rodents and humans (Iadarola et al., 1988; Millan et al., 1988, 1985; Samuelsonn et al., 1993; Xu et al., 2004; Podvin et al., 2016; Liu et al., 2019). Following the onset of pain, the increase in dynorphin parallels the development of hyperalgesia and KOPR antagonism can facilitate hyperalgesic responses (Millan et al., 1987; Xu et al., 2004). This suggests that the dynorphin-kappa system is actively recruited under pain conditions to suppress nociceptive transmission. However, the ability of KOPR activity to suppress hyperalgesic responses may be dependent on the cell populations activated by dynorphin. For example, spinally restricted dynorphin signaling at KOPR expressed in astrocytes, rather than neurons, can produce nociceptive responses (Chartoff and Mavrikaki, 2015; Cahill et al., 2022b). In this regard, astrocytic KOPR activation can trigger hypertrophy in spinal astrocytes to facilitate the persistence of pain and the development of MOPR analgesic tolerance (Donnelly et al., 2020).

Pain can also trigger dynorphin-mediated KOPR activity in supraspinal regions. Pain induced adaptations to KOPR function within mesolimbic pathways may represent a primary mechanism by which pain can lead to the emergence of negative affect and altered motivational states. Indeed, pain conditions increase dynorphin expression and KOPR activity in multiple supraspinal sites including the VTA and NAc (Narita et al., 2005; Tejeda et al., 2017; Liu et al., 2019; Massaly et al., 2019; Navratilova et al., 2019; Wawrzczak-Bargieła et al., 2020). Although genetic deletion of KOPR or KOPR antagonism fails to alter pain-induced hyperalgesia, these manipulations can effectively restore pain-induced anhedonia and aversion (Narita et al., 2005; Tejeda et al., 2017; Liu et al., 2019; Massaly et al., 2019; Navratilova et al., 2019; Vergara et al., 2020). Recent evidence suggests that KOPR activity in NAc may be important for the transition from acute to chronic pain. Using hind paw injections of prostaglandin E2 to induce a persistent hyperalgesic state in rats, KOPR manipulations did not affect mechanical sensitivity during the induction phase (14 daily injections) (Vergara et al., 2020). Rather, intra-NAc KOPR agonists or antagonists facilitated or inhibited the persistence of hyperalgesia, respectively (Vergara et al., 2020). The findings suggest that the KOPR system may play an important role in pain chronification (Borsook et al., 2016).

Dynorphin recruitment under conditions of pain and the ability of KOPR activity to drive the transition from acute to chronic pain, suggest that KOPR may also be important for the development of comorbidities associated with persistent pain states such as negative affect and motivational deficits (Al-Hasani et al., 2015; Hipólito et al., 2015; Taylor et al., 2015; Elman and Borsook, 2016; Liu et al., 2019; Massaly et al., 2019). In general, KOPR agonists produce aversion and are associated with negative affect across species. In humans, KOPR agonists have psychotomimetic effects and produce dysphoria and hallucinations (Pfeiffer et al., 1986; Ranganathan et al., 2012) while increasing circulating stress hormone levels of cortisol (Ur et al., 1997). Similarly, in rodent models, both systemic and intracranial injections of KOPR agonists are sufficient to produce a conditioned place aversion (CPA) (Chefer and Ba, 2013; Tejeda et al., 2013) and increases in circulating levels of the stress hormone, corticosterone (Hayes and Stewart, 1985; Iyengar et al., 1986). These findings indicate that dynorphin-mediated activation of KOPR is acutely aversive and stimulates HPA axis activity, a putative mechanism contributing to negative affect associated with pain conditions. In support of this, increases in NAc dynorphin are found in suicidal individuals (Hurd et al., 1997) and animal models of depression (Carlezon and Krystal, 2016; Tejeda and Bonci, 2019). Importantly, these effects appear to be driven by the ability of KOPR activity to attenuate DA release in the NAc (Chefer and Ba, 2013; Conway et al., 2019; Escobar et al., 2020).

Dynorphin recruitment in mesolimbic pathways under conditions of pain leads to motivational deficits. For example, our lab showed that inflammatory pain increases KOPR function and recruits dynorphin-containing neurons in the NAc shell (Massaly et al., 2019). In this work, we found that the recruitment of NAc shell dynorphin neurons and activity at KOPR is both necessary and sufficient to drive pain-induced motivational deficits for natural rewards (Massaly et al., 2019). These effects also translate to motivational deficits for opioid drug reward. In models of neuropathic or inflammatory pain, morphine-induced conditioned place preference (CPP) scores are attenuated but can be restored by intra-NAc infusions of KOPR antagonists (Narita et al., 2005; Liu et al., 2019). Moreover, pain reduced opioid-evoked DA release in the NAc, an effect restored by intra-systemic KOPR antagonism (Narita et al., 2005; Liu et al., 2019). This suggests that pain-induced recruitment of dynorphin significantly decreases opioid reward processing. Importantly, KOPR antagonism does not impact opioid reward or dopamine release in the absence of pain (Liu et al., 2019), further implicating the state-dependent role of dynorphin. Opioid exposure, in the absence of pain, can perturb KOPR function in a manner similar to pain. For example, opioid self-administration or chronic opioid exposure increases prodynorphin (dynorphin precursor) levels in the NAc (Nylander et al., 1995; Trujillo et al., 1995; Solecki et al., 2009; Schlosburg et al., 2013). Based on this, pain patients on long-term opioid therapies may have compounding effects of pain and opioid use on KOPR dysfunction, exacerbating motivational deficits, negative affect, and leading to increased risk for maladaptive opioid use. Consistent with this idea, genetic polymorphisms to the prodynorphin gene have been linked with increased risk for OUD (Clarke et al., 2012).

The role of the KOPR system in pain-related mood disturbances and negative affect make this system an appealing target from a treatment perspective (Roeckel et al., 2016; Jassar et al., 2019; Llorca-Torralba et al., 2019b). Although systemic KOPR agonists can produce analgesia, many undesirable effects including hallucinations, impaired stress-coping skills, and deficits in reward-driven motivation, limit their clinical utility as therapeutic alternatives to traditional exogenous opioids (Jarcho et al., 2012; Davis et al., 2017; Jones and McCance-Katz, 2019; Toubia and Khalife, 2019; Emery and Akil, 2020). However, pharmacotherapies with partial agonist properties at KOPR have been examined in clinical trials for treatment of alcohol use disorder (AUD). Nalmefene, a MOPR inverse agonist and weak partial KOPR agonist can effectively reduce alcohol consumption and heavy drinking days (Barrio et al., 2018; Miyata et al., 2019), while improving emotional processing in AUD patients (Vollstädt-Klein et al., 2019). On the other hand, considering the upregulated KOPR signaling in supraspinal sites driving negative affective states under pain conditions, the development of KOPR antagonists may yield promising therapeutic potential for the treatment or prevention of neuropsychiatric disorders comorbid with pain (Ghozland et al., 2002; Liu et al., 2019; Escobar et al., 2020;

Ji et al., 2021; Cahill et al., 2022b). Buprenorphine is a KOPR antagonist/partial agonist, a partial MOPR and NOPR agonist, and DOPR antagonist with higher efficacy in the periphery than centrally (Bloms-Funke et al., 2000; Lutfy and Cowan, 2004). As such, this treatment provides higher levels of analgesia while sparing many of the negative side-effects associated with traditional opioid medications (Cowan et al., 1977; Lutfy and Cowan, 2004; Koppert et al., 2005; Gudin and Fudin, 2020). The ability of buprenorphine to reduce depressive symptoms has been demonstrated in patients with treatment resistant depression (Karp et al., 2014) and patients with comorbid depression and OUD (Yovell et al., 2016; Ahmadi et al., 2018a). Adding further support to this strategy, buprenorphine is also effective in reducing pain severity in experimentally-induced pain (Koppert et al., 2005) and pain patients (Pergolizzi and Raffa, 2019; Gudin and Fudin, 2020). In patients with OUD and pain symptoms, combinatorial therapeutic approaches with buprenorphine and naloxone can effectively reduce pain severity (Worley et al., 2017, 2015; Shulman et al., 2020). The ability of similar strategies to curb opioid use and craving are less consistent (Blondell et al., 2010; Ahmadi et al., 2018b; Parida et al., 2019). However, evidence suggests that the efficacy of buprenorphine as a substitution therapy for OUD is dependent on the dose and rate of tapering (Walsh et al., 1994; Sturgeon et al., 2020), but concerns remain for the potential for abuse (Cicero et al., 2018). To advance KOPR targeting strategies, it will be critical for future research to dissociate the analgesic properties of spinal KOPR and the emotional component of pain mediated by supraspinal KOPR. Biased ligands and peripherally restricted pharmacotherapeutics targeting KOPR will be important developments for treating the mood disruptions in the context of pain.

δ Opioid receptor

The DOPR system plays an important role in pain, analgesia, and negative affective states (Quirion et al., 2020). Similar to KOPR, the functional role of the DOPR system may be selectively dependent on pain states. In rodent models of inflammatory or neuropathic pain, DOPR expression increases in the dorsal horn of the spinal cord and DRG neurons (Cahill et al., 2003; Morinville et al., 2004a; Kabli and Cahill, 2007). The recruitment of DOPR in pain conditions appears to have an inhibitory influence over nociception because genetic deletion of DOPR, but not MOPR, exacerbates and prolongs thermal and mechanical sensitivity in mice with inflammatory pain (Gavériaux-Ruff et al., 2008). Similarly, conditional knock-out of DOPR in the peripheral nociceptive neurons exacerbates mechanical sensitivity in conditions of inflammatory or neuropathic pain (Gaveriaux-Ruff et al., 2011). Moreover, systemic, or local DOPR agonism effectively reduces mechanical and thermal hyperalgesia in wild-type, but not DOPR knock-out, mice, adding further support to the anti-nociceptive role of DOPR (Gaveriaux-Ruff et al., 2011). Importantly, the role of DOPR in nociception is dependent on the presence of pain. In the absence of pain, DOPR activity has negligible effects on analgesia; but in the presence of neuropathic or inflammatory pain, DOPR agonists can reduce thermal and mechanical pain sensitivity (Cahill et al., 2001; Gendron et al., 2007a,b; Normandin et al., 2013). DOPR agonists have also been shown to attenuate migraine associated-pain in preclinical models *via* signaling through calcitonin gene-related peptide (Moye et al., 2021). The weak antinociceptive effects of DOPR agonists in pain naïve animals results from low levels of DOPR expression in plasma membrane. In conditions of pain, the density of DOPR increases at the membrane and cell surface in spinal cord regions and DRG neurons (Quirion et al., 2020). The ability of pain to increase DOPR trafficking is a potential cellular mechanism to explain the pain selective analgesic properties of DOPR agonists. DOPR trafficking is controlled by constitutive pathways involving dynamic remodeling of actin filaments of the cytoskeleton (Mittal et al., 2013) or regulated signaling pathways involving G-protein receptor kinases (GRKs) (Quirion et al., 2020), but the precise mechanisms of DOPR trafficking remain unclear. The DOPR system can modulate nociceptive components of pain not only through neuronal mechanisms, but astrocytic mechanisms as well. For example, deletion of astrocytic DOPR decreases cold allodynia in neuropathic pain while mechanical allodynia is not affected (Reiss et al., 2021). In contrast, DOPR activity in somatostatin-expressing neurons of the dorsal horn of the spinal cord can reduce mechanical, but not thermal, sensitivity in neuropathic pain models (Wang et al., 2018). Therefore, DOPR can modulate distinct elements of the nociceptive experience based on their activity in different cellular populations.

The DOPR system has also received a lot of attention for its role in emotional regulation of mood disorders like anxiety and depression. For example, genetic ablation of DOPR or DOPR antagonists has anxiogenic effects in animal models, while DOPR agonists produce opposite effects (Filliol et al., 2000; Saitoh et al., 2005; Narita et al., 2006a,b; Perrine et al., 2006; Bilkei-Gorzo et al., 2007; Chu Sin Chung and Kieffer, 2013). Similarly, DOPR agonists are associated with higher latency for immobility in the forced swim task, a measure of depressive-like behavior in rodent models (Filliol et al., 2000; Jutkiewicz et al., 2006; Torregrossa et al., 2006), suggesting that pain-related recruitment of DOPR may function to offset mood dysregulation in pain. Unlike MOPR, DOPR activity is not rewarding in the absence of pain. DOPR agonists can elicit CPP in mice with peripheral nerve injury, but not sham controls, while DOPR antagonists selectively produce CPA in mice with pain (Cahill et al., 2022a). This demonstrates the pain state-dependent role of DOPR and suggests that DOPR activation acts as negative reinforcer by alleviating pain rather than producing positive reinforcement.

Given the ability of the DOPR system to modulate analgesic responses and negative affect while sparing any properties that may lead to abuse, DOPR have been investigated for their potential role in curbing opioid use (Quirion et al., 2020). Exogenous opioid exposure can regulate DOPR trafficking in a similar way to the induction of pain. For example, morphine exposure increases DOPR expression at the cell surface of DRG or cortical neurons (Cahill et al., 2001; Morinville et al., 2004b; Gendron et al., 2006). DOPR may also play an important role in the development of analgesic tolerance to exogenous opioids because genetic deletion of DOPR or DOPR antagonists can prevent the development of analgesic tolerance to morphine (Zhu et al., 1999; Abul-Husn et al., 2007; Beaudry et al., 2015). However, the role of DOPR in regulating opioid reward is less clear. DOPR knock-out or DOPR antagonists can facilitate morphine-induced locomotor sensitization (Chefer and Shippenberg, 2009; Billa et al., 2010), a measure of drug responsivity manifesting after repeated drug exposures. However, similar DOPR manipulations have been shown to reduce morphine CPP (Chefer and Shippenberg, 2009; le Merrer et al., 2009, 2011; Billa et al., 2010). These effects may not be attributed to reductions in opioid reinforcement, *per se,* as these manipulations fail to alter morphine self-administration (David et al., 2008; le Merrer et al., 2011). Instead, DOPR may play an important role in drug-cue associated learning.

DOPR signaling is necessary for cued value-based decisions making, particularly within the NAc shell (Laurent et al., 2014, 2012). This effect is driven by distinct anatomical regulation of DA transmission in the NAc by DOPR (Saigusa et al., 2017), such that DOPR agonists in the NAc core increase extracellular DA, while decreasing DA release in the NAc shell (Hirose et al., 2005; Hipólito et al., 2008; Saigusa et al., 2017). Adding another layer of complexity to DOPR-mediated effects on DA release, is that distinct DOPR subtypes (DOPR-1 and DOPR-2) can differentially regulate DA release through their interactions with MOPR. While stimulation of either subtype can have an inhibitory influence over MOPR-mediated slow increases DA release, the precise mechanisms underlying these effects are less clear. For example, stimulation of DOPR-1, not DOPR-2, can activate MOPR causing rapid increases in extracellular DA. However, DOPR agonists can also facilitate DA release independent of MOPR or DOPR, possibly by regulating sodium channel activity (Murakawa et al., 2004; Hirose et al., 2005; Saigusa et al., 2017). In contrast, DOPR-2, not DOPR-1, may play an important role in the development of analgesic tolerance (Beaudry et al., 2015). Future research delineating the precise role of DOPR in mesolimbic circuits will be crucial to exploit on the therapeutic potential of targeting the DOPR system for pain and opioid abuse. Interestingly, gene polymorphisms to the DOPR encoding gene have been linked with increased risk for drug dependence, further strengthening the need for untangling the DOPR system from the behavioral to the genetic level (Zhang et al., 2008; Crist et al., 2013). Moreover, because DOPR agonists have lower abuse liability than MOPR agonists (Stevenson et al., 2005), the DOPR system may represent a useful target for managing pain states during long-term opioid therapy. While the analgesic properties and anxiolytic effects of DOPR agonists are desirable for improving mood states of chronic pain patients, it should be noted that activation of DOPRs can lead to convulsions which may limit their clinical utility (Pradhan et al., 2011). As such, advancing clinical use of DOPR-based ligands will likely be dependent on the development of biased-ligands or dimer-specific drugs capable of DOPR heteromized with other GPCRs (Chu Sin Chung and Kieffer, 2013). Nevertheless, the DOPR system represents a promising target for the development of chronic pain therapies with improved analgesia and minimal unwanted side-effects attributed to traditional opioid medications.

Nociceptin/orphanin FQ opioid receptor

The role of the NOPR system in pain is complex (Toll et al., 2016). In animal models of inflammatory pain, neuropathic pain, and fibromyalgia, NOPR expression and respective endogenous peptide, nociceptin/orphanin FQ (N/OFQ), are upregulated in DRG neurons, spinal tissue, and supraspinal sites (Andoh et al., 1997; Briscini et al., 2002; Dagnino et al., 2019). The ability of NOPR to regulate nociception is related to crosstalk between the NOPR system and stress systems and anatomical distinctions in NOPR function in spinal versus supraspinal sites. Early studies found that intracerebroventricular administration of N/OFQ reduced hot plate and tail flick latencies, suggesting a pro-nociceptive role of supraspinal NOPR activity (Meunier et al., 1995). However, subsequent studies determined that this pro-nociceptive effect was solely related to stress-induced analgesia (Mogil et al., 1996a,b; Morgan, 1997; Rizzi et al., 2001, 2007), a phenomenon triggering the release of endogenous opioids. The pro-nociceptive effects supraspinal N/OFQ are driven partially by antagonistic effects at MOPR, DOPR, and KOPR (Mogil et al., 1996a,b) as well as non-opioid components of stress-induced analgesia (Rizzi et al., 2001). On the contrary, intrathecal administration of N/OFQ produces anti-nociceptive effects and potentiates the effects of morphine (Xu et al., 1996; Yamamoto et al., 1997), indicating the role of NOPR signaling in pain is anatomically specific. Intrathecal administration of N/OFQ or NOPR agonists reduce pain sensitivity in animal models of neuropathic and inflammatory pain (Hao et al., 1998; Ko and Naughton, 2009; Tzschentke et al., 2017). Similar effects are observed with systemic NOPR agonists on mechanical allodynia in preclinical models of cancer-induced bone pain (Sliepen et al., 2021).

NOPR function also varies depending on the persistence of pain. Genetic deletion of NOPR does not alter acute pain

sensitivity but exacerbates hyperalgesic responses in conditions of persistent inflammatory pain (Depner et al., 2003; Rizzi et al., 2011). However, significant differences in NOPR supraspinal distribution and localization is observed between species, particularly between preclinical animal models and non-human primates/humans (Florin et al., 2000; Berthele et al., 2003). As such, the effects of NOPR manipulations in preclinical models of pain may not directly translate to clinical populations (Spetea et al., 2022). Because cellular adaptations within the NOPR system and anatomical distribution of NOPR vary across species and different pain models, future research is required to uncover how recruiting/silencing NOPR signaling can efficiently treat pain symptoms in a more individualized setting.

When considering the clinical utility of targeting the NOPR system for treating opioid abuse in pain patients, it is important to highlight that NOPR activity is neither rewarding nor aversive (Devine et al., 1996). This significantly adds to the therapeutic potential of targeting the NOPR system since NOPR manipulations mitigate abuse potential while sparing negative side-effects. NOPR agonists reduce extracellular release of DA in the NAc (Murphy et al., 1996; Lutfy et al., 2001a), suggesting an inhibitory influence of NOPR activity over drug reward. Indeed, intracerebroventricular administration of N/OFQ or NOPR agonists block the acquisition of CPP for morphine, cocaine, alcohol, and methamphetamine (Ciccocioppo et al., 2000; Kotlińska et al., 2002, Kotlinska et al., 2003; Sakoori and Murphy, 2004; Zaveri et al., 2018). This evidence further solidifies the therapeutic potential of the NOPR system in mitigating opioid abuse and substance use disorders in general. Recent studies found that local administration of N/OFQ in the central amygdala attenuates escalation of oxycodone self-administration (Kallupi et al., 2020). These effects may be attributed to site-specific NOPR regulation as intracerebroventricular administration of N/OFQ fails to reduce heroin self-administration (Walker et al., 1998.). Further adding to this complexity is that the effects of NOPR manipulations have inconsistent effects on alcohol self-administration (Ciccocioppo et al., 1999, 2004; Kuzmin et al., 2004; Economidou et al., 2008). One possibility is that NOPR function may be important for drug-associated memory formation given that NOPR activity can negatively impact memory (Moulédous, 2019). In this regard, NOPR activity may impact the formation of drug-context association (conditioned place preference) rather than impact drug reinforcement and thus, instrumental drug-seeking behavior. This would align with findings demonstrating that NOPR agonists effectively block the acquisition of morphine CPP, but not its expression (Shoblock et al., 2005). The precise mechanisms underlying the effects of pain and opioid use on NOPR function remain unclear, but emerging evidence indicates that NOPR agonists, like cebranopadol, have high analgesic efficacy in chronic pain, delayed development of analgesic tolerance, and lower abuse potential (Linz et al., 2014; Tzschentke et al., 2019). Therefore, it will be important for ongoing research endeavors to fully characterize the role of NOPR in the context of pain and opioid misuse liability and determine whether this opioid system is a therapeutic target with clinical utility.

Opioid system dysfunction by exogenous opioids

Chronic exogenous opioid use can lead to the development of tolerance, a progressive decrease in opioid efficacy which can be mitigated by increasing opioid doses (Lee et al., 2011). Pain patients on long-term opioid therapy typically require increasing doses of opioids to achieve the same level of analgesia (Williams et al., 2001; Zernig et al., 2007; Hayes et al., 2020). In addition to analgesia, tolerance to other opioid-induced effects, like euphoria, sedation, nausea, respiratory depression, and constipation, can also develop over time, albeit not at the same rate (Hayhurst and Durieux, 2016). For example, the development of analgesic and euphoric tolerance occurs on a faster time scale than tolerance to respiratory depression (Ling et al., 1989; Volkow et al., 2018), which contributes to the heightened risk of overdose for opioid users with escalating opioid doses (Kaplovitch et al., 2015; Hayes et al., 2019, 2020). Furthermore, the rate at which tolerance develops often depends on genetic variability and differential responses to different opioid ligands, duration of exposure, and route of administration (Dumas and Pollack, 2008; Ballantyne and Koob, 2021).

Tolerance

The development of tolerance stems from desensitization of the opioid system and inflammatory immune responses within peripheral and central nervous systems (Zhu et al., 1999; Dumas and Pollack, 2008; Koch and Höllt, 2008; Matsui et al., 2014; Corder et al., 2017; Lueptow et al., 2018; Eidson and Murphy, 2019). Following activation, opioid receptors can be phosphorylated by GPCR kinases, which triggers G-protein uncoupling and binding of β-arrestins (Dumas and Pollack, 2008; Zhou et al., 2021). β-arrestin pathway signaling causes desensitized receptors to remain inactive at the plasma membrane, facilitates their endocytosis and subsequent degradation or recycling. As such, these cellular mechanisms represent a critical component in facilitating the development of tolerance at multiple levels (Hutchings et al., 1997; Bohn et al., 2000; Koch and Höllt, 2008; Zhou et al., 2021). Biased agonists, that preferentially activate G-protein signaling cascades with minimal β-arrestin pathway activity, have received great interest as therapeutic alternatives with the thought that such ligands may minimize the development of tolerance and other unwanted side-effects (Ballantyne and Chavkin, 2020). In

mice with genetic deletion of the β-arrestin2 isoform, acute morphine prolongs analgesia while reducing the unwanted side-effects of respiratory depression and constipation, while chronic morphine treatment reduces MOPR desensitization and the development of tolerance (Bohn et al., 1999, 2000; Raehal et al., 2011). These findings led to the development of functionally selective MOPR agonists, like oliceridine, which exhibit preference for G protein-biased signaling and produce less respiratory depression in preclinical models compared to non-selective agonists (DeWire et al., 2013). However, subsequent studies found that opioid-induced respiratory depression and constipation may occur independent of β-arrestins (Kliewer et al., 2020) and G-protein selectivity may worsen some side effects (Kliewer et al., 2019). Although negative side-effects remained during clinical trials (Hertz, 2018), the risk for respiratory depression with oliceridine was lower than morphine (Dahan et al., 2020). Similar findings were found for another biased-MOPR agonist, PZM21 (Graeme Henderson et al., 2018), further highlighting the need to better understand biased opioid ligand signaling mechanisms and their role tolerance.

NOPR signaling appears to play facilitative role in the development of tolerance in the context of pain. As previously mentioned, NOPR expression increases after the induction of pain in spinal and supraspinal sites (Andoh et al., 1997; Briscini et al., 2002; Dagnino et al., 2019) which, under conditions of chronic pain, can suppress hyperalgesic responses (Depner et al., 2003; Rizzi et al., 2011). Based on this, it is somewhat surprising that N/OFQ potentiates the development of opioid tolerance. Genetic ablation of the endogenous peptide, nociceptin, N/OFQ, its receptor (NOPR), or blocking NOPR signaling using an exogenous antagonist, prevents and reverses the development of morphine tolerance (Ueda et al., 1997; Lutfy et al., 2001b; Chung et al., 2006; Scoto et al., 2010). These likely are attributed to the antagonistic properties of N/OFQ at other opioid receptors (Mogil et al., 1996b). NOPR can also undergo desensitization after chronic or acute stimulation (Donica et al., 2013). While these findings suggest that pain-induced upregulation of the NOPR system underlies attenuated analgesic responses to exogenous opioids, they also suggest that targeting the NOPR system may be a useful target to treat vulnerabilities in opioid tolerance, escalation, and abuse in pain patients.

The development of tolerance can also develop in response to the recruitment of neuroinflammatory mediators. Long-term opioid use triggers neuroinflammatory responses in the CNS to increase neuronal excitability which can contribute to tolerance (Eidson and Murphy, 2019; Zhang et al., 2020; Zhou et al., 2021). In particular, the ventrolateral PAG (vlPAG) is a critical hub in which descending control over nociceptive signaling is negatively affected by chronic opioid use. Chronic intra-vlPAG opioid agonist administration is sufficient to produce tolerance to systemically administered opioids. Similarly, blocking vlPAG opioid receptor-mediated signaling can prevent the development of tolerance to chronic systemic administration of exogenous opioids (Lane et al., 2004; Morgan et al., 2006; Meyer et al., 2007; Loyd et al., 2008; Macey et al., 2009; Bobeck et al., 2012; Eidson and Murphy, 2019). Opioid-induced activation of toll-like receptor 4 (TLR4) in astrocytes and microglia within the spinal cord or PAG triggers inflammatory responses through activation of nuclear factor kappa B (NFκB) and the release of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukins, IL-1β and IL-6 (Raghavendra et al., 2002; Eidson et al., 2016; Liang et al., 2016; Eidson and Murphy, 2019; Wang et al., 2020). This release in cytokines leads to down-regulation of GABA receptors resulting in increased function of glutamate receptor systems. Consequently, hyper-excitability in nociceptive pathways acts to oppose the analgesic actions of opioids, resulting in tolerance (DeLeo et al., 2004; Eidson and Murphy, 2019; Zhou et al., 2021). Based on the role of cytokines in opioid tolerance, significant efforts have been directed toward the development of treatments that may inhibit opioid-induced cytokine production (Namba et al., 2021). For example, manipulations inhibiting TNF signaling through TLR4 can prevent morphine tolerance and associated hyperexcitability (Shen et al., 2011; Eidson et al., 2016; Wang et al., 2020). As such, modulation of TNF signaling represents a promising adjunctive therapy to curb the development of opioid tolerance. Taken together, opioid tolerance manifests through adaptations to endogenous opioid and inflammatory systems, but a better understanding of the relationship between these systems will facilitate our ability to identify novel therapeutic targets to overcome the development of opioid tolerance.

Opioid-induced hyperalgesia

In contrast to the development of tolerance, chronic opioid use can also result in opioid-induced hyperalgesia (OIH), a paradoxical increase in pain sensitivity either at the initial source of pain or elsewhere (Chu et al., 2008; Hay et al., 2009; Roeckel et al., 2016). While the phenomenon of tolerance represents a reduction in drug potency and creates a rightward shift in analgesic opioid dose response curves, OIH increases pain sensitivity modeled by a significant downward shift in analgesic dose response (Chu et al., 2008). It is, thus, distinct from tolerance in that escalating opioid doses may exacerbate the development of OIH in the long-term. However, both tolerance and OIH are associated with hyperexcitability in glutamatergic systems and up-regulation of pro-inflammatory molecules at spinal synapses and supraspinal regions, like the RVM (Bederson et al., 1990; Kaplan and Fields, 1991; Kovelowski et al., 2000; Vanderah et al., 2001). OIH is a pro-nociceptive process that can be observed independently of tolerance through acute exposure to ultra-low opioid doses. However, the development of OIH is more often observed after

the development of tolerance, following chronic exposure to higher, analgesic doses (Drdla et al., 2009; Silverman, 2009; Lee et al., 2011; Hayhurst and Durieux, 2016; Roeckel et al., 2016).

Opioid-induced hyperalgesia is driven by cellular adaptations in pronociceptive signaling pathways, particularly within glutamatergic systems (Lee et al., 2011). Opioid agonists increase cellular excitability underlying OIH by inhibiting glutamate transporter systems (Mao et al., 2002). The resulting abundance in synaptic glutamate can lead to NMDA receptor-dependent long-term potentiation (LTP) at primary afferents and second-order spinal neurons resulting in sensitization of pain signaling pathways (Drdla et al., 2009; Silverman, 2009; Heinl et al., 2011; Drdla-Schutting et al., 2012; Roeckel et al., 2016; Corder et al., 2018). Adding to this, previous reports from our lab found that OIH is driven by insertion of GluA4-containing AMPA receptors in the dorsal horn of the spinal cord (Cabañero et al., 2013). Similar to the development of tolerance, OIH is also associated with opioid-dependent production and release of nociceptive signaling molecules from microglia and astrocytes such as pro-inflammatory cytokines, chemokines, ATP, nitric oxide, and others detailed elsewhere (Chu et al., 2008; Lee et al., 2011; Roeckel et al., 2016). Consequent release of the neuropeptide, cholecystokinin (CCK) in the RVM has been shown to have 'anti-opioid' actions that facilitate pronociceptive pathways contributing to OIH (Kaplan and Fields, 1991; Kovelowski et al., 2000; Friedrich and Gebhart, 2003; Heinricher and Neubert, 2004). The NMDA receptor-dependent hyperexcitability associated with OIH has been targeted in efforts to mitigate the impact of opioids on central sensitization. For example, low-dose ketamine (non-selective NMDA receptor antagonist) administration in conjunction with opioid analgesics can prevent the development of OIH in animal models and clinical patients with postoperative pain (Célèrier et al., 2000; Maher et al., 2017). Similarly, methadone, a potent MOPR agonist and weak NMDA receptor antagonist, has been examined as a substitute for opioid therapies and can effectively reduce opioid-induced OIH (Sjøgren et al., 1994; Shimoyama et al., 1997; Davis and Inturrisi, 1999; Axelrod and Reville, 2007). While the efficacy of methadone maintenance treatment (MMT) is less reliable in patients with opioid dependence or a prior history of abuse, MMT reduce instances of heroin use, drug craving, and criminal activity (Dole and Nyswander, 1965, 1976; Shi et al., 2008; Mattick et al., 2009; Ward et al., 2009; Lee et al., 2011). Despite this, moral reservations among some groups precipitated shifts in the treatment goals initially outlined for long-term MMT advising sufficient dosing and instead, goals were centered around achieving abstinence and using less-than-effective doses, which compromised treatment outcomes and funding for MMT research (Dole and Nyswander, 1976; Ward et al., 2009). As such, OIH remains a barrier to effective treatment with opioids. Further research delineating the mechanisms mediating the physiological and behavioral effects of opioids and whether pain affects these properties will help facilitate the development of novel and safer pharmacotherapies to improve patient care and well-being.

Pain, opioids, and reward

The mesolimbic pathway integrates both aversive and rewarding properties of external stimuli (Bromberg-Martin et al., 2010). Activation of the mesolimbic pathway by rewarding stimuli results in phasic DA release from the VTA into the NAc to reinforce goal-directed behaviors (Fibiger et al., 1987; Berridge and Robinson, 1998; Becerra and Borsook, 2008; Pignatelli and Bonci, 2015). As described previously, opioids reliably activate mesolimbic DA pathway and thus promote motivational salience (Matsui et al., 2014; Galaj et al., 2020; Doyle and Mazei-Robison, 2021). In conditions of pain, the ability of opioids to trigger comparable responses is significantly reduced. Furthermore, the motivational salience of opioid reward may be driven by hedonic pleasure (positive reinforcement) or pain relief (negative reinforcement) (Koob, 2020). Similar to exogenous opioids in pain-naïve conditions, relief from pain itself can elicit increases in DA release and reinforce motivated behaviors (Martin et al., 2006; Leknes et al., 2011; Navratilova et al., 2015; Eikemo et al., 2021). As such, the presence of pain may perpetuate maladaptive patterns of opioid use.

Pain disrupts mesolimbic DA function contributing to maladaptive effects on reward processing. Deficits in DA signaling, or administration of DA receptor antagonists reduce approach behaviors and hedonic responses to rewarding stimuli (Frank et al., 2016; Nguyen et al., 2019). In rodent models of inflammatory and nerve injury pain, motivated behaviors for natural and drug rewards, such as opioids, are significantly impaired (Schwartz et al., 2014; Hipólito et al., 2015; Taylor et al., 2015; Massaly et al., 2019). This pain-induced decrease in motivation is strongly correlated with blunted DA signaling in the mesolimbic pathway (Cahill et al., 2013; Schwartz et al., 2014; Hipólito et al., 2015). These findings parallel clinical studies in which pain-induced negative emotional states positively correlates with reductions in DA neurotransmission and maladaptive changes in NAc function (Lee and Tracey, 2010; Jarcho et al., 2012; Martikainen et al., 2015; Makary et al., 2020). Importantly, pain-related alterations in DA signaling are also associated with deficits in emotional and sensory processing. For example, deficits in DA receptor binding potential in the NAc are observed in patients with lower back pain, which can predict the severity of negative affect and pain (Baliki et al., 2010; Martikainen et al., 2015). In line with this, DA transporter activity, a mechanism important for clearing DA from the synaptic cleft, is increased in the NAc of animal models of chronic neuropathic

or inflammatory pain (Ren et al., 2015, 2021; Selley et al., 2020). Moreover, morphine-induced DA release in the NAc is suppressed by sciatic nerve ligation (Niikura et al., 2008). These changes in mesolimbic DA function strongly impact reward thresholds which may contribute to pain-related occurrences of negative affect and enhanced vulnerability for opioid abuse (Massaly et al., 2019, 2021). Supporting this, pain patients are more likely to initiate and continue opioid treatment if they have a cooccurring mood disorder (Halbert et al., 2016).

Opioid abuse susceptibility in pain states is likely exacerbated by a rightward shift in opioid reinforcement thresholds due to pain-related deficits in mesolimbic pathway function. In lower back pain patients, the propensity for risky monetary behavior is associated with altered connectivity of the NAc (Berger et al., 2014). The severity of pain is also associated with increased impulsivity in humans and rodent models (Wakaizumi et al., 2019; Cunha et al., 2020). These would suggest that pain patients are predisposed to developing problematic opioid use. Although it is recognized that chronic pain patients receiving prescription opioids are at high risk for opioid dependence (Ballantyne, 2015), the prevalence of maladaptive opioid use in pain patients has been difficult to determine based on confounding outcome measurements (i.e., mortality) and imprecise or poorly defined terminology (i.e., "abuse," "misuse," "addiction") (Vowles et al., 2015). Opioid "misuse," or use contrary to the prescribed pattern, occurs in up to 29% of pain patients receiving opioid medications while "addiction," or continued use despite negative consequences, can occur in up to 12% (Vowles et al., 2015). Opioid "abuse," or aberrant drug taking behavior often predictive of maladaptive opioid use has been reported in 46–81% of pain populations (Butler et al., 2004; Wilsey et al., 2008; Vowles et al., 2015). However, there remains a general consensus that high-quality research on this relationship is lacking (Ballantyne, 2015; Voon et al., 2017; Nadeau et al., 2021). Nevertheless, qualitative evidence from clinical literature indicates that negative outcomes associated with opioid use can be instigated by pain severity (Grol-Prokopczyk, 2017; Zajacova et al., 2021), duration of opioid use (Chung et al., 2019; Jantarada et al., 2021), escalating opioid doses (Zernig et al., 2007; Kaplovitch et al., 2015), comorbid anxiety and depression (Peciña et al., 2018; Emery and Akil, 2020; Rogers et al., 2020), discontinuation of opioid medications (Mark and Parish, 2019; Stein et al., 2021), and inherent risk factors like sex (Manubay et al., 2015; McHugh, 2020) or genetics (Kendler et al., 2003; Agarwal et al., 2017). Evidence from patients with pain and long-term opioid use have been critical in identifying potential risk factors for maladaptive opioid use but have yielded minimal impacts on either public health concern.

Determining the level of synergy between pain, long-term opioid use, and opioid misuse can be difficult for many reasons, but preclinical pain models of opioid self-administration provide a translational means to better understand how pain may provoke motivational shifts to alter opioid misuse liability. Although pain-induced dysfunction of mesolimbic reward pathways produces clear deficits in motivation for natural rewards (Massaly et al., 2019, 2021; Reiner et al., 2019), the effects of pain on opioid motivation are more complex. Evidence from self-administration studies suggest that the ability of pain to effect opioid self-administration is related to the chronicity of pain, selected opioid/dose, and the duration of daily opioid exposure. For example, chronic arthritic pain has biphasic effects on rates of oral fentanyl self-administration, that interestingly, follow the time-course of pain progression (Colpaert et al., 2001, 1982). Specifically, one week after the onset of pain there are no effects on fentanyl consumption but, during successive weeks, fentanyl intake dramatically increases—peaking at 2–3 weeks – and declines to baseline levels several weeks later. Importantly, the time course of fentanyl consumption rates parallels the time course of progressive pain sensitivity (Colpaert et al., 1982, 2001). Similarly, spinal cord injury has time-dependent effects on long-access morphine self-administration in rats. In this regard, pain reduces morphine intake 24 h after the induction of pain, then peaks at 14–21 days before normalizing 35–42 days later (Woller et al., 2014). These findings indicate that the persistence of pain is an important driver of opioid consumption. Adding further support to this, acute pain manipulations with capsaicin or lactic acid do not alter rates of fentanyl or heroin self-administration, but persistent inflammatory pain-induced reductions in fentanyl vs. food choice procedures match controls by one week after the induction of pain (Reiner et al., 2021). Notably, a small study found that arthritic pain reduced self-administration of relatively high doses of morphine with 24-h access for weeks following pain onset (Lyness et al., 1989) while another found that multiple forms of chronic pain attenuated oral fentanyl self-administration and discrimination in mice (Wade et al., 2013). These findings allude to the notion that pain may produce a shift opioid dose-response. Consistent with this, our lab found that inflammatory pain reduces heroin intake at low doses, but increases intake when doses are high (Hipólito et al., 2015). Our findings suggest that these effects are driven by deficits in VTA DA cell excitability (Hipólito et al., 2015) and this is exemplified by evidence showing that pain reduces the ability of low-dose opioids to facilitate VTA intracranial self-stimulation (Ewan and Martin, 2011). Spinal nerve ligation also produces a rightward shift in dose-response for multiple opioids, but the time-dependency of these effects has not been examined (Martin et al., 2007). Taken together, evidence from preclinical pain models of opioid abuse suggest that chronic pain can increase motivation for high opioid doses in a time-dependent manner that parallels the progression of pain. It will be important for

future studies to evaluate whether the time- and dose-dependent effects of pain on opioid consumption are related to time-dependent disruptions in mesolimbic pathway function.

Conclusions

Pain conditions, chronic opioid use, and withdrawal from chronic opioid use disrupt the endogenous opioid system function at spinal and supraspinal levels to negatively impact pain thresholds, opioid sensitivity, mood, and reward sensitivity. These physiological and behavioral alterations, particularly among opioid systems and mesolimbic reward pathways, may contribute to persistent use of opioid medications in an attempt to alleviate adverse physical and emotional states, thereby creating a susceptibility for opioid misuse. In addition, other mediating factors outside the scope of this review contribute to individual variabilities in pain perception and opioid sensitivity like sex differences (Huhn et al., 2018; Pisanu et al., 2019), genetic (Tremblay and Hamet, 2010; Mogil, 2012), and epigenetic mechanisms (Liang et al., 2015; Browne et al., 2019) and likely influence proclivity for opioid abuse in the context of pain. Neuroadaptive processes produced by pain conditions and long-term opioid use have compounding effects on negative outcomes, like the development of tolerance or opioid-induced hyperalgesia. An understanding of the synergy between these processes remains incomplete, but the ability to curb the opioid crisis and the prevalence of pain relies heavily on the ability to identify safer pharmacotherapeutic alternatives derived from a better comprehension of pain- and opioid-induced adaptations to opioid systems and functional neurocircuitry.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mu-opioid receptor and receptor tyrosine kinase crosstalk: Implications in mechanisms of opioid tolerance, reduced analgesia to neuropathic pain, dependence, and reward

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Despite the prevalence of opioid misuse, opioids remain the frontline treatment regimen for severe pain. However, opioid safety is hampered by side-effects such as analgesic tolerance, reduced analgesia to neuropathic pain, physical dependence, or reward. These side effects promote development of opioid use disorders and ultimately cause overdose deaths due to opioid-induced respiratory depression. The intertwined nature of signaling via μ-opioid receptors (MOR), the primary target of prescription opioids, with signaling pathways responsible for opioid side-effects presents important challenges. Therefore, a critical objective is to uncouple cellular and molecular mechanisms that selectively modulate analgesia from those that mediate side-effects. One such mechanism could be the transactivation of receptor tyrosine kinases (RTKs) via MOR. Notably, MOR-mediated side-effects can be uncoupled from analgesia signaling via targeting RTK family receptors, highlighting physiological relevance of MOR-RTKs crosstalk. This review focuses on the current state of knowledge surrounding the basic pharmacology of RTKs and bidirectional regulation of MOR signaling, as well as how MOR-RTK signaling may modulate undesirable effects of chronic opioid use, including opioid analgesic tolerance, reduced analgesia

to neuropathic pain, physical dependence, and reward. Further research is needed to better understand RTK-MOR transactivation signaling pathways, and to determine if RTKs are a plausible therapeutic target for mitigating opioid side effects.

KEYWORDS

mu-opioid receptor, opioid signaling, pain, tolerance, neuropathic pain, physical dependence, reward, receptor tyrosine kinase

Introduction

The opioid epidemic has reached unprecedented proportions globally. In the United States alone, overdoses caused by opioids have claimed the lives of over hundreds of thousands of people, with rates of lethal overdoses expected to double in the next five years (Holland et al., 2021; Pickard and Lee, 2021). Prescription opioids are major contributors to the current opioid crisis, despite serving as the mainstay treatment for severe and chronic pain. Safe use of opioids is hampered by potentially severe side-effects including respiratory depression and the development of dependence and addiction (Benyamin et al., 2008; Pattinson, 2008; Henry et al., 2015; Hayhurst and Durieux, 2016; Algera et al., 2019). Emergence of these side-effects is promoted by escalating doses of opioids in chronic pain patients to mitigate the development of analgesic tolerance (Collett, 1998; Benyamin et al., 2008; Henry et al., 2015). High opioid doses are also necessary in neuropathic pain patients to overcome the minimal analgesic efficacy of current opioid-based therapies (Przewlocki and Przewlocka, 2001; Balayssac et al., 2009; Donica et al., 2014; Puig et al., 2020b). Such chronically high opioid doses promote physical dependence, causing deleterious physiological symptoms upon opioid withdrawal (Azolosa et al., 1994; Epstein et al., 2006; Burma et al., 2017), and ultimately prevents the discontinuation of opioid treatment. As a result, patients are forced to choose between effective pain treatments and the risk of physical dependence and/or addiction. With high doses, patients also risk developing respiratory depression (decreased respiration), the main cause of overdoses death (Pattinson, 2008; Algera et al., 2019). Opioid addiction has resulted in severe social and steep economic costs of hundreds of billions of dollars annually (The Council of Economic Advisers, 2017) and spurred a growing effort on finding new strategies to treat pain effectively and safely. One focus is toward finding a safe and "ideal" analgesic drug that would be free of addiction potentiating side-effects and have a low lethality. Unfortunately, to date, no safer alternative with equal analgesic efficacy to opioids has been found (Stuart et al., 2018). Many other proposed strategies involve reducing opioid dosage by locally targeting injured tissue (and limit central penetration), or reducing opioid prescriptions including establishing multimodal pain treatment regimens (as opposed to opioid monotherapy), opioid prescription monitoring, and restricted prescribing guidelines (Saloner et al., 2018; Mir et al., 2019; Franz et al., 2021). Yet this has not been enough. Therefore, it is imperative to continue efforts toward preserving long-term opioid analgesia, while mitigating side-effects. To this end, a better understanding of the molecular mechanisms underlying opioid signaling is needed.

Opioid receptors currently characterized include μ-opioid receptor (MOR), κ-opioid receptor (KOR), δ-opioid receptor (DOR), and opioid receptor like-1 (ORL1). These opioid receptors (ORs) belong to the class A (rhodopsin family) family of G protein-coupled receptors (GPCRs) which are coupled to inhibitory $G\alpha_{i/o}$ G proteins. These GPCRs function to reduce neuronal excitability primarily by increasing potassium conductance and inhibiting voltage-gated calcium channels (Al-Hasani and Bruchas, 2011). Prescription opioids specifically modulate analgesia through MOR (Matthes et al., 1996; Loh et al., 1998), which is concentrated in structures essential for conductance of pain-related signaling including peripheral sensory neurons, spinal cord, brainstem and central brain nuclei (Mansour et al., 1994a,b, 1995a,b; Basbaum et al., 2009; Scherrer et al., 2009). Activation of MOR expressed on pain processing neurons via endogenous (e.g., endorphin) or exogenous (e.g., morphine or fentanyl) opioids directly inhibits these cells' activity and controls analgesia (Al-Hasani and Bruchas, 2011).

Mechanisms of opioid analgesic tolerance and side-effects are still poorly understood (Adhikary and Williams, 2022). Traditionally, tolerance was thought to occur via the direct modulation of MOR signaling and trafficking (Williams et al., 2013). More recent evidence suggests that MOR-mediated side-effects can be uncoupled from analgesia, suggesting distinct signaling pathways for opioid-induced side effects versus analgesia (Puig and Gutstein, 2017; Paul et al., 2021). Separable pathways suggests that specific therapeutic strategies can be developed to selectively target side-effects without altering analgesia. This is further complicated by the fact that, apart from observational clinical studies, in contrast to animal experiments, practically no rigorously controlled clinical trials have unequivocally demonstrated pharmacodynamic tolerance to opioids in human patients

(Collett, 1998; Henry et al., 2015), hampering the clinical translatability of earlier preclinical models.

Though the precise mechanisms for the above opioid-related signaling pathways remain to be determined, important clues have emerged which involve the receptor tyrosine kinase (RTK) family (Wang et al., 2012). More specifically, RTK signaling selectively regulates analgesic tolerance to MOR selective agonists (Puig et al., 2020a,b). Emerging evidence also suggests that RTKs could be involved in reduced opioid analgesia against neuropathic pain (Donica et al., 2014; Puig et al., 2020b), physical dependence (Rezamohammadi et al., 2020; Dorval et al., 2022), and reward (Koo et al., 2014; Fetterly et al., 2021). Together, these studies suggest that targeting opioid side-effects with RTK inhibitors could constitute a promising strategy to improve opioid safety. This review summarizes current knowledge about signaling interactions and crosstalk between MORs and RTKs. Furthermore, we discuss the implications of these mechanisms in opioid-mediated side-effects, with a focus on tolerance, reduced neuropathic pain analgesia, physical dependence, and reward. Finally, we discuss the potential clinical use of RTK inhibitors. Though RTK inhibitors are FDA-approved cancer chemotherapy drugs (Karaman et al., 2008; Gialeli et al., 2014; Roskoski, 2018), we present the possibility that these medications can be repurposed as a novel therapy for chronic pain and to improve opioid safety.

Overview of mu-opioid receptor signaling

Brief overview of mu opioid receptor signaling transduction pathways

As a canonical GPCR, MOR recruits $G\alpha_{i/o}$ G proteins upon stimulation. These inhibitory G proteins are composed of a monomeric $\alpha_{i/o}$ subunit and a dimeric $G_{\beta\gamma}$ complex and are characterized by their sensitivity to pertussis toxin (Connor and Christie, 1999). At rest, the G proteins exist as an inactive $G_{\alpha/\beta\gamma}$ heterotrimeric complex that is GDP-bound. However, upon receptor activation by opioid ligands, changes in receptor conformation lead to the dissociation of G_{α} and $G_{\beta\gamma}$ subunits via GDP/GTP exchange, which triggers intracellular signaling through downstream signaling effectors (**Figure 1A**). Canonical signaling pathways of $G\alpha_{i/o}$ include inhibition of adenylyl cyclase (AC), the enzyme responsible for production of cyclic adenosine monophosphate (cAMP)—a critical second messenger of ORs. The resulting decrease in intracellular cAMP diminishes activity of protein kinase A (PKA) and PKA-dependent processes including activation of the C-AMP Response Element-binding protein (CREB) transcription factor. $G\alpha_{i/o}$ signaling also positively regulates the activity of G protein-gated inwardly rectifying potassium (GIRK) channels, causing cellular hyperpolarization (Navarro et al., 1996). In parallel, $G_{\beta\gamma}$ negatively regulates Ca^{2+} currents via inhibition of P/Q-type, N-type, or L-type Ca^{2+} channels, further contributing to overall inhibition of cellular activity (for review see: (Al-Hasani and Bruchas, 2011; Williams et al., 2013)). To illustrate, in pain circuitry, release of $G_{\beta\gamma}$ subunits in presynaptic neurons results in inhibition of N-type Ca^{2+} channels for negatively modulating neurotransmitter release, while $G_{\beta\gamma}$ subunits in postsynaptic neurons activate GIRKs, preventing neuronal depolarization (Chieng and Christie, 1994; Zamponi et al., 1997). Together, these mechanisms activated by MOR agonists result in analgesia via modulation of neuronal transmission in circuits conveying nociception. Following G protein signal transduction, G protein receptor kinases (GRKs) are recruited for phosphorylation of MOR on 11 potential phosphorylation sites present on the carboxyl terminal domain of the receptor, including serine (S), threonine (T), and tyrosine (Y) residues (Doll et al., 2011; Lau et al., 2011). Several GRKs (e.g., GRK2, GRK3, GRK5, GRK6) selectively phosphorylate different MOR phosphorylation sites, modulating signal transduction in a ligand and context-dependent manner (Lemel et al., 2020). Of note, other kinases, such as protein kinase C (PKC) or calcium/calmodulin-dependent protein kinase II (CaMKII), also phosphorylate MOR on selective phosphorylation sites in a ligand-dependent manner (Kelly et al., 2008). Additionally, MOR phosphorylation initiates receptor desensitization via receptor recruitment of β-arrestin2 (**Figure 1B**; Whistler and Von Zastrow, 1998; Martini and Whistler, 2007). This activates clathrin-mediated endocytosis of the MOR-β-arrestin2 complex, resulting in MOR internalization and recycling which terminates receptor signaling at the plasma membrane. The MOR-β-arrestin2 complex also recruits specific transduction signal proteins including kinases such as src, phosphoinositide 3-kinases (PI3K), or Mitogen-Activated Protein Kinases (MAPK), including extracellular signal-regulated kinases 1 and 2 (ERK 1 and 2), or c-Jun N- terminal Kinases (JNK) 1–3 (Pierce et al., 2001) (for full review of pathways see Williams et al., 2013; Jean-Charles et al., 2017). Finally, MOR signaling can be terminated by degradation via ubiquitination pathways (Chaturvedi et al., 2001; Petäjä-Repo et al., 2001).

Proposed mechanisms of opioid-mediated side-effects

Mu-opioid receptor signaling is essential for opioids to induce analgesia and their side-effects, as global deletion of the gene encoding MOR (*Oprm1*) completely blocks opioid analgesia, reward, and physical dependence in rodents (Matthes et al., 1996; Loh et al., 1998). Indeed, most signaling pathways downstream of MOR are critical for the development and maintenance of opioid side-effects (Al-Hasani and Bruchas, 2011; Williams et al., 2013; Allouche et al., 2014; Zhou et al., 2021). Historically, mechanisms explaining side-effects

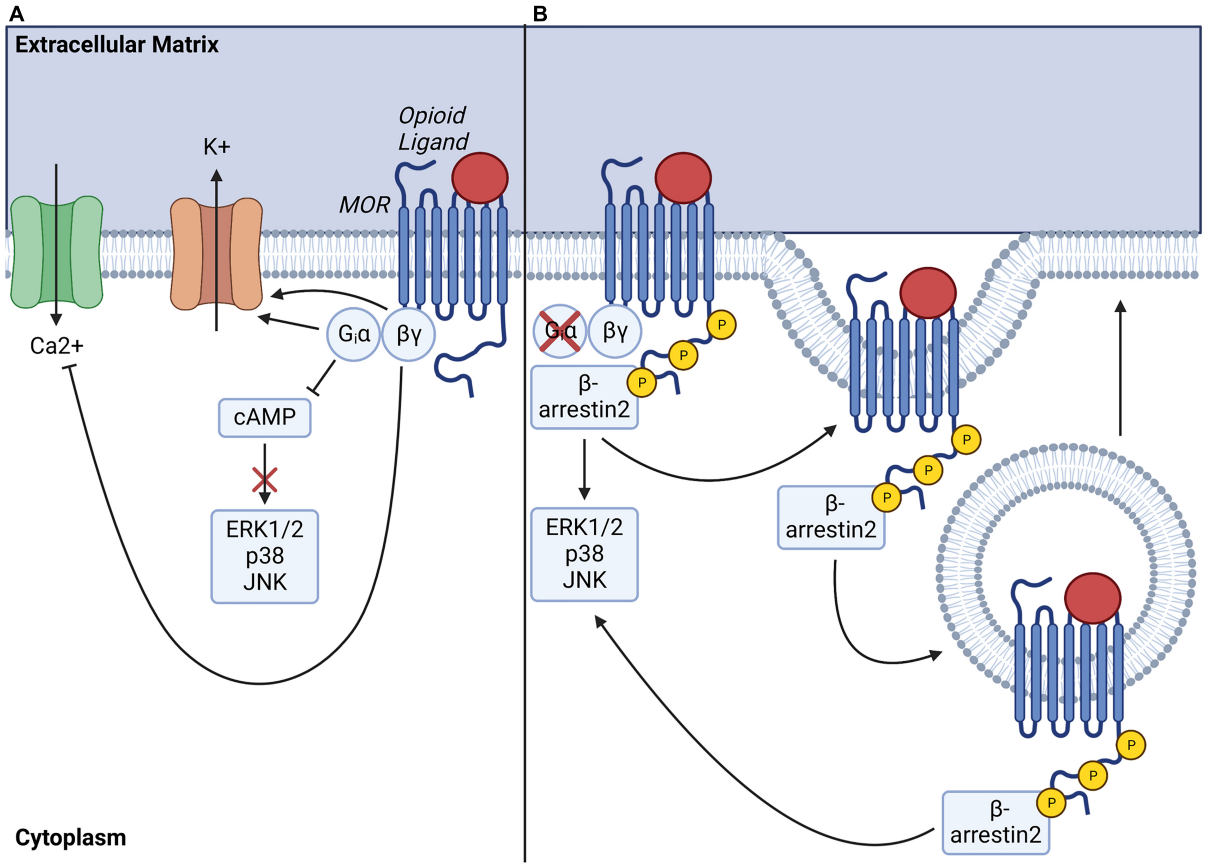

FIGURE 1
Mu-opioid receptor (MOR) signaling transduction pathways, internalization, and recycling. **(A)** Ligand-activation of MOR activates $G\alpha_{i/o}$-induced inhibition of adenylate cyclase, resulting in decreased intracellular cAMP levels and depleted downstream signaling. $G\alpha_{i/o}$ also serves to activate G protein gated inwardly rectifying potassium channels, leading to efflux of potassium ions while $\beta\gamma$ heterodimers simultaneously perturb calcium influx by inhibiting voltage gated calcium channels, overall inhibiting intracellular signaling and cellular activity. **(B)** Ligand-activation of MOR also eventually leads to phosphorylation of MOR c-terminal tail by G protein receptor kinases (GRKs), which enables docking of β-arrestin2 and initiates MOR endocytosis for further receptor degradation or recycling. Note that recruitment of β-arrestin2 can also drive activation of downstream signaling effectors, including ERK, p38 or JNK pathways.

of morphine were generalized to all MOR ligands, however, it has been difficult to find a single unifying mechanism that could explain side-effect profiles shared by all MOR agonists (Raehal and Bohn, 2011; Raehal et al., 2011; Whistler, 2012). This is likely since MOR ligands differ in their potencies, pharmacokinetics, and receptor internalization. Such drug-specific differences may also lead to varying recruitment of signaling effectors and pathways (Duttaroy and Yoburn, 1995; Keith et al., 1996, 1998; Trafton et al., 2000; Bohn et al., 2004; Kenakin, 2011; Posa et al., 2016; Schmid et al., 2017). Relatedly, different MOR ligands can stabilize the receptor in distinct conformations unique to each drug. As a result, different ligands can preferentially activate distinct signaling cascades that are biased toward either G protein versus β-arrestin2 pathways (Alvarez et al., 2002; Kenakin, 2011). Biased signaling downstream of MOR was proposed to drive the distinction between opioid side-effects and analgesia. In such a model, β-arrestin2 signaling preferentially mediates opioid-induced side effects while G protein signaling preferentially mediates the analgesic properties of these drugs (Bohn et al., 1999, 2000, 2002, 2003, 2004; Raehal and Bohn, 2011; Schmid et al., 2017). Consequently, much research has focused on identifying opioid ligands with higher intrinsic efficacy for stimulating G protein signaling downstream of MOR, while not triggering activation of the β-arrestin2 pathway (Soergel et al., 2014; Manglik et al., 2016). Although several G protein-biased compounds provide efficacious analgesia (Singla et al., 2019; Viscusi et al., 2019), adverse effects remain (Hill et al., 2018; Conibear and Kelly, 2019). Additionally, despite different signaling bias, all prescription opioids cause side-effects such as tolerance. G protein-biased MOR ligands thus cannot fully explain the mechanisms responsible for analgesia versus side-effects (Gillis et al., 2020).

Ultimately, a major thrust of opioid research is to uncouple the signaling mechanisms that selectively regulate analgesia from mechanisms that regulate undesired side-effects. New approaches proposing mechanisms that may not involve traditional canonical MOR signaling pathways may be key in addressing this issue. We propose that RTK signaling may be a common signaling pathway recruited downstream of MOR by all opioid agonists beyond their signaling bias. Here, we will present evidence suggesting that RTK signaling selectively modulates opioid side-effects but not analgesia. Therefore, we hypothesize that targeting RTKs offers a novel strategy to prevent and/or treat opioid side-effects without altering analgesia a critical objective for the field.

Overview of receptor tyrosine kinase signaling

Receptor tyrosine kinases are a subclass of tyrosine kinases expressed at the cell surface which respond with high affinity to selective soluble polypeptide growth factors, cytokines, and hormones. RTKs constitute 20 sub-families (Robinson et al., 2000), including the ErbB family comprising the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor family (PDGFR), vascular-endothelial growth factor receptor family (VEGFR), tropomyosin receptor family (Trk), fibroblast growth factor receptor family (FGFR), ephrin receptor family (EphR), and insulin receptor family (IR) (**Table 1**). Structurally, RTKs are composed of single transmembrane glycoproteins, with the N-terminal extracellular domain containing the ligand-binding sequence, and the C-terminal intracellular domain containing multiple tyrosine residues which form the protein kinase catalytic core of these receptors (Du and Lovly, 2018; **Figure 2**). Ligand activation of RTKs elicits non-covalent oligomerization of monomeric RTKs and promotes formation of homo- or heterodimers. This process leads to *trans*-autophosphorylation (Honegger et al., 1989; Favelyukis et al., 2001) of key tyrosine residues on the interacting receptors. This activates downstream signaling via recruitment of selective docking proteins possessing Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains (Pawson, 2004); SH2 and PTB-domain-containing proteins include insulin receptor substrate-1 (IRS1), Grb2-associated binder (Gab1), and FGFR substrate 2 (FRS2α/FRS2β). These downstream proteins, lacking intrinsic kinase activity, serve as scaffolds to organize signaling complexes and trigger intracellular signaling cascades. Most docking proteins like Gab1 can be recruited by multiple RTKs. However, some are specific to a subset of receptors. For example, FRS2α and FRS2β are only involved in FGFR-, and Trk-mediated signaling (Schlessinger, 2000). This confers activation of specific signaling pathways by different subsets of RTKs and possibly enables signaling specificity. Pathways activated following docking protein recruitment include phospholipase Cγ (PLCγ), phosphoinositide 3-kinases

TABLE 1 Receptor tyrosine kinases identified to modulate opioid-mediated behaviors.

Receptor tyrosine kinase (RTK)	RTK-MOR crosstalk	Analgesic tolerance	Resistance of neuropathic pain to opioid analgesia	Opioid dependence	Opioid reward
Epidermal growth factor receptor (EGFR)	Belcheva et al., 2001; Belcheva et al., 2003; Belcheva et al., 2005; Miyatake et al., 2009; Zhao et al., 2013; Phamduong et al., 2014; Yang et al., 2021	Puig et al., 2020b	Martin et al., 2017; Puig et al., 2020b		
Fibroblast growth factor receptor (FGFR)		Fujita-Hamabe et al., 2011		Blackwood et al., 2019	
Platelet-derived growth factor receptor (PGFR)	Wang et al., 2012; Weber et al., 2013; Li et al., 2020	Wang et al., 2012; Puig and Gutstein, 2017; Puig et al., 2020a	Narita et al., 2005; Donica et al., 2014		
Insulin Receptor (IR)	Mclaughlin and Chavkin, 2001; Li et al., 2003				Li et al., 2003; Xu et al., 2012
Ephrin B	Liu et al., 2011	Liu et al., 2011	Han et al., 2008	Xia et al., 2014	
Tyrosine receptor kinase B (TrkB)				Peregud et al., 2016; Rezamohammadi et al., 2020	Freeman et al., 2003; Koo et al., 2012; Koo et al., 2014; Jorjani et al., 2021
Fms-like tyrosine kinase (FLT3)			Rivat et al., 2018		
Vascular endothelial growth factor receptor (VEGFR)		Lopez-Bellido et al., 2019			

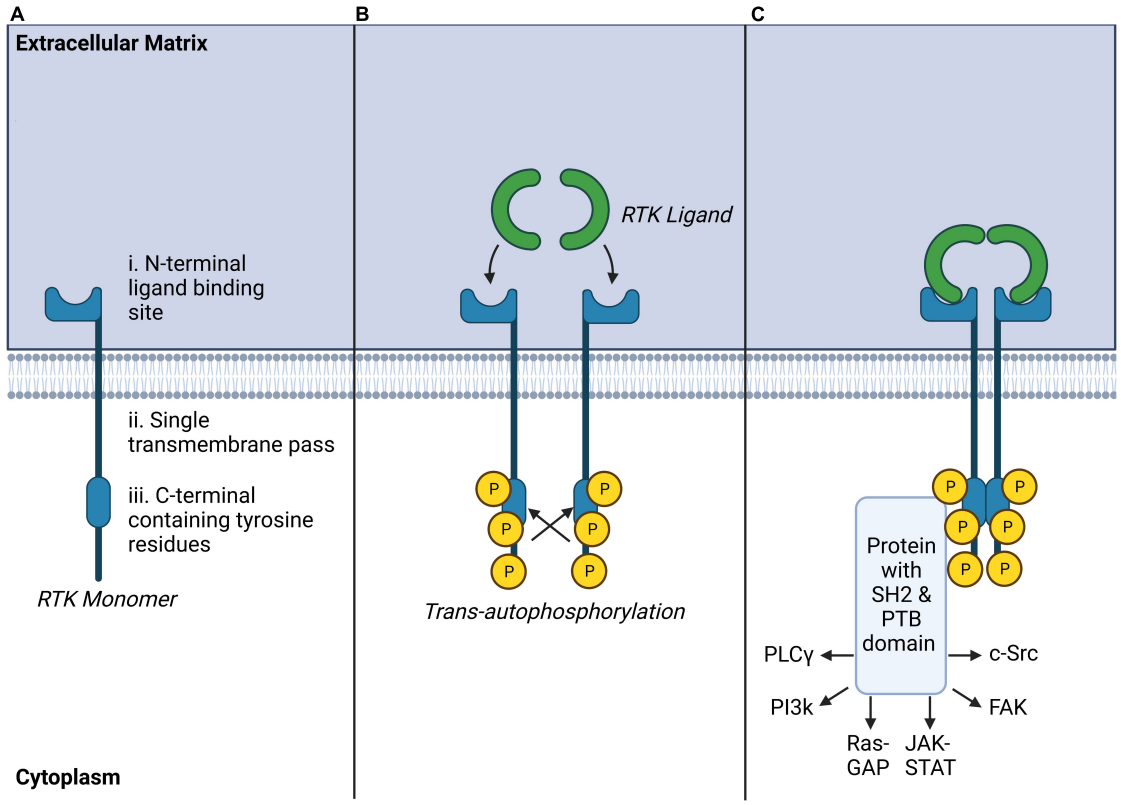

FIGURE 2
Receptor tyrosine kinase (RTK) structure, ligand binding and autophosphorylation, and common downstream signaling pathways. **(A)** RTK monomers are single transmembrane crossing peptides with extracellular ligand binding sites and tyrosine-rich intracellular effector regions. **(B)** RTK ligands bind as homo or heterodimers to RTKs inducing *trans*-autophosphorylation of opposing intracellular tyrosine residues. **(C)** Ligand-bound RTKs typically recruit protein complexes with SH2 and PTB domains which may activate a number of secondary intracellular messengers known to modulate other transmembrane receptors, intracellular signaling, or transcriptional regulation.

(PI3K), mitogen-activated protein kinase/p38 (MAPK/p38), Ras-GTPase-activating protein (Ras-GAP), Janus kinase/signal transducer and activator of transcription (JAK/STAT), proto-oncogene c-Src, or focal adhesion kinase (FAK) signaling cascades (for a review see: (Lemmon and Schlessinger, 2010; Du and Lovly, 2018).

Historically, RTK signaling pathways were found to be involved in cell proliferation, differentiation, migration, or metabolic changes (Lemmon and Schlessinger, 2010), and were also associated with cancer development (Du and Lovly, 2018). Most, if not all, RTK signaling effectors are also activated by opioid receptors. Numerous protein kinases including, ERK, JNK, p38, PKC, AKT, and CaMKII are utilized by both MOR and RTKs (Lemmon and Schlessinger, 2010; Williams et al., 2013). The ability of multiple receptors to concurrently activate signaling effectors raises the possibility of complex crosstalk between these receptors or even receptor cross-activation by the same molecule. Importantly, nearly all downstream pathways utilized by RTK receptors including MAP kinase cascades (Mckay and Morrison, 2007), PI3K (Haglund et al., 2007), PKC (Heckman and Wade, 2018), Akt (Choudhary et al., 2009), or ubiquitination (Haglund et al., 2003) play roles in opioid signaling in analgesia, tolerance, and dependence. It remains unclear, however, how these pathways pertain to opioid behaviors and side-effects (Mouledous et al., 2007; Chen et al., 2008a; Macey et al., 2009; Wang et al., 2009; Gregus et al., 2010). These discrepancies could be related to cellular context and, most importantly, they may involve modulation of signaling via differential engagement of RTK signaling in response to specific opioids.

Mu-opioid receptors-receptor tyrosine kinases crosstalk

General mechanisms of G protein-coupled receptors-receptor tyrosine kinases transactivation

Crosstalk between GPCRs and RTKs can amplify signaling pathways downstream of one or both receptors in a process

known as GPCR-RTK transactivation (Daub et al., 1996). This mechanism enables the integration of signal transduction between GPCRs and RTK signaling networks at signaling hubs shared between the respective receptor signaling pathways (Ragunathrao et al., 2019). Two major pathways of GPCR-RTK transactivation have been identified which involve either extracellular RTK ligand release (ligand-dependent) or intracellular recruitment of signaling effectors such as phosphotyrosine kinases (ligand-independent) (for review see Wetzker and Bohmer, 2003; **Figure 3**.

Ligand-dependent transactivation requires GPCR activation of matrix regulatory proteins such as membrane-bound matrix metalloproteinases (MMPs) or A Disintegrin and Metalloproteases (ADAMs) which contribute to the shedding of ligands. Several different MMPs or ADAMs are involved in the proteolytic ectodomain shedding of membrane bound RTK ligands from the extracellular matrix (ECM), which in turn, may transactivate several different RTKs (Cattaneo et al., 2014). This has been mostly described for EGFR as MMPs can cleave the heparin binding EGFR (Hb-EGF) to activate EGFR (Kilpatrick and Hill, 2021). Though the precise mechanisms of GPCR-mediated activation of MMPs or ADAMS are not fully understood, studies have implicated kinases such as c-src and PKC or calcium influx as activators of these proteases **Figure 3A**, for review, see Cattaneo et al. (2014). Notably, GPCR effectors like $G_{\beta\gamma}$ (Overland and Insel, 2015) and β-arrestin2 (Noma et al., 2007; Oligny-Longpré et al., 2012).

Ligand-independent transactivation pathways involve complex intracellular signaling cascades which recruit kinases like Src or PI3K (Di Liberto et al., 2019) to phosphorylate selective tyrosine residues on RTKs (**Figure 3B**). This mode of GPCR-RTK transactivation can also require association of the two receptors via protein complex formation (for review see Wetzker and Bohmer, 2003). Of importance, GPCR-RTK heterodimerization may completely change GPCR signal transduction mechanisms and even promote a switch in the associated G protein. This is of particular interest because MOR signals via pertussis-toxin-insensitive stimulatory $G\alpha_s$ proteins following chronic morphine exposure or neuropathic pain (Chakrabarti et al., 2005, 2010; Chakrabarti and Gintzler, 2007; Tsai et al., 2009). Therefore, involvement of RTKs in G protein switching downstream of MOR is a possibility that remains to be investigated.

Other ligand-independent transactivation involves atypical mechanisms of GPCR-RTK crosstalk via reactive oxygen species (ROS), such as nitric oxide (NO) (**Figure 3C**). ROS production by GPCRs could block protein-tyrosine phosphatases, activate phosphotyrosine kinases and modulate phosphorylation of RTK tyrosine residues (Cattaneo et al., 2014). Such a mechanism may be particularly relevant to opioid actions since ROS modulate MOR-mediated behaviors in rodents (Doyle et al., 2013). This therefore raises the possibility that RTKs could be involved in these ROS-mediated signaling pathways.

Mu-opioid receptors-receptor tyrosine kinases transactivation *in vitro*

Most *in vitro* studies investigating RTK transactivation by MORs have focused on EGFR or PDGFRβ. In immortalized cell lines transfected with MOR, acute treatment with selective MOR agonists such as [D-Ala(2),MePhe(4),Gly-ol(5)]enkephalin (DAMGO) or morphine resulted in transactivation of EGFR (Belcheva et al., 2001, 2003, 2005; Phamduong et al., 2014) or PDGFRβ (Weber et al., 2013) as shown by phosphorylation of these RTKs. Interestingly, transactivation of RTKs by MOR activates downstream effector signaling at levels comparable to activation to direct activation of the RTKs themselves (Belcheva et al., 2001; Weber et al., 2013; Phamduong et al., 2014), and MOR-RTK transactivation can be abolished by pre-treatment with selective RTK inhibitors (Belcheva et al., 2001; Chen et al., 2008b; Weber et al., 2013). In cultured cells, mechanisms of MOR-EGFR and MOR-PDGFRβ transactivation were shown to require release of EGF (Belcheva et al., 2001, 2003, 2005; Phamduong et al., 2014) or of PDGF-B (Wang et al., 2012; Weber et al., 2013), respectively. Consistent with mechanisms of ligand-dependent GPCR-RTK transactivation, MOR-EGFR and MOR-PDGFRβ transactivation also require MMP activity (Belcheva et al., 2001). MMP activation by MOR may involve calmodulin (CaM), a Ca^{2+} sensor and binding protein. In resting conditions, CaM prevents MMP activity at the plasma membrane (PM) in HEK293 cells (Belcheva et al., 2001). Acute treatment with MOR agonist DAMGO promotes CaM translocation from the plasma membrane (PM) to MOR intracellular domains, lifting CaM inhibition on MMP via mechanisms involving activation of phospholipase C (PLC) and PKCε signaling (Belcheva et al., 2001, 2005; Miyatake et al., 2009; **Figure 4A**).

Other signaling effectors of MOR are involved in MOR-RTK transactivation. MOR-EGFR transactivation requires both $G\alpha_{i/o}$ and β-arrestin2. Indeed, opioid-mediated EGFR phosphorylation can be attenuated via pertussis toxin or by siRNA-mediated β-arrestin2 silencing in cultured rat astrocytes (Miyatake et al., 2009). In addition to canonical MOR transduction pathways, other common signaling effectors between GPCRs and RTKs can take part in MOR-RTK transactivation. PI3K inhibitors abolish EGFR activation by DAMGO-activated MORs in cultured rat astrocytes, suggesting involvement of this kinase in MOR-RTK transactivation (Belcheva et al., 2005). Similarly, JNK inhibitors block MOR-PDGFRβ transactivation in rat spinal neurons (Li et al., 2020). Together, these studies indicate that MOR-RTK transactivation likely involves a complex network of converging signaling pathways (**Figure 4A**). It is important to note that most studies of mechanisms of MOR-RTK transactivation have employed acute MOR agonist treatments. However, longer MOR agonism may have different effects on RTK activity (Belcheva et al., 2003; Miyatake et al., 2009). Over hours, longer term treatment

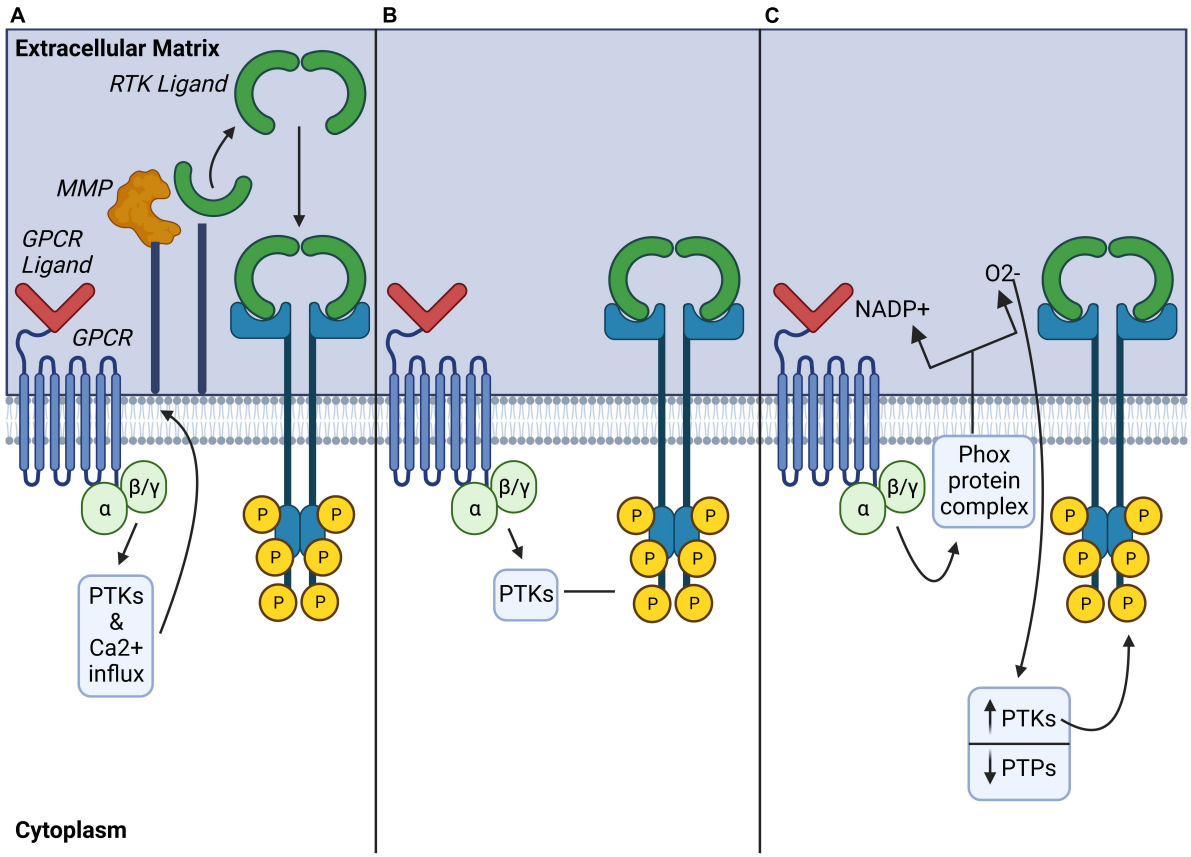

FIGURE 3
Ligand-dependent, ligand-independent, and atypical mechanisms of GPCR modulation of RTKs. **(A)** Ligand-dependent transactivation: Activated GPCRs induce a variety of downstream signaling pathways including activation of phospho-tyrosine kinases (PTKs), or increase of the influx of Ca^{2+}, which activates matrix metalloproteinases (MMP) to cleave cell membrane-bound RTK ligands. **(B)** Ligand-independent transactivation: Activated GPCRs may also recruit intracellular PTKs to directly phosphorylate tyrosine residues on the intracellular domain of RTKs and induce their activation in a ligand-independent manner. **(C)** Atypical transactivation: Phox protein complexes activated by GPCRs generate reactive oxygen species which modulate phospho-tyrosine kinases (PTK) and phosphotyrosine phosphatases (PTP) activity to promote phosphorylation of intracellular RTK tyrosine residues. GPCR, G protein coupled receptor; MMP, matrix metalloproteinase; PTK, phosphotyrosine-kinase; PTP, phosphotyrosine phosphatases.

with MOR agonists DAMGO, enkephalin, or morphine induces EGFR phosphorylation as well as both downregulation and decreased ERK phosphorylation. These mechanisms are β-arrestin2- and $G\alpha_{i/o}$-dependent and not observed with acute opioid treatments on the order of minutes, suggesting that acute versus longer-term events cause temporally distinct effects on signaling (Belcheva et al., 2003; Miyatake et al., 2009). Because most opioid-mediated side-effects occur after long-term opioid treatment, further studies to understand the specific alterations of MOR-RTK transactivation mechanisms by long-term opioid MOR stimulation are still needed.

Intriguingly, mechanisms of MOR-EGFR transactivation identified *in vitro* in immortalized cell lines do not differ between opioids with different ability to internalize MOR (Belcheva et al., 2001). Belcheva and colleagues (Belcheva et al., 2001) found that EGFR was phosphorylated by MOR whether it was activated by morphine (low internalizing (Sternini et al., 1996), DAMGO (highly internalizing synthetic opioid peptide (Keith et al., 1998) or endomorphin (highly internalizing endogenous opioid peptide (Mcconalogue et al., 1999). In addition, mechanisms of MOR-EGFR transactivation by these agonists all required similar mechanisms of CaM recruitment and PKC signaling, although they had been characterized as opioids with different signaling bias toward G protein and β-arrestin recruitment (Schmid et al., 2017). Together this implies that RTK transactivation mechanisms may be independent from MOR-ligands bias.

Receptor tyrosine kinases transactivation of mu-opioid receptors

In addition to modulation of RTK signaling by GPCRs, RTKs can also modulate GPCR-mediated signaling, suggesting

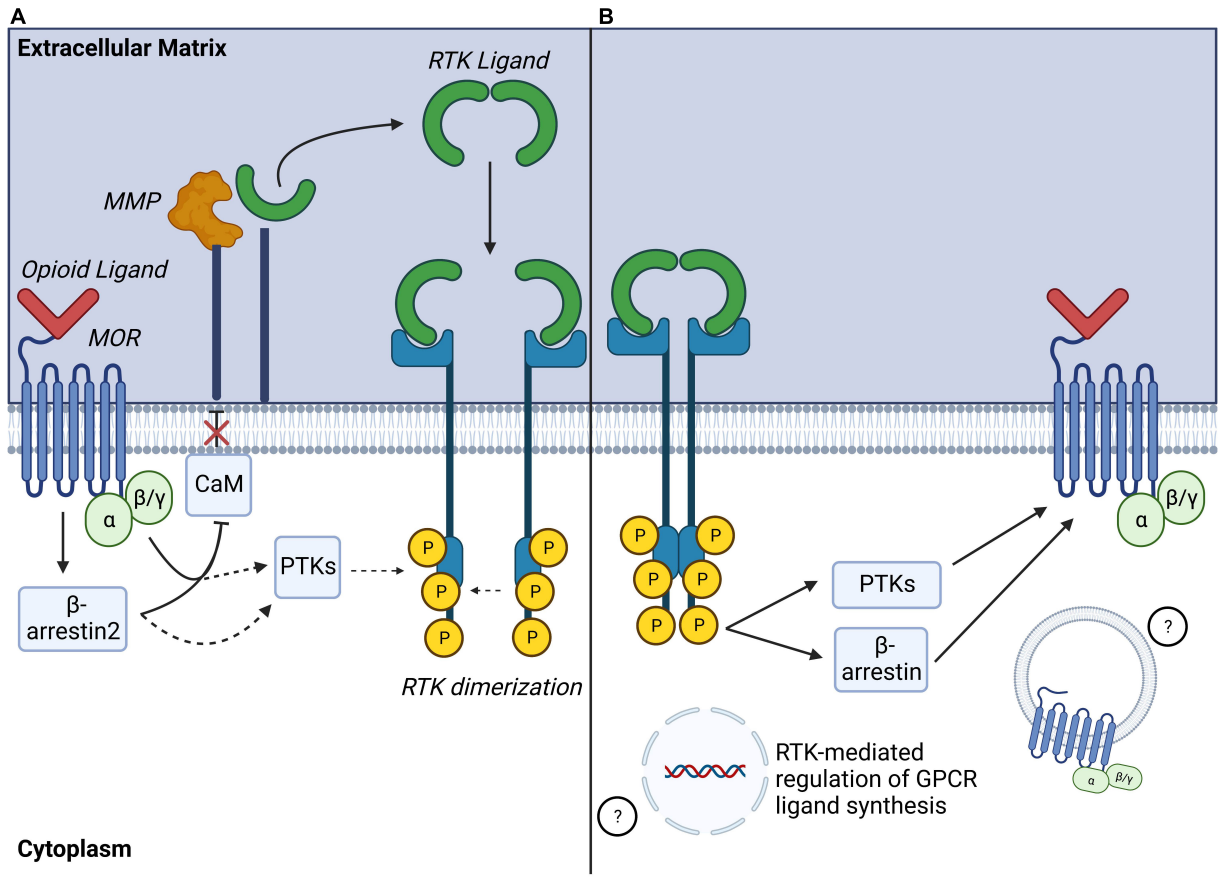

FIGURE 4
Identified mechanisms of MOR-RTK crosstalk. **(A)** Identified mechanisms of MOR-RTK transactivation: Activated MOR can induce MMP activation via mechanisms including disinhibition Calmodulin (CaM), leading to ligand shedding and ligand-dependent RTK activation. Other ligand-independent mechanisms may involve recruitment of intracellular phosphotyrosine kinases (PTKs) to phosphorylate RTK tyrosine residues. G protein and β-arrestin2 signaling may also be involved in MOR-RTK transactivation. **(B)** Identified mechanisms of RTK modulation of MOR signaling: Phosphorylated RTK may modulate activation of GPCR via a recruitment of PTKs, or β-arrestins activity. RTK activation may also lead to altered GPCR ligand gene expression or MOR internalization. MOR, mu-opioid receptor; MMP, matrix metalloproteinase; RTK, receptor tyrosine kinase; GPCR, G protein coupled receptor.

that the relationship between GPCRs and RTKs is reciprocal (Delcourt et al., 2007; **Figure 4B**). General mechanisms of GPCR transactivation by RTKs or "GPCR highjacking" (Delcourt et al., 2007) can involve recruitment of GPCR signaling effectors like GRKs (García-Sáinz et al., 2010; Sun et al., 2018), β-arrestins (Dalle et al., 2001; Povsic et al., 2003; Hupfeld and Olefsky, 2007) or activation of RTK downstream kinases including PI3K (Molina-Munþoz et al., 2006), Akt, or c-Src (Baltensperger et al., 1996; Doronin et al., 2002; Gavi et al., 2007). These mechanisms either require physical interactions between GPCRs and RTKs or transcriptional regulation of GPCR ligand synthesis (Delcourt et al., 2007). Relevant to this review, accumulating studies show that RTK signaling influences MOR signal transduction by modulation of phosphorylation. In cultured *Xenopus laevi* oocytes co-transfected with MOR and the insulin receptor (IR), pretreatment with insulin potentiated DAMGO-activated GIRK inward currents via MAPK signaling and possible dephosphorylation of MOR tyrosine residues, Y-106, or Y-166. Thus, indirectly demonstrating that IR signaling modulates MOR-signaling efficacy (Mclaughlin and Chavkin, 2001). In contrast, concomitant activation of MOR by DAMGO and activation of EGFR by EGF in HEK293 cells promotes MOR phosphorylation on Y-166 in a src-dependent manner, resulting in negative regulation of MOR-G protein coupling (Clayton et al., 2010). This suggests that regulation of MOR phosphorylation by opioids may be modulated by RTK-dependent activity. In separate studies, EGFR activation by EGF caused recruitment and translocation of G-coupled protein receptor kinase 2 (GRK-2) to the plasma membrane where it phosphorylated MOR on Serine-residues 363 and 375 (S-363, S-375), and Threonine-residue-370 (T-370), and enabled DAMGO-mediated MOR internalization (Chen et al., 2008b).

Together, these studies highlight that RTKs can modulate MOR phosphorylation, signaling and internalization.

Involvement of receptor tyrosine kinase signaling opioid-mediated behaviors

While much of the literature has focused on MOR-RTK transactivation *in vitro*, this phenomenon is also relevant physiologically *in vivo*, particularly in the development and maintenance of deleterious opioid side-effects caused by MOR agonists. Reviewed here is evidence that several RTKs (**Table 1**) play major roles in mediating opioid side-effects such as analgesic tolerance, resistance of neuropathic pain to opioid analgesia, physical dependence or reward.

Receptor tyrosine kinase signaling and opioid analgesic tolerance

In the clinic, opioid analgesic tolerance is defined by a gradual loss of analgesic efficacy to a fixed dose of an opioid. As a result, escalation of opioid doses occurs over time to maintain analgesic benefit (Henry et al., 2015; Hayhurst and Durieux, 2016). MOR signaling is essential in the mechanisms of tolerance (Williams et al., 2013; Adhikary and Williams, 2022) and MOR is expressed in structures strongly implicated in tolerance and pain mechanisms including dorsal root ganglia (DRG) neurons and neurons of the spinal cord substantia gelatinosa (Mansour et al., 1988, 1995a; Scherrer et al., 2009; Corder et al., 2017; Puig and Gutstein, 2017). RTKs are similarly expressed alongside MOR in the spinal cord and DRG, including PDGFRβ (Sasahara et al., 1991; Eccleston et al., 1993), EGFR (Werner et al., 1988; Huerta et al., 1996), VEGFR2 (Spliet et al., 2004; Herrera et al., 2009), or Ephrin type-B receptor 1 (EphRB1) (Liu et al., 2011). Putative roles for RTK signaling in opioid tolerance were first shown in mice with global deletion of EphRB1 (Liu et al., 2011) as they failed to develop tolerance to spinal morphine administration. Similarly, we and others found that systemic or intrathecal co-administration of morphine alongside RTK inhibition via inhibitors of PDGFRβ (Wang et al., 2012; Li et al., 2020; Puig et al., 2020a), EGFR (Puig et al., 2020b), or VEGFR-2 (Lopez-Bellido et al., 2019) completely blocked tolerance. Together, these studies show that spinal RTK signaling is essential in morphine tolerance development. In addition, supraspinal inhibition of the RTK, FGFR, via intracerebroventricular (i.c.v.) injection also blocks tolerance to morphine injected subcutaneously (Fujita-Hamabe et al., 2011). Thus, other supraspinal structures of the pain circuitry may additionally contribute to RTK-mediated tolerance behaviors.

Precluding spinal signaling from one RTK at a time is sufficient to fully ablate tolerance. This apparent signaling redundancy raises the possibilities that: (1) spinal RTKs may work in parallel to transduce complex signaling cascades that specifically mediate tolerance and (2) that all signaling cascades recruited by RTKs are essential for tolerance. Interestingly, RTKs including EGFR and PDGFR-β were shown to heterodimerize *in vitro* (Habib et al., 1998; Saito et al., 2001). Heterodimerization could also happen *in vivo,* and co-transactivation of several spinal RTKs by MOR may be involved in mechanisms of tolerance. However, inhibition of RTKs individually alters tolerance in different ways depending on the RTK. For example, PDGFRβ inhibition only masks the expression of morphine tolerance (Wang et al., 2012), while EGFR inhibition completely blocks its development (Puig et al., 2020b). Importantly, these results have been reproduced by several independently conducted studies, highlighting the robustness of these findings (Wang et al., 2012; Li et al., 2020; Puig et al., 2020a).

In addition, we found that PDGFRβ inhibition blocks tolerance to several opioid analgesics used in the clinic including fentanyl, sufentanil, hydromorphone, and oxycodone (Puig et al., 2020a). Interestingly, these opioids have profoundly different pharmacokinetic and pharmacodynamic properties and have different signaling bias (Keith et al., 1998; Schmid et al., 2017). These findings show functional dissociation between MOR endocytosis, ligand signaling bias and tolerance, challenging the long-held hypotheses that mechanisms of MOR internalization (Whistler et al., 1999; Finn and Whistler, 2001) or of recruitment of β-arrestin2 (Bohn et al., 2000, 2004) are at the core of tolerance signaling. Instead, it suggests that PDGFRβ signaling could be a core mediator of opioid analgesic tolerance (Puig et al., 2020a). This is further supported by the fact that tolerance occurs independently of opioid-induced MOR internalization, and PDGFRβ inhibition does not modify levels of internalization while preventing tolerance (Puig et al., 2020a).

The precise RTK signaling pathways activated by opioid-stimulated MOR that mediate tolerance remain completely unknown. However, a recent *in vivo* study suggested that they could involve JNK signaling downstream of PDGFRβ (Li et al., 2020). Mechanisms of MOR-RTK transactivation in the spinal cord to mediate tolerance are similarly unclear. However, they seem to involve RTK ligand-dependent signaling pathways (Liu et al., 2011; Wang et al., 2012). Therefore, MOR may recruit RTK signaling in either an autocrine or a paracrine manner and RTKs may not necessarily need to be co-expressed with MOR. In addition, RTKs that have been involved in tolerance are closely phylogenetically related (Brunet et al., 2016). Indeed, VEGFR-2 and PDGFRβ share

a direct ancestor gene, the RTK PDGF/VEGF receptor (Pvr) (Lopez-Bellido et al., 2019). This implies that involvement of RTKs in opioid tolerance could be a phylogenetically conserved function.

Receptor tyrosine kinase signaling and reduced opioid analgesia to neuropathic pain

Neuropathic pain results from lesions or diseases of the somatosensory system that lead to a combination of inflammation and nerve compression (Dworkin et al., 2003, 2010). NP can also result from nerve damage as a consequence of prolonged chemotherapy (Murnion, 2018; Finnerup et al., 2021). Due to a lack of better alternatives, opioids are commonly used for NP (Quasthoff and Hartung, 2002; Chong and Bajwa, 2003; Lynch et al., 2004; Balayssac et al., 2009). Nevertheless, opioids are not very effective in treating NP as several clinical studies have shown that, despite high opioid dosage, NP could not be alleviated following opioid administration (Chaparro et al., 2012; Cooper et al., 2017). Although combination therapy between opioids and gabapentin, a blocker of voltage-gated calcium channels, proved effective, it has been associated with severely debilitating side-effects including nausea, constipation, and vomiting (Chaparro et al., 2012). Therefore, new therapeutic strategies and targets are needed for ameliorating NP.

NP can be modeled in rodents by inducing nerve injury on spinal nerves via spinal nerve ligation (SNL) (Kim and Chung, 1992) or chronic contraction injury (CCI) of the sciatic nerve (Bennett and Xie, 1988). In these models, low doses of opioids fail to produce analgesia. Several studies have established the impact of signaling from different RTKs in neuropathic pain development and maintenance in rodents including signaling by PDGFRα (Narita et al., 2005), FLT3 (Rivat et al., 2018), EphRB1 (Han et al., 2008), EGFR (Martin et al., 2017), or TrkA (Ugolini et al., 2007). In addition, a clinical study demonstrated that targeted inhibition of EGFR significantly reduces pain in male and female NP patients (Kersten et al., 2019). Groundbreaking recent discoveries also established direct involvement of RTK signaling in NP resistance to opioid analgesia. Pharmacological inhibition of either PDGFRβ (Donica et al., 2014) or EGFR (Puig et al., 2020b) restores analgesia to a dose of morphine previously ineffective on mechanical allodynia caused by SNL. This shows that PDGFRβ or EGFR inhibition is sufficient to restore morphine analgesic properties that were abolished by alterations caused by nerve injury. Importantly, administration of the same doses of PDGFRβ or EGFR inhibitors alone does not have any analgesic effect. In fact, we estimated that the dose of EGFR inhibitor used to restore morphine analgesia is ∼20-fold lower than the dose previously needed to induce analgesia (Nair and Jacob, 2016; Puig et al., 2020b). These results emphasize that RTK inhibitors restore morphine-mediated analgesia rather than causing analgesia by themselves. This indicates that recruitment of PDGFRβ and EGFR signaling by nerve injury activates signaling pathways that may block opioid analgesic signaling during NP. Moreover, these mechanisms resemble findings in the context of opioid tolerance (Wang et al., 2012; Puig et al., 2020a,b), and imply that convergent mechanisms between opioid tolerance and NP involve RTK signaling. Based on these observations, it was speculated that injured nerves release growth factors such as PDGF-B to activate PDGFRβ signaling and induce morphine-resistant states (Donica et al., 2014). It has also been proposed that this endogenous PDGF-B release by injured nerves, is similar to the release of PDGF-B in response to opioid administration, leading to activation of MOR to mediate tolerance (Wang et al., 2012). In conclusion, RTK signaling mediating opioid analgesic resistance may form a mechanistic link between neuropathic pain development and opioid tolerance (Mao et al., 1995; Mayer et al., 1999; Joseph et al., 2010; Donica et al., 2014; Puig et al., 2020b).

Receptor tyrosine kinase signaling and opioid dependence

There is a complex bidirectional relationship between RTK gene expression and opioid dependence. Dorval and colleagues showed that mice that overexpress FGF21, an FGFR ligand (FGF21-Tg mice, 50-fold overexpression), have a reduced preference to morphine in a conditioned place preference paradigm (Dorval et al., 2022). Further, naloxone-precipitated physical dependence behavior, (i.e., number of vertical jumps post-naloxone injection) is depressed in FGF21-Tg mice compared to wildtype littermates, suggesting that acute morphine physical dependence is regulated by FGF21 activity. Interestingly, morphine analgesia and tolerance development were not altered in FGF21-Tg mice, showing that FGF21 plays a role in opioid dependence but not in analgesia or tolerance. These findings are consistent with previous studies showing that oxycodone self-administration is associated with elevated striatal *fgf2*, *fgfr2*, and *fgfr3*mRNA levels during incubation of oxycodone seeking (Blackwood et al., 2019). Furthermore, these changes in FGF receptor gene expression are associated with elevated *c-fos* mRNA expression in the dorsal striatum, and elevated *junB* mRNA levels in these same regions. Given that the striatum is an important region of the reward circuitry, Blackwood and collaborators (Blackwood et al., 2019) hypothesized that incubation of oxycodone seeking, a behavior correlated with future dependence, is mediated at least in-part by FGF2-dependent signaling. However, the mechanisms of FGF receptor-driven opioid dependence remain unknown.

Other studies have also indicated that brain-derived neurotrophic factor (BDNF), as well as its receptor, tropomyosin

receptor kinase B (TrkB), may also play integral roles in opioid dependence and withdrawal development. BDNF, TrkB, IGF1, and IFG1R mRNA levels were found to be elevated in rodents frontal cortex in a model of physical dependence to morphine. In addition, BDNF was upregulated in hippocampus and midbrain (Peregud et al., 2016). Recruitment of BDNF-TrkB signaling by MOR during exposure to opioids or during withdrawal may be mediated via mechanisms of atypical GPCR-RTK transactivation involving a ROS, nitric oxide (NO) (as illustrated in **Figure 3C**). Withdrawal associated elevation of BDNF and TrkB and their respective receptors is markedly lower in animals pretreated with the nitric oxide synthase (NOS) inhibitor L-N^G-nitroarginine methyl ester (L-NAME). L-NAME-treated animals also exhibited depressed amounts of phosphorylated TrkB following abstinence from morphine (Peregud et al., 2016). Confirming the role of TrkB signaling in withdrawal behaviors, a recent study showed that rats pre-treated with ANA-12, a TrkB antagonist, displayed greater drug dependence and significantly more spontaneous withdrawal behaviors after a chronic treatment with morphine. Furthermore, BDNF levels in the cerebrospinal fluid (CSF) of ANA-12 treated animals are depressed during morphine dependence, and elevated during withdrawal (Rezamohammadi et al., 2020). Together these studies show that FGFR and TrkB activation may have protective effects against physical dependence, highlighting that this should be carefully considered in the process of testing RTK targeting therapies to treat opioid side-effects.

One hallmark of chronic opioid use which occurs upon opioid withdrawal is opioid-induced hyperalgesia (OIH). Of interest, Ephrin receptors, the most prominent subfamily of RTKs, which are commonly associated with neuron-neuron and neuron-glia interactions have been implicated in OIH. In a rat model of remifentanil-induced hyperalgesia, remifentanil-induced decrease of mechanical and thermal pain threshold has been correlated with elevated spinal Fos protein levels. Interestingly, these effects were reversed by inhibition of either EphB ligand (via EphB1-Fc) or the NMDA receptor (NMDAR) (via MK801) (Xia et al., 2014). Further, intrathecal injection of ephrinB/EphB agonist, was sufficient to induce significant hyperalgesia in a NMDAR-dependent manner (Xia et al., 2014), showing that activation of ephrinB/EphB pathways are sufficient to mediate OIH development via NMDAR (Xia et al., 2014). Importantly, other RTKs are also known to be involved in NMDAR-mediated OIH, including BDNF-TrkB signaling in the spinal dorsal horn. Notably, previous work demonstrated that morphine-induced hyperalgesia occurs because of MOR-dependent BDNF release leading to a downregulation of K^+/Cl^- co-transporter (KCC2) in rat spinal lamina neurons (Ferrini et al., 2013). The resulting Cl^- dysequilibrium serves as a driver of hyperalgesia which is reversible by inhibition of BDNF-TrkB *or* via prevention of KCC2 downregulation (Ferrini et al., 2017). Further, this reversible anion transport dysfunction induces a dampening of GABAergic and glycinergic spinal signaling and elevated NMDAR activity (Li et al., 2016).

Receptor tyrosine kinase signaling and opioid reward

RTK signaling and opioid reward involve midbrain dopamine neurons in the ventral tegmental area (VTA) which project to the nucleus accumbens (NAc) in the striatum. In the context of opioids, morphine promotes activation of striatal D_1 receptor (D1R)-expressing MSNs which increase reward behaviors and decreases dopamine D_2 receptor-expressing (D2R) MSNs which promote aversion. TrkB is expressed in both $D1R^+$ and $D2R^+$ MSNs (Freeman et al., 2003; Baydyuk et al., 2011) and most evidence about involvement of RTK signaling in opioid reward derives from work analyzing TrkB and morphine administration. Indeed, there is decreased conditioned place preference (CPP) for morphine when the selective TrkB antagonist, ANA-12, is injected into the NAc of rats (Jorjani et al., 2021). However, it was also shown that selective knockout of TrkB from $D1R^+$ MSNs of the NAc in mice, enhances morphine CPP while knockout of $D2R^+$ MSNs produces no change (Koo et al., 2014). Moreover, knocking out TrkB in the VTA produces a similar effect as TrkB knockout in $D1R^+$ MSNs in the NAc with enhanced morphine CPP (Koo et al., 2014). Overall, these findings suggest that TrkB-based RTK signaling in $D1R^+$ versus $D2R^+$ MSNs mediates opposing actions that together modulate opioid-induced behaviors and that these actions are dependent on striatal dopamine release from projections of midbrain dopaminergic neurons.

IR signaling has also been implicated in opioid reward. In the hippocampus and hypothalamus, morphine induces IR phosphorylation in wildtype, but not MOR knockout mice, suggesting that MORs are able to transactivate IRs in these structures (Li et al., 2003). Additionally, given the important role of glutamatergic neurotransmission in drug reward (Britt et al., 2012), the increases in presynaptic glutamate release in the NAc in response to IR activation (Fetterly et al., 2021) may provide a further RTK-mediated mechanism for opioid actions. In contrast, insulin growth factor like receptor (IGFR) activation decreases presynaptic glutamate release in the same neuronal population, demonstrating differential effects depending on the RTK. Additional involvement of IR in opioid reward is supported by work showing that prolonged morphine-activated MORs *in vitro* can cause desensitization of IR signaling to Akt and ERK cascades (Li et al., 2003), both of which have been implicated in reward (Shi et al., 2014; Zamora-Martinez and Edwards, 2014). Inhibition of ERK in the NAc shell prevents development of morphine CPP (Xu et al., 2012). While, in a separate study, Russo and colleagues showed that downregulation of Akt and IR subunit 2, an essential component

of functional IR signaling, in the VTA results in reward tolerance as shown by decreased CPP behaviors over time (Russo et al., 2007). Together, these results further reinforce the involvement of IRs in modulation of MOR-mediated reward signaling within the NAc. Further studies to establish if this could be generalized to other opioids remain necessary. For example, PDGFRβ is also expressed in brain regions involved in reward and addiction such as mPFC, NAc and dStr (Balayssac et al., 2009; Bor et al., 2017) and PDGFRβ levels were shown to be altered in the striatum and midbrain of rodents with disrupted dopaminergic signaling, a central component for reward signaling (Masuo et al., 2004). Overall, this work suggests RTKs could be promising, yet understudied, candidates to mitigate morphine reward, especially IRs.

Clinical implications

Treating human disease with RTK-targeted therapies is an established standard of care as a therapeutic strategy for cancer. Indeed, RTK inhibitors serve as the gold standard treatment of malignancies (Savage and Antman, 2002; Elisei et al., 2013; Sim et al., 2018). These medications are being explored for putative efficacy in other, non-oncological conditions. Specifically, EGFR and PDGFR inhibitors have recently received attention for their clinical therapeutic potential against pain. Several case reports have described analgesic effects by EGFR inhibitors in patients with severe pain (Kersten and Cameron, 2012; Kersten et al., 2015). Patients with either cancer pain (Moryl et al., 2006; Macey et al., 2009) or different types of neuropathic pain (Kersten et al., 2015) were treated with EGFR inhibitors which significantly improved their pain score after a few days. Most interestingly, in a clinical study led by Kersten and collaborators (Kersten et al., 2013), half the patients who experienced immediate pain relief following administration of the EGFR inhibitor cetuximab also decreased their required opioid doses. The authors concluded that cetuximab reversed opioid tolerance. Similarly, PDGFR-β inhibitor imatinib induced analgesia in cancer patients (Stankovic Stojanovic et al., 2011; Kutlar, 2013). Based on the promise of these recent clinical studies and case reports, it is possible that the improved pain relief observed with RTK inhibitors is due to the reversal of pre-existing opioid tolerance. We propose that combined treatment with opioids and RTK inhibitors may decouple the intertwined pathways mediating analgesia and tolerance.

Conclusion

Uncoupling analgesia from undesirable effects of opioids by RTK inhibitors could therefore enable patients to maintain opioid efficacy at smaller doses, mitigating the risk of side-effects associated with chronic opioid use. Importantly, in rodents, efficacious doses to mitigate opioid tolerance appear to be significantly lower than those required to treat cancers. This holds the promise that RTK doses required for effective prevention of opioid side-effects in humans should not have a deleterious impact that could outweigh advantages of RTK inhibitors. Nevertheless, more work is clearly needed to better understand how RTK inhibitors work in the context of tolerance. It is still unknown if RTK inhibitors could be used to treat opioid withdrawal symptoms or prevent rewarding properties of opioids in humans. It is imperative that future studies assess the power of concurrent opioid-RTK inhibitors treatments both in the clinic and in pre-clinical models of pain. If successful, RTK inhibitors may represent a promising new class of drugs to treat pain more safely in conjunction with opioids and therefore positively impact the lives of millions living with chronic pain.

Author contributions

MG, NS, and BW contributed equally by generating a first draft of this manuscript. LP, ZF, and RL contributed to the writing of this manuscript. SP initiated the manuscript, supervised collection of cited references, and contributed to first and final drafts. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The role of endogenous opioid neuropeptides in neurostimulation-driven analgesia

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Due to the prevalence of chronic pain worldwide, there is an urgent need to improve pain management strategies. While opioid drugs have long been used to treat chronic pain, their use is severely limited by adverse effects and abuse liability. Neurostimulation techniques have emerged as a promising option for chronic pain that is refractory to other treatments. While different neurostimulation strategies have been applied to many neural structures implicated in pain processing, there is variability in efficacy between patients, underscoring the need to optimize neurostimulation techniques for use in pain management. This optimization requires a deeper understanding of the mechanisms underlying neurostimulation-induced pain relief. Here, we discuss the most commonly used neurostimulation techniques for treating chronic pain. We present evidence that neurostimulation-induced analgesia is in part driven by the release of endogenous opioids and that this endogenous opioid release is a common endpoint between different methods of neurostimulation. Finally, we introduce technological and clinical innovations that are being explored to optimize neurostimulation techniques for the treatment of pain, including multidisciplinary efforts between neuroscience research and clinical treatment that may refine the efficacy of neurostimulation based on its underlying mechanisms.

KEYWORDS

pain, analgesia, opioid, μ-opioid receptor, neurostimulation, neuromodulation, deep brain stimulation (DBS), spinal cord stimulation (SCS)

Introduction

Over 20% of people worldwide suffer from chronic pain disorders (Goldberg and McGee, 2011). In response to an unmet need for effective pain management, opioid drugs have been widely adopted. Opioid drugs harness the body's endogenous opioid receptors, which are dispersed throughout the central and peripheral nervous system to modulate pain perception. While prescription opioids often provide effective pain relief, they have undesirable and potentially dangerous side effects including abuse liability and respiratory depression. Their contribution to the ongoing opioid epidemic and the enormous negative impact of chronic pain underscore the need for safe and effective pain therapies (Manchikanti et al., 2012). Neurostimulation therapies are potential alternatives for managing medically refractory pain. However, these therapies are hampered by inconsistent pain relief across patients and diminishing analgesic effects over time (Kumar K. et al., 1998). To optimize these therapies and predict patient responses, we must first understand the mechanisms of action underlying their therapeutic effects. The purpose of this review is to summarize the evidence suggesting current neurostimulation therapies may provide analgesia in part by driving endogenous opioid mechanisms. We conclude by discussing opportunities for multidisciplinary research to shed new light on mechanisms of neurostimulation-induced pain relief.

Chronic pain

Chronic pain is a condition often defined by the presence of long-standing pain that persists beyond recovery of the injured tissue. In humans, chronic pain is clinically defined as pain that persists for longer than 6 months (Russo and Brose, 1998), without regard to tissue healing. One type of severe chronic pain for which neurostimulation techniques are often used is neuropathic pain, which is defined by the International Association for the Study of Pain as "pain caused by a lesion or disease of the somatosensory system" (Jensen et al., 2011). In the United States, an estimated 20.5% of adults suffer from a chronic pain condition, with 10% experiencing high-impact chronic pain that limits work and diminishes quality of life (Yong et al., 2022). This figure is mirrored by an estimated global prevalence of chronic pain of 18% (Sá et al., 2019). Many patients experiencing chronic pain are inadequately treated, with estimates ranging from 40 to 77% depending on pain etiology and study parameters (Deandrea et al., 2008; Majedi et al., 2019). Due to its high prevalence worldwide, there is a clear and urgent need for safe and effective therapies for managing chronic pain.

Opioid analgesics

Prescription opioids have major drawbacks that limit their tolerability, effectiveness, and safety. Opioids produce disorienting psychoactive effects which can interfere with daily activities. Opioid use can cause constipation which produces significant discomfort. Repeated opioid use leads to adaptations in opioid receptor signaling, such as receptor desensitization, internalization, and augmented downstream signaling pathways, which are thought to differentially contribute to tolerance and limit effectiveness in treating pain (von Zastrow et al., 2003; Gintzler and Chakrabarti, 2006; Martini and Whistler, 2007). Activation of opioid receptors in circuits that control breathing induces strong respiratory depression that leads to death at high doses, with opioid-related deaths rising steadily over the past 20 years and continuing at epidemic levels (Rudd et al., 2016; Scholl et al., 2019). Coupled with the rewarding aspects of opioid signaling that reinforce drug consumption, respiratory depression is the most dangerous aspect of opioid analgesics, as it is responsible for the large number of opioid overdose deaths. There is thus an urgent demand for novel effective and tolerable treatment paradigms to lessen suffering of chronic pain patients, a mission that has been recently prioritized by the US National Institutes of Health (Collins et al., 2018).

Endogenous opioids

Opioid receptors are expressed throughout the nervous system, including the cortex, midbrain, brainstem, spinal cord, and in the presynaptic terminals of the primary afferents of the dorsal root ganglion (le Merrer et al., 2009). Due to its prominence as the primary target of opioid analgesics, most studies of pain revolve around the μ-opioid receptor (MOR). However, the δ- and κ-opioid receptors (DORs and KORs) are also important in pain modulation (Fields, 2004; Corder et al., 2018). MORs are activated by the endogenous opioid neuropeptides enkephalin, beta-endorphin, and dynorphin. Enkephalins, of which there are two forms that differ in their C-terminal amino acid ([Met^5]-enkephalin and [Leu^5]-enkephalin), also activate DORs with similar affinity (Toll et al., 1998; Gomes et al., 2020). Beta-endorphin, which includes [Met^5]-enkephalin at its N-terminus, is usually considered MOR-selective but can also activate DORs and KORs, with notable signaling bias toward downstream G-protein signaling compared to beta-arrestin signaling at MORs observed *in vitro* (Gomes et al., 2020). Several opioid peptides that can be described as short, C-terminally extended forms of [Met^5]-enkephalin have also been isolated from mammalian brains; one of which (Met-enkephalin-Arg-Phe) has been recently demonstrated to act at MORs when released endogenously (Trieu et al., 2022). Several dynorphin peptides of different

length and sequence are prominent in the mammalian nervous system. Although dynorphins are usually considered KOR agonists due their high affinity for KORs (especially the longer forms), they can also activate MORs and DORs at physiologically relevant concentrations (Toll et al., 1998; Gomes et al., 2020).

It is generally assumed that endogenous opioids produce pain relief through MOR activation. The most unequivocal experimental manipulation in humans implicating endogenous opioids in pain is the administration of naloxone, which is a non-specific opioid antagonist that acts on MORs, DORs, and KORs in a similar concentration range. Thus, endogenous opioids may impart some of their antinociceptive effects through activation of DORs and KORs, in addition to MORs.

Pain processing circuits and their expression of opioid receptors

Pain information is processed by two broad pathways: the ascending nociceptive pathway and the descending pain modulatory system (DPMS). The ascending pathway begins in peripheral nociceptors, which encode painful stimuli and synapse onto projection neurons and interneurons in the spinal cord dorsal horn (DH). Ascending pathways include the spinothalamic, spinomesencephalic, and spinoreticular tracts, which target the thalamus, midbrain areas such as the periaqueductal gray (PAG), and the brainstem reticular formation, respectively. Within the spinothalamic tract, subdivisions that target the lateral thalamus and onto the somatosensory cortices and insula are considered to mediate the sensory-discriminative aspects of pain (i.e., the sensory experience of pain involved in reflexive pain behaviors such as limb withdrawal in response to noxious stimuli). Spinothalamic subdivisions that target the medial thalamus and onto the anterior cingulate cortex (ACC) are thought to contribute to the affective percept of pain (i.e., the emotional-motivational experience of pain which is non-reflexive). The descending pain modulatory pathway begins in the PAG. Canonically, ventrolateral PAG (vlPAG) projects to the rostroventral medulla (RVM), which in turn sends projections to the DH to gate spinal outflow of incoming pain information. A brief overview of key brain areas that encode and modulate pain for the understanding of neurostimulation-induced analgesia follows. Schematics of the location, circuitry, and opioid receptor expression in brain areas within the descending and ascending pathways most relevant for current neurostimulation techniques for the treatment of chronic pain are shown in **Figure 1**.

Descending pathway

Periaqueductal gray

The PAG, a heterogenous midbrain region known for its roles in divergent behaviors such as defensive responses and vocalization (Behbehani, 1995), represents the first major hub in the DPMS. In the context of the pain, PAG receives and consolidates top-down input from numerous cortical and subcortical regions, including the prefrontal cortex (PFC), ACC, anterior insula, and amygdala (Hardy and Leichnetz, 1981; Bingel et al., 2006; Lu et al., 2016; Cheriyan and Sheets, 2018; Li and Sheets, 2018; Rozeske et al., 2018; Huang et al., 2019; Zhu et al., 2021). In addition to the RVM and nearby noradrenergic nuclei, the PAG displays broad ascending efferent projections to brain regions such as the thalamus, hypothalamus, and ventral tegmental area (Cameron et al., 1995a,b; Linnman et al., 2012; Ntamati et al., 2018). Though human tractography studies indicate some differences in PAG cortical connectivity between rodents and humans, midbrain and hindbrain connectivity is conserved, which is critical to our understanding of neurostimulation techniques that may harness descending pain modulatory mechanisms (Ezra et al., 2015; Menant et al., 2016).

In the rodent, the anatomy and function of the PAG opioid system has been extensively studied and recently reviewed by Bagley and Ingram, 2020. The canonical circuit by which opioids signal in the PAG follows a disinhibitory mechanism: MORs are highly expressed on local vlPAG GABAergic interneurons that provide tonic inhibition onto PAG projection neurons. In the presence of endogenous or exogenous opioids, these inhibitory inputs are suppressed by MOR signaling, leading to the disinhibition of glutamatergic PAG-RVM projections (Lau and Vaughan, 2014). The resultant activation of descending GABAergic, opioidergic and serotonergic RVM neurons directly inhibits spinal cord neurons to suppress nociception (Salas et al., 2016; Weiwei et al., 2021).

In line with this hypothesis, vlPAG microinfusion of glutamate receptor agonists and GABA receptor antagonists produces antinociception in rodents (Moreau and Fields, 1986; Jones and Gebhart, 1988; Jensen and Yaksh, 1989). More recently, modern chemogenetic methods in behaving rodents indicate that activation of glutamatergic vlPAG neurons or inhibition of GABAergic neurons is antinociceptive, while inhibition of glutamatergic neurons or activation of GABAergic neurons is pronociceptive, although the opioid dependence of this analgesia was not examined (Samineni et al., 2017). Local opioid infusion in the PAG, especially vlPAG, has long been noted for its strong antinociceptive properties in rodents (Yaksh, 1979; Jones and Gebhart, 1988; Jensen and Yaksh, 1989). MORs can also be found, however, in a subpopulation of PAG projection neurons (Wang and Wessendorf, 2002; Bagley and Ingram, 2020), suggesting that this accepted circuitry may not account for non-canonical or bidirectional signaling from PAG to RVM, which may involve competing facilitation and inhibition. Indeed, about half of RVM-projecting PAG neurons are actually hyperpolarized by MOR agonists (Osborne et al., 1996; Umana et al., 2017).

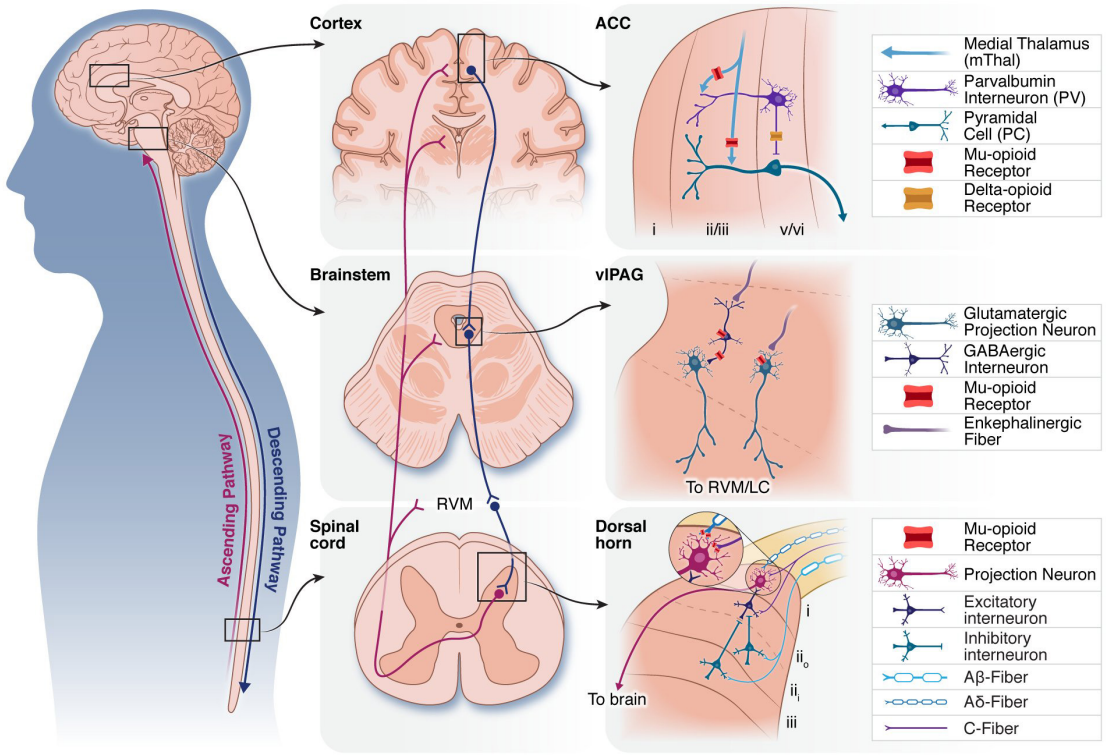

FIGURE 1
Overview of three neural structures that have been targeted by neurostimulation therapies. Schematic of ascending (purple) and descending (blue) pain modulatory pathways **(left)**. Middle: Macro level anatomy of the cortex, brainstem and spinal cord, showing key nodes in the ascending and descending pain modulatory pathways. Connections between the brainstem and spinal cord *via* the RVM are indicated. Right: Select synaptic connections and microcircuitry of the ACC, vlPAG and DH are shown. Mu-and delta-opioid receptors are expressed on cell bodies and pre-synaptic terminals of neurons throughout the pain neuraxis to modulate ascending and descending pain pathways. ACC, anterior cingulate cortex; RVM, rostroventromedial medulla; vlPAG, ventrolateral periaqueductal gray; LC, locus coeruleus; DH, dorsal horn.

Using functional imaging in humans, PAG activity has been implicated in a multitude of functions, from pain-and placebo-related conditions to homeostatic bodily processes and the manifestation of negative emotional states in panic and depression (Zhao, 2008; George et al., 2019). For a comprehensive review of human functional imaging of PAG, we recommend the meta-analysis provided by Linnman et al., 2012. In brief, many studies have found pain-induced PAG activation in response to noxious stimuli such as heat, cold, pressure, and light touch on allodynic regions, as well as in chronic pain conditions such as neuropathic pain. PAG fMRI indicates its functional connectivity at rest with ACC and RVM (Kong et al., 2010), and this ACC-PAG interaction correlates with attentional analgesia and can be disrupted by opioid antagonists (Oliva et al., 2022). Placebo conditioning in humans increases PAG activity during the anticipation of a painful stimulus (Wager et al., 2004) and induces coupling of ACC and PAG activity that is sensitive to systemic naloxone (Eippert et al., 2009). Due to the abundance of opioid receptors expressed, PAG is thought to play a key role in pain modulation produced by exogenous and endogenous opioids. In humans, PET imaging of [11C]-carfentanil indicates a decrease in radiotracer binding and therefore an increase in PAG endogenous opioid signaling in response to pain (Zubieta et al., 2005) and placebo analgesia (Scott et al., 2008).

Rostroventral medulla

Rostroventral medulla (RVM) receives inputs from PAG and sends projections to the DH to modulate spinal signaling through GABAergic, serotonergic, and opioidergic mechanisms (Millan, 2002; François et al., 2017). RVM neurons are categorized as ON, OFF, and neutral cells based on their electrophysiological responses to noxious stimuli and during nocifensive responses. RVM receives input from the PAG and has recently been shown to receive synaptic connections from the parabrachial nucleus (Chen et al., 2017). RVM outputs relevant for pain modulation include the spinal cord and midbrain and brainstem noradrenergic nuclei (Clark and Proudfit, 1991a).

Like PAG, RVM is a known locus of exogenous and endogenous opioids in pain modulation (Bagley and Ingram, 2020). RVM neurons express opioid receptors in serotonergic

and non-serotonergic neurons that project to the spinal cord (Gutstein et al., 1998; Wang and Wessendorf, 1999). Supporting a role for endogenous opioids, all three opioid receptor types are also expressed by terminals in the neuropil around RVM neurons (Kalyuzhny et al., 1996; Gutstein et al., 1998). RVM receives input from enkephalinergic terminals and some RVM neurons are enkephalinergic, including a subset of spinally-projecting GABAergic neurons (Khachaturian et al., 1983; Zhang et al., 2015). In addition to enkephalins, RVM receives dynorphinergic input from PAG and contains KOR-expressing spinally-projecting neurons that inhibit pain and itch *via* descending mechanisms (Nguyen et al., 2022). RVM may also contain dynorphin-expressing neuronal cell bodies (Menetrey and Basbaum, 1987). Application of opioids to the RVM leads to the increase in activity of antinociceptive OFF-cells and the decrease in spiking of pronociceptive ON-cells (Heinricher et al., 1994) as well as strong antinociception in rodents (Dickenson et al., 1979; Azami et al., 1982).

Noradrenergic cell groups

Rodent intrathecal pharmacological studies have long implicated spinal noradrenergic signaling as a key component in supraspinal influence on pain suppression (Yaksh, 1979; Proudfit and Hammond, 1981; Hammond and Yaksh, 1984; West et al., 1993). The locus coeruleus (LC) (A6), brainstem (A5), and midbrain (A7) noradrenergic cell groups display projections to the spinal cord in parallel with the RVM (Westlund et al., 1983, 1984; Clark and Proudfit, 1991b,c, 1993; Proudfit and Clark, 1991; Bruinstroop et al., 2012; Li et al., 2016; Hirschberg et al., 2017) and receive anatomical input from canonical DPMS nuclei PAG and RVM (Clark and Proudfit, 1991a; Bajic and Proudfit, 1999).

Locus coeruleus (LC) highly expresses opioid receptors (Pert et al., 1976) and LC neuron activity is directly suppressed by both endogenous and exogenous opioids (Williams et al., 1982). Opioid receptor expression in LC, A5, and A7 neurons appears to be limited to MORs (Williams and North, 1984; North et al., 1987; Guajardo et al., 2017), although a subset of presynaptic terminals in these areas have been shown to express DORs (Arvidsson et al., 1995; van Bockstaele et al., 1997; Holden et al., 1999; Erbs et al., 2015). Additionally, LC and the pericoerulear region are densely innervated by enkephalin-expressing terminals (Drolet et al., 1992). Microinfusion of morphine directly into the LC is antinociceptive in rodents (Bodnar et al., 1988).

Spinal cord

The spinal cord, especially the DH, is the ultimate target of the DPMS. Release of neuromodulators and neurotransmitters in the DH from descending sources modulates spinal outflow of ascending nociceptive information arriving from the periphery. Aδ and C nociceptive fibers terminate onto DH superficial laminae I projection neurons that respond to high threshold stimulation, as well as onto deeper layer V wide dynamic range projection neurons. Most neurons in the laminae II-III, however, are not supraspinally-projecting, but instead are excitatory or inhibitory interneurons that signal locally in the spinal cord. It is thought that descending fibers from the midbrain and brainstem can terminate onto primary afferent terminals, spinal interneurons, and spinal projection neurons to modulate the spinal circuit response to incoming pain information at multiple levels (Mannion and Woolf, 2000; D'Mello and Dickenson, 2008). In addition to neurotransmitters, spinal pain transmission is also modulated by a complicated combination of other neurochemicals such as neurokinins, CGRP, somatostatin, and opioids (Dickenson, 1995).

Endogenous opioid peptides and receptors play a substantial role in spinal cord pain-related activity. The rat spinal cord predominantly expresses MORs, but also exhibits some DORs and very low KOR expression. Within each of these receptor subtypes, all show predominant expression on presynaptic terminals entering the DH, with a smaller proportion on postsynaptic neurons (Besse et al., 1990; Dickenson, 1995). Recordings from DH neurons during intrathecal morphine application show that C and Aδ fibers that convey noxious information are the most highly inhibited by morphine, while the pain evoked activity of larger Aβ mechanosensory fibers is only mildly opioid-modulated (Dickenson and Sullivan, 1986; Heinke et al., 2011). Intrathecal application of enkephalin is analgesic (Yaksh et al., 1977), presumably due to activation of the same opioid receptors affected by morphine. Enkephalin- and dynorphin-immunoreactive cell bodies and fibers are present in the DH, suggesting that endogenous opioid peptides are released in the DH locally and by descending mechanisms (Seybold and Elde, 1980; Harlan et al., 1987; Marvizón et al., 2009; François et al., 2017). However, parsing the contribution of local and descending opioid release has been experimentally challenging.

Ascending pathway

Thalamus

The thalamus receives nociceptive information directly from the spinal cord and relays it to the cortex (Ab Aziz and Ahmad, 2006). The spinothalamic tract conveys information about non-noxious and noxious stimuli to the lateral and medial thalamus. The lateral thalamic ventral posterolateral (VPL) and ventral posteromedial (VPM) nuclei project to the somatosensory cortex and relay tactile, proprioceptive, and nociceptive signals from the body and face, respectively (Monconduit et al., 1999; Alitto and Usrey, 2003). Medial thalamic nuclei receive additional nociceptive information from ascending spinal tracts. These nuclei transmit information thought to be related to the affective components of pain to areas involved in emotional processing, such as the ACC and the insular cortices (Friedman and Murray, 1986). A study in rats found a functional correlation between medial thalamus and

ACC activity during electrical stimulation, supporting the idea that thalamus conveys information on the affective components of pain through this projection (Shyu et al., 2004). Among the medial thalamic nuclei, the mediodorsal nucleus (MD) is the major source of inputs to the ACC. Also implicated in pain processing is the medial thalamic nucleus submedius (Sm), which projects to the ventrolateral orbital cortex (VLO) and on to the PAG, a pathway that has been shown to mediate antinociception (Zhang et al., 1995; Huang et al., 2021). Imaging and electrophysiology studies in both animals and humans have also found that, like ACC, the MD is hyperactive in chronic pain conditions (Whitt et al., 2013; Meda et al., 2019). In mice with neuropathic pain, optogenetic activation of MD inputs to ACC induces behavioral avoidance and is considered aversive (Meda et al., 2019).

A meta-analysis of published fMRI data in humans with acute, experimentally-induced and chronic pain showed that the thalamus is active in both conditions (Friebel et al., 2011). Chronic pain patients show altered thalamic regional cerebral blood flow (rCBF) and several imaging studies suggest that altered thalamic activity is involved in the development of neuropathic pain (Witting et al., 2001; Casey et al., 2003). Studies in animal models of neuropathic pain have also shown a correlation between chronic pain and changes in biochemistry and immediate early gene expression in the thalamus (Narita, 2003).

Opioid receptors are widely expressed in the thalamus. High levels of MOR mRNA are observed in several thalamic nuclei, including the medial habenula, laterodorsal, paraventricular, centromedial, and reuniens nuclei. DOR mRNA expression is also observed in the thalamus, but KOR mRNA expression is limited to fewer nuclei in the paraventricular and zona incerta (Mansour et al., 1994; Erbs et al., 2015). In rodent brain slices, thalamic output to ACC and dorsal striatum is suppressed in the presence of a MOR agonist, indicating the sensitivity of thalamic output to opioids and suggesting the attenuation of noxious information relay to cortex during opioid treatment (Birdsong et al., 2019). In rodents, pharmacological blockade of MORs in the dorsal midline thalamus induced a fear memory extinction deficit (Bengoetxea et al., 2020), while stimulation of MORs caused increased locomotor activity associated with decreased freezing extinction. These data suggest that targeting dorsal midline thalamus MORs could have therapeutic effects on stress-related and anxiety disorders. Animal research using both electrophysiology and EEG points to the medial thalamus as the primary site of morphine action (Linseman and Grupp, 1980). Indeed, morphine microinfused in the medial or intralaminar thalamic nuclei has been shown in a small number of rodent studies to produce analgesia (Carr and Bak, 1988; Wang et al., 2006; Erfanparast et al., 2015). Consistently, studies in both humans measuring [11C]diprenorphine binding *via* PET imaging and rodents have found lower opioid receptor availability in chronic pain conditions in the thalamus, ACC, posterior temporal and orbitofrontal cortices, as well as in the posterior midbrain (Thompson et al., 2018).

Anterior cingulate cortex

The ACC refers to a subregion of frontal cortex with heterogenous subdivisions that are differentially involved in the affective, cognitive, and emotional components of pain processing (Bush et al., 2000; Vogt, 2005; Heilbronner and Hayden, 2016). In humans, ACC receives inputs from the anterior insular cortex (aI) (Peltz et al., 2011; Wiech et al., 2014) and amygdala (Sharma et al., 2020). It receives ascending noxious sensory information mainly *via* the medial thalamic nuclei (Xiao and Zhang, 2018). The ACC pain-aversive response can be increased by inputs from the primary somatosensory cortex on a subset of ACC neurons (Singh et al., 2020). Several pieces of evidence suggest that projections from ACC to the brainstem, specifically through the PAG or by way of the medial thalamic nuclei, are important for the cortical contribution to opioid analgesia and to placebo analgesia (Hardy and Leichnetz, 1981; Royce, 1983; Devinsky et al., 1995). ACC also sends reciprocal projections to the amygdala (Allsop et al., 2018) and insular cortex; while functional connectivity between these regions is associated with negative affective states (Shao et al., 2018), the role of this circuitry in the emotional and affective components of pain remains to be determined.

Early single neuron recordings in cingulotomy patients showed that ACC neurons respond selectively to mechanical and thermal painful stimuli, but not to innocuous stimuli (Hutchison et al., 1999). Likewise, single-unit recordings in rabbits demonstrate that ACC neurons which respond to noxious stimuli have diffuse receptive fields covering the entire body (Sikes and Vogt, 1992). In non-human primates, ACC neurons were reported to encode the integration of nociception, specifically the anticipation of pain following cutaneous electric stimulation (Koyama et al., 1998). Interestingly, ACC activation has also been observed during placebo-induced analgesia (Wager et al., 2004), though this activation may occur in a different substructure than that activated by noxious stimuli. Subsequent human fMRI and PET studies further confirm that ACC is activated by noxious stimuli (Kwan et al., 2000) and the response magnitude correlates with stimulus intensity and changes in the perceived unpleasantness of painful stimuli (Vogt et al., 1996; Rainville et al., 1997; Tölle et al., 1999). Together, these findings confirm that nociceptive stimuli activate ACC across species.

Arguing against a simple role for the ACC in nociception, patients with ACC lesions experience reduced pain-related unpleasantness and reduced avoidance of noxious stimuli, but their ability to identify intensity and location of noxious stimuli remains intact (Foltz and White, 1962; Ballantine et al., 1967; Wayne Hurt et al., 1974). Similarly, microinjection of excitatory amino acids into the ACC in naïve rodents elicits conditioned place aversion without altering pain thresholds (Johansen and

Fields, 2004), while ACC lesions eliminate the aversiveness of neuropathic pain but not stimulus-evoked hypersensitivity (Qu et al., 2011). These findings argue against the role of ACC in nociceptive processing *per se*. Instead, several studies in both humans and rodents have shown that ACC contributes to the unpleasantness of pain (Seminowicz et al., 2009; Fuchs et al., 2014; Bliss et al., 2016). Functional and structural alterations of ACC, such as hyperactivation and reduction of gray matter, have been observed in neuropathic patients and are associated with emotional and psychological pain (Rodriguez-Raecke et al., 2009; Bushnell et al., 2013).

Early human studies reported high [3H]diprenorphine binding in the ACC of healthy subjects but a reduction in patients with central post-stroke pain (Willoch et al., 1999), suggesting that opioids can directly impact aspects of pain processing by binding ACC opioid receptors (Vogt et al., 1995; Jones et al., 1999). Further receptor-imaging studies confirm the involvement of ACC in opioid-dependent analgesia and, intriguingly, suggest a role in placebo analgesia (Petrovic et al., 2002). PET studies performed with [11C]Carfentanil observed endogenous ACC opioid release during placebo analgesia and the consequent endogenous opioid-induced ACC activation correlated with a reduction in pain affect during a sustained painful stimulus (Zubieta et al., 2001). Consistent with this, rodent ACC morphine microinjection selectively suppresses pain affect but not withdrawal responses (LaGraize et al., 2006; Gomtsian et al., 2018).

Opioid receptors are abundantly expressed in the ACC, with MOR expression most prominent in superficial layers (Vogt et al., 1995). MORs are expressed by both cortical neurons and afferent axons from subcortical regions. Presynaptic MORs are predominant on thalamic axonal projections to the ACC (Vogt et al., 1995). This distribution pattern led to the idea that endogenous opioids can regulate nociception by inhibiting the thalamocortical afferents in the ACC or by modulating the activity of interneurons and projection neurons (Navratilova et al., 2015). This model has been recently expanded upon by examining the thalamo-cortico-striatal circuit (Birdsong et al., 2019), whose involvement in pain processing was first described by Rainville et al. (1997). Thalamic inputs to ACC are potently inhibited by MOR agonists, but ACC inputs to dorsomedial striatal neurons are not affected. In contrast, DOR agonists disinhibit ACC pyramidal neurons and allow for the excitation of ACC inputs onto striatal medium spiny neurons. These mechanisms are mediated by different receptors and suggest that opioid-mediated attenuation of nociceptive information transfer to ACC from thalamus may be a primary mechanism by which opioids reduce the negative affective component of pain.

Prefrontal cortex

While most frequently studied in the context of executive cognitive function, recent evidence has begun to implicate the PFC in processing acute nociceptive stimuli and in the development of chronic pain. Within the PFC, the dorsolateral PFC (dlPFC) is considered a master regulator of higher order cognitive functions and is also involved in the cognitive and affective modulation of pain (Lorenz et al., 2003), including placebo analgesia (Petrovic et al., 2002). Functional imaging in humans with acute and chronic pain reveal that PFC activity correlates with the activity of pain-implicated regions above, including ACC, insula, and thalamus (Apkarian et al., 2005). Further, it has been posited that PFC-PAG output and reciprocal PFC connections with the amygdala play a role in antinociception, whereas thalamocortical PFC input and PFC output to the basal ganglia may contribute to pain chronicity (Ong et al., 2019). Previous fMRI studies have found that the magnitude of placebo-induced dlPFC activity correlates with an increase in PAG activity, supporting the idea that this circuit is involved in expectancy-based placebo (Wager et al., 2004, 2007). The prelimbic cortex in rodents is often included in definitions of the rodent PFC, and while not considered homologous to dlPFC in primates (Laubach et al., 2018), recent work has revealed a role for this structure in pain processing. Specifically, inflammatory pain decreases both basal firing rate and evoked nociceptive responses in prelimbic neurons (Dale et al., 2018), while inhibition of prelimbic neurons and their outputs to the nucleus accumbens enhances pain responses (Zhou et al., 2018).

The effects of opioids in the PFC are less well-characterized. Rodent PFC neuronal activity has been shown to be opioid sensitive (Williams and Zieglgänsberger, 1981; Giacchino and Henriksen, 1998), while in humans, PET imaging implicates PFC endogenous opioid signaling in placebo-induced analgesia (Wager et al., 2007). Caution is required, however, when attempting to draw parallels between the rodent and human PFC as expansion over the course of evolution has led to more distinct functions and subregions within the human PFC as compared to the rodent (Carlén, 2017; Laubach et al., 2018), with rodents lacking a specific homologue of the dlPFC. Nonetheless, important findings for the implications for PFC in pain signaling may still be gleaned by carefully designing and interpreting experiments and corroborating findings across experimental models.

Neurostimulation therapies for chronic pain

It is now well-established that the widespread adoption of prescription opioids for the treatment of chronic pain has been instrumental in driving the ongoing opioid epidemic. The continuing burden of untreated chronic pain on patients underscores the need for safe and effective pain therapies. Neurostimulation therapies that target peripheral or central pain mechanisms are promising alternatives for managing medically refractory pain. However, these therapies are hampered by inconsistent pain relief across patients and

frequently diminishing analgesic effects over time. Across all neurostimulation therapies, we do not currently understand the physiological mechanisms of action by which these therapies provide pain relief. A clear understanding of the mechanisms of stimulation-induced analgesia is crucial to improve the efficacy of these therapies.

Overview of neurostimulation for chronic pain

Neurostimulation therapies (Figure 2) are non-addictive, reversible strategies for managing intractable chronic pain. Neurostimulation therapies aim to modulate neural activity through targeted delivery of electrical stimuli to specific regions of the nervous system. In the clinical context, the term "neuromodulation" commonly refers to electrical neurostimulation therapies, but may also refer to targeted drug delivery (e.g., intrathecal pumps), radiofrequency ablation therapies, or modulation of neural activity *via* ultrasound, which are outside the scope of this review. We use the terms "neuromodulation" and "neurostimulation" interchangeably to describe therapies which use electrical stimulation of the nervous system to treat neurological disorders.

Neurostimulation therapies range in invasiveness. Non-invasive therapies, such as transcranial direct current stimulation (tDCS), place electrodes on the scalp or magnetic coils proximal to the head. Invasive neurostimulation therapies, such as deep brain stimulation (DBS) or spinal cord stimulation (SCS), involve placing small electrode arrays in the body near the neural structure of interest, which are connected to implantable pulse generators. After electrode placement, a clinician programs the stimulus pulse (i.e., sets the stimulus pulse amplitude, duration, and frequency) to maximize therapeutic effect while minimizing unwanted side effects.

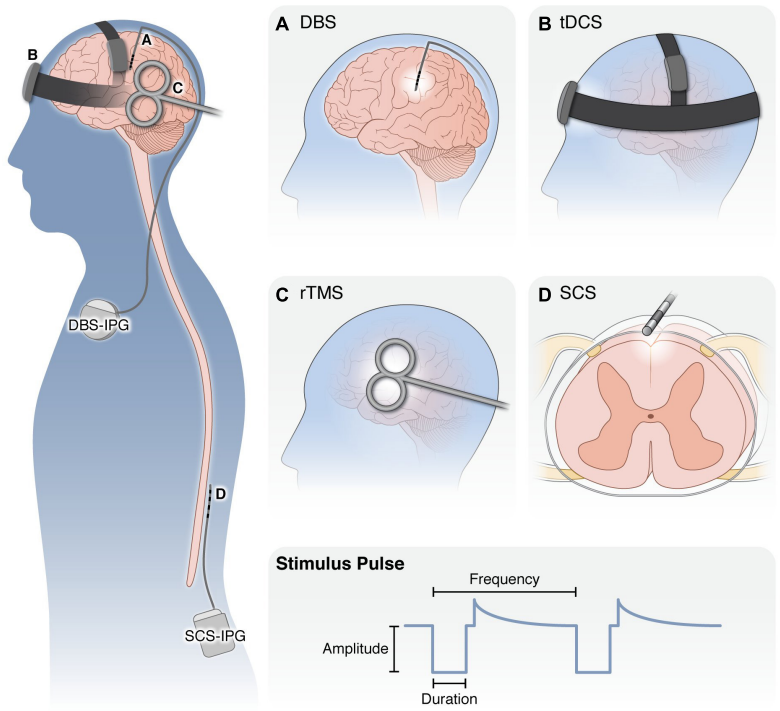

FIGURE 2
Overview of neurostimulation modalities for the treatment of chronic pain. (Left) Schematic of application of neurostimulation devices for the treatment of chronic pain. **(A)** DBS electrodes are surgically targeted to specific brain nuclei (i.e., ACC, midline thalamus, PAG) with an external pulse generator. Following optimization of stimulation settings, the pulse generator and leads are internalized under the clavicle to deliver electrical stimulation to the brain. **(B)** With tDCS, small amounts of electric current are applied externally *via* electrodes held in place against the scalp. **(C)** rTMS is applied with an external electromagnetic coil to generate an electromagnetic field in the underlying cortical regions. Both tDCS and rTMS are applied for 20–60 min over repeated sessions without requiring anesthesia. **(D)** SCS employs implanted electrodes in the epidural space to apply electrical current to the spinal cord. Similar to DBS, SCS patients undergo a trial period to ensure adequate pain relief before the pulse generator and leads are internalized in the posterior flank. (Bottom) For all modalities, several properties of the stimulus waveform can be modulated, including the waveform shape, pulse amplitude, duration, and frequency, as well as whether it is applied continuously, in regular burst patterns or in a closed-loop manner in response to neural activity or patient control. DBS, deep brain stimulation; tDCS, transcranial direct current stimulation; rTMS, repeated transcranial magnetic stimulation; SCS, spinal cord stimulation; IPG, implanted pulse generator.

These stimulation parameters may be adjusted at follow-up visits to ensure consistent therapeutic benefit.

Neurostimulation has emerged in the past 60 years as an effective therapeutic approach to treating pain and other disorders (Bittar et al., 2005; Moisset et al., 2020). Based on the premise that pain percept is encoded by aberrant patterns of neural activity, the objective of neurostimulation is to alter neural activity in a way that minimizes the experience of pain. Melzack and Wall's Gate Control Theory of Pain formed the scientific basis for the first modern uses of electrical stimulation-induced pain relief in humans (Melzack and Wall, 1965). This theory suggests that driving the activity of large-diameter afferents may produce pain relief by increasing the activity of inhibitory interneurons in the spinal cord DH. Only 2 years after the publication of the Gate Control Theory, Wall and Sweet demonstrated analgesia *via* peripheral nerve stimulation (Wall and Sweet, 1967), and Shealy and colleagues demonstrated analgesia *via* electrical stimulation of the dorsal columns of the spinal cord (Shealy et al., 1967). Conventional neurostimulation theory suggests that extracellular electrical stimulation induces action potentials (APs) in myelinated axons at lower stimulus amplitudes than other neural structures (e.g., non-myelinated axons, cell bodies) (Rattay, 1986, 1999; McIntyre and Grill, 1999). Therefore, electrical stimulation of peripheral nerves and the dorsal columns likely provides analgesia by driving the activity of myelinated tactile afferent axons and feed-forward pain-gating circuitry (Mendell, 2013; Braz et al., 2014; Duan et al., 2018).

The past several decades have produced many innovations in stimulation-induced analgesia. Therapies such as spinal cord stimulation (SCS) are most commonly indicated for neuropathic limb pain conditions, such as failed back surgery syndrome and complex regional pain syndrome. Modern neurostimulation approaches have also been investigated to treat central chronic pain syndromes, such as post-stroke and phantom limb pain (Bittar et al., 2005; Moisset et al., 2020). Furthermore, novel stimulation targets (e.g., deep brain stimulation (DBS) of the ACC (Spooner et al., 2007)) and stimulus pulse paradigms [e.g., burst SCS (de Ridder et al., 2013)] are hypothesized to modulate the neural activity associated with the affective component of pain, rather than affecting circuits associated with the sensory component (e.g., the spinal cord DH). Recent years have seen numerous promising innovations in neurostimulation for pain, and these modalities of exogenous electrical stimulation likely have broad effects across the pain neuraxis, which are not limited to circuits being directly stimulated. This property poses additional challenges to understanding the specific therapeutic mechanisms underlying each neurostimulation technique. Therefore, understanding how different neurostimulation therapies affect specific circuits, such as opioidergic circuits, is crucial to understanding the mechanisms that will ultimately be necessary for optimizing the design and implementation of each therapy.

Spinal cord stimulation

Spinal cord stimulation (SCS) is the most common neurostimulation therapy, with more than 50,000 SCS systems implanted each year (Sdrulla et al., 2018). SCS is primarily indicated for chronic neuropathic pain of the trunk or limbs which is refractory to conventional medical management (Kumar et al., 2007). SCS is achieved by implanting an electrode array in the dorsal epidural space, either *via* percutaneous implantation of a cylindrical electrode array, or by implanting a paddle electrode array which requires a laminectomy (Sears et al., 2011). Traditionally, SCS is applied with stimulus pulse frequencies between 40 and 60 Hz, pulse durations between 200 and 600 μs, and pulse amplitudes on the order of several Volts or milliamps for voltage-and current-controlled stimulation, respectively (Kumar R. et al., 1998; Kapural et al., 2015; Malinowski et al., 2020). Recent innovations in SCS technology apply novel stimulus pulse paradigms, particularly with regards to stimulus pulse frequency (Lempka and Patil, 2018). However, few studies have provided evidence regarding the involvement of endogenous opioid mechanisms in analgesia achieved with these novel SCS therapies. Therefore, we will focus our discussion on the possible opioidergic mechanisms of conventional SCS. Furthermore, to the extent that peripherally-targeted neurostimulation therapies such as peripheral nerve stimulation (PNS) (Helm et al., 2021) and dorsal root ganglion stimulation (DRGS) (Deer et al., 2017) engage the CNS, they are hypothesized to directly stimulate similar neural targets as conventional SCS (Lin et al., 2020; Graham et al., 2022). Accordingly, in addition to potentially modulating action potential propagation in nociceptors, these therapies likely engage similar central analgesic mechanisms as with conventional SCS.

Conventional SCS applied with pulse frequencies between ∼40 and 60 Hz evokes paresthesia (i.e., tingling or pins-and-needles sensations) in the area of the body targeted by stimulation. The goal of stimulator programming is to overlap these evoked paresthesias with the patient's painful region (North et al., 1991). Conventional SCS induces bidirectionally propagating action potentials (APs) in Aβ axons in the dorsal columns (Struijk et al., 1991; Holsheimer, 2002; Zhang et al., 2014; Lempka et al., 2020; Rogers et al., 2022). Antidromically propagating APs enter the dorsal horn caudal to the spinal level where SCS is applied, where they likely provide pain relief by activating feed-forward pain-gating circuitry in the spinal cord. Orthodromically propagating APs are likely responsible for SCS-induced paresthesia (Moffitt et al., 2009) and enter the brain at the brainstem dorsal column nuclei. It is possible that SCS simultaneously engages the endogenous opioid system both *via* orthodromically propagating APs to the brain and antidromically propagating APs into the DH.

Several brain structures related to the endogenous opioid system have been implicated in the supraspinal mechanisms

of action of SCS, such as the PAG, RVM, and thalamic VPL nucleus (Sivanesan et al., 2019). Many studies have examined the role of the DPMS, particularly the GABAergic and serotonergic components, in SCS-induced analgesia (Cui et al., 1996; Song et al., 2009, 2011). Early work in four patients suggested that SCS-induced analgesia is not reversed by naloxone administration, suggesting opioid-independent mechanisms (Freeman et al., 1983). However, this study examined a limited number of patients, and subsequent preclinical work has demonstrated RVM activation during SCS, a structure known to be crucial in endogenous opioid release (Dejongste et al., 1998), leaving the role of opioidergic circuits in SCS-induced analgesia unclear.

In more recent preclinical work, SCS applied to the cervical spinal cord caused dynorphin release in spinal segments caudal to the stimulation site (Ding et al., 2008), suggesting a potential role for segmental opioid release in SCS. In addition, SCS-induced analgesia in rats can be abolished by systemic naloxone, with both SCS-frequency and naloxone-dose dependent effects (Sato et al., 2013). A naloxone dose of 3 mg/kg/h reversed the effects of 4 Hz SCS, but the dose had to be increased to 10 mg/kg/h to reverse the analgesic effects of 60 Hz SCS. Interestingly, administering the DOR antagonist naltrindole abolished analgesia induced by 60 Hz but not by 4 Hz SCS. Finally, a recent preclinical study simultaneously applied SCS and the cholecystokinin (CCK) receptor antagonist proglumide (Inoue et al., 2017). While CCK receptor antagonists typically enhance opioid-dependent analgesia, co-application of SCS and proglumide did not provide enhanced analgesia compared to a single therapy alone. Taken together, these data present a murky picture regarding opioid-dependent analgesia during SCS, warranting continued study into both the involvement of endogenous opioids in SCS-induced analgesia and how SCS pulse parameters influence the engagement of these mechanisms.

Deep brain stimulation

Deep brain stimulation (DBS) is a surgical therapy whereby electrode arrays are implanted in discrete nuclei in the brain. Current is then passed through these electrode contacts through a fully implanted pulse generator to manipulate brain activity. Due to its invasiveness, DBS is typically reserved as a late-stage intervention after pharmacological and behavioral treatments have proven ineffective. Brain regions targeted for DBS are often historically identified as sites at which surgical lesions provide some relief for a disorder. Relative to ablative surgery, DBS is reversible and individually programmable, enabling stimulation parameters to be titrated for each patient. Although most commonly used for treatment of movement disorders, indications for DBS have recently expanded to include major depressive disorder, obsessive compulsive disorder, Tourette syndrome, cluster headache, and chronic pain. We focus our discussion on three brain sites that have been targeted clinically for pain relief and highlight evidence for involvement of opioidergic mechanisms in the therapeutic effects of DBS applied to these brain targets.

Periaqueductal gray-deep brain stimulation

When targeting PAG, DBS electrodes are placed bilaterally or contralaterally to the site of pain. Some studies indicate that even unilateral electrode placement provides a largely generalized pain relief described as a feeling of warmth and analgesia (Hosobuchi et al., 1977; Boccard et al., 2015). Across multiple case studies, PAG-DBS has proven effective in patients with "nociceptive pain" (Kumar and Wyant, 1985; Levy et al., 1987; Gybels and Kupers, 1990; Kumar et al., 1990), referring to pain generated through ascending dorsal horn input, such as peripheral neuropathic pain, spinal cord injury, plexopathy or phantom limb pain (Prévinaire et al., 2009; Subedi and Grossberg, 2011). Conversely, PAG-DBS exhibits much lower efficacy in centrally generated pain (e.g., post-stroke pain or headache) (Levy et al., 1987; Kumar et al., 1990; Gray et al., 2014; Kashanian et al., 2020). PAG-DBS was largely abandoned in 2000 after two large scale clinical trials (206 total patients) failed to meet clinical endpoints (Coffey, 2001). However, several design and interpretation issues have been raised concerning these studies, including the absence of randomization or placebo control, heterogeneity of the initial pain condition, and attrition of patients from the study which reduced statistical power to detect treatment differences (Shirvalkar et al., 2020). Critically, most data on PAG-DBS has been collected in case series or small clinical trials, without proper randomization or double blinding, the latter of which is arguably unfeasible due to PAG-DBS-induced paresthesia. Though its popularity has decreased, PAG-DBS is still used clinically to treat patients who are treatment refractory with good overall outcomes (Boccard et al., 2013). In the future, patient selection will be a key focus point for refinement to optimize treatment efficacy (Farrell et al., 2018; Frizon et al., 2020).

The therapeutic effects of PAG-DBS are frequency-dependent, with frequencies between 5 and 25 Hz being more efficacious than frequencies above 50 Hz (Nandi et al., 2002; Hentall et al., 2016). Interestingly, patients tended to prefer stimulation frequencies as low as 0.67 Hz (Jermakowicz et al., 2017) and between 5 and 35 Hz (Nandi and Aziz, 2004) when given the opportunity to blindly tune the parameters of their own DBS. It is interesting to note that pain-relieving stimulation in the 5 to 25 Hz range is within the physiological firing frequency of PAG neurons (Yu et al., 2021) and stands in sharp contrast to frequencies classically used to treat movement disorders, which are typically above 100 Hz (Creed, 2018). This supports the interpretation that intermittent activation of PAG descending projections with DBS applied at a physiological

firing rate could induce its effects through downstream opioid release.

In rodents with nerve injury, electrical stimulation of vlPAG was effective in reducing spontaneous pain behaviors and mechanical allodynia even 30–40 min after stimulation (Lee et al., 2012). A similar study using acute noxious stimuli found that unilateral vlPAG stimulation produces significant bilateral analgesia in rodents (Wang N. et al., 2016). Both studies state that the mechanism of this analgesia is still unclear, although opioids have been identified as a probable factor due to the concentration of MORs in PAG (Wang and Wessendorf, 2002; Loyd et al., 2008) and the finding that naloxone reverses some of the PAG stimulation-induced analgesia (Mayer et al., 1971; Akil et al., 1976; Morgan et al., 1991). Further downstream, the role of endogenous opioid release in the RVM for antinociception achieved by pharmacological and electrical activation of PAG has been assayed in preclinical models. PAG microinjection of GABA receptor antagonists (to cause PAG disinhibition), morphine, and non-opioid painkillers leads to antinociception that can be blocked by RVM microinfusion of naloxone (Llewelyn et al., 1984; Aimone and Gebhart, 1986; Kiefel et al., 1993; Roychowdhury and Fields, 1996; Vasquez and Vanegas, 2000). The role of endogenous opioid activity in the spinal cord with activation of DPMS by PAG and RVM electrical stimulation is still unclear; these stimulation interventions produce antinociception that can be blocked by intrathecal naloxone in some studies, while others have found a lack of an effect on antinociception by spinal opioid antagonism (Aimone et al., 1987; Miller and Proudfit, 1990; Morgan et al., 1991).

Clinical studies also suggest a role of endogenous opioids in PAG-DBS-induced analgesia. Early studies found that treatment with systemic naloxone blocks the analgesic effects of PAG-DBS in humans (Adams, 1976; Hosobuchi et al., 1977). A more recent study investigating dlPAG DBS-produced local field potentials also found that naloxone reversed the analgesia while increasing the 30–60 Hz band power measured at the same site, but this experiment was restricted to only two human subjects (Pereira et al., 2013). However, in a study of 45 patients with electrodes implanted in the PAG or periventricular gray (PVG), the attenuation of PAG-DBS pain relief by naloxone was similar in magnitude in both active and sham DBS conditions, suggesting the effect of naloxone may not specifically block PAG-DBS, but may instead enhance subjective pain ratings independent of stimulation (Young and Chambi, 1987). A study utilizing PET imaging to observe PAG opioid release found an increase in endogenous release during DBS, but it was not correlated with subjective analgesia (Sims-Williams et al., 2017). Furthermore, upon naloxone treatment, analgesia was still observed, with no significant effect to ongoing pain scores.

Additionally, it has been reported that patients may develop tolerance to chronic PAG-DBS stimulation and cross tolerance to opioids such that morphine becomes less effective after chronic PAG stimulation, suggesting occlusion of descending pain modulatory pathways and endogenous opioid release (Hosobuchi, 1986). However, other studies of PAG-DBS in humans have found tolerance to stimulation in other brain regions that are not presumed to function through endogenous opioid signaling and a lack of cross tolerance to morphine in chronic PAG-DBS (Young et al., 1985; Young and Chambi, 1987; Duncan et al., 1991). Finally, initial reports of endogenous opioid release driven by PAG stimulation in humans found increased enkephalin and beta-endorphin in cerebrospinal fluid of patients that had a positive, pain-relieving response to stimulation (Akil et al., 1978; Hosobuchi et al., 1979). Follow-up studies, however, found that this effect may be due to artifacts in immunoreactivity assays caused by contrast media (Dionne et al., 1984; Fessler et al., 1984). As a result of these collective studies, involvement of endogenous opioid peptides in PAG-DBS-driven analgesia remains unresolved.

Thalamus-deep brain stimulation

Compared to PAG-DBS, DBS in the sensory thalamus is thought to be more effective for deafferentation pain (Bittar et al., 2005), which is caused by damage to the peripheral or central nervous system that causes the loss of normal incoming pain signals. Examples of this type of pain include post-stroke pain, spinal cord injury, and facial anesthesia dolorosa (Hosobuchi et al., 1973; Adams et al., 1974). The theory behind the effectiveness of sensory thalamus DBS for this type of pain is that deafferentation pain is caused by a lack of normal proprioceptive information reaching the thalamus, which is combated by direct stimulation of VPL and VPM (Duncan et al., 1991). Additionally, stimulation may modulate the altered firing patterns in the sensory thalamus that are found in chronic pain patients (Dostrovsky, 2000; Moisset et al., 2020). When targeting sensory thalamus, stimulating electrodes are typically placed contralaterally and somatotopically according to the location of the painful area, and stimulation produces paresthesia in that area that masks pain (Hosobuchi et al., 1973; Boccard et al., 2015; Moisset et al., 2020). Comparatively, studies of sensory thalamic-DBS often use higher stimulus pulse frequencies than PAG-DBS, with frequencies falling between 50 and 100 Hz (Bittar et al., 2005; Moisset et al., 2020).

Deep brain stimulation (DBS) of medial thalamic centromedian-parafascicular nuclear complex (CM-Pf) has been attempted in humans under the assumption that this stimulation may activate descending pain modulatory opioidergic or non-opioidergic mechanisms, as well as drive a sensory feedforward loop with cortical targets (Andy, 1980; Duncan et al., 1991). While this manipulation appeared to be effective in a small cohort of patients with painful dyskinesia (Andy, 1980), other studies have produced variable results on reported painfulness and report a variety of potentially unpleasant side effects (Thoden et al., 1979; Hollingworth et al., 2017). Interestingly, a recent case study in 3 patients refractory to conventional neuromodulatory therapies found

potential therapeutic benefits of dual stimulation of CM-Pf and PAG/PVG using a single electrode at different frequencies (Hollingworth et al., 2017).

The different electrical stimulation parameters of successful PAG-and thalamic-DBS strongly suggest that these two therapies exert their effects through distinct neural mechanisms. Early neurostimulation trials provide further evidence for this distinction. Specifically, responsiveness to morphine is used throughout the literature to select patients for PAG-or thalamic-DBS. Patients that respond moderately well to morphine are selected for PAG-DBS, while those that do not respond well to high doses of morphine are still able to find pain relief *via* thalamic-DBS whereas PAG-DBS would be ineffective (Hosobuchi, 1986). Along these lines, centrally generated pain is attenuated by thalamic-DBS, whereas PAG-DBS is not effective. These findings, coupled with the observation of low-threshold spontaneous discharge patterns in midline thalamic nuclei associated with pain states (Andy, 1983), lead to the hypothesis that thalamic-DBS produces a "functional lesion" by inducing depolarization block and inactivating low threshold discharging neurons surrounding the stimulation electrode. This "functional lesion" mechanism has also been proposed to account for the anti-dyskinetic effects of subthalamic nucleus-DBS applied for Parkinson's disease, which shows pathological burst activity that correlates with onset of motor symptoms (Lobb, 2014). If an analogous mechanism of thalamic-DBS were confirmed, it presents the opportunity to trigger thalamic-DBS in response to nociceptive-related spontaneous discharge patterns of thalamic nuclei. Such closed-loop stimulation protocols have been increasingly adopted with STN-DBS for Parkinson's disease and have the advantage of reduced off-target effects and extended battery life by requiring only intermittent stimulation.

Anterior cingulate cortex-deep brain stimulation

In contrast to PAG and thalamus which have been targeted with electrical stimulation for pain relief for over 30 years, DBS of dorsal ACC (dACC) has only recently emerged as treatment for neuropathic pain. In an initial case report (Spooner et al., 2007), a single patient with neuropathic pain resulting from a spinal cord injury received bilateral dACC-DBS electrodes and a unilateral electrode in the PVG. In this patient, DBS applied to the dACC at 130 Hz provided superior pain relief, mood improvement, and reduction in medication usage compared to PVG-DBS applied at 20 Hz. This treatment resulted in reduced pain as assessed *via* visual analog scale (VAS) pain ratings and pain medication usage. This patient also showed improved mood in terms of reduction of fear, anxiety, and depression, suggesting that dACC stimulation works at least in part by targeting pain affect.

Anterior cingulate cortex (ACC) stimulation in rodents can produce diverse behavioral effects depending on stimulus pulse frequency and which neuronal subtypes are stimulated. Unilateral electrical stimulation of the rodent ACC with intermittent trains of 100 Hz pulses (200 ms inter-train interval) induced fear-like freezing responses (Tang et al., 2005). Optogenetically activating ACC Thy1 + neurons at 20 Hz induced anxiodepressive behaviors, but did not increase the hindpaw withdraw threshold to mechanical stimuli (Barthas et al., 2015). Optogenetic activation at 10 Hz of CaMKII + excitatory ACC neurons (which partially overlap with the Thy1 + population) increased paw withdrawal thresholds in naïve mice, while inhibition reversed inflammatory pain-induced behavior (Kang et al., 2015). Further, nociceptive responses have been demonstrated to be attenuated in rodents following optogenetic and chemogenetic activation of subsets of ACC interneurons (Gu et al., 2015; Kang et al., 2015; Shao et al., 2021). These findings suggest that heterogeneity in both function, topography, and cellular architecture contribute to the diverse behavioral responses produced by ACC stimulation.

Clinical applications of ACC-DBS are typically applied at stimulation frequencies of approximately 130 Hz and stimulus pulse widths around 450 μs (Boccard et al., 2014, 2017). The efficacy of ACC-DBS has been shown for patients suffering from failed back surgery syndrome, poststroke pain, brachial plexus injury, cervical spinal cord injury, head injury, and pain of unknown origin (Boccard et al., 2014). Interestingly, some patients receiving ACC-DBS do not report significant reductions in pain as measured by numerical rating scales. However, many ACC-DBS patients report improvements in metrics related to the affective component of pain as well as overall improvements in quality of life and describe their pain as being "separate from them" or "not distressing" (Boccard et al., 2017).

Due to its novelty, there are few published studies on ACC-DBS mechanisms of action. However, the ACC projects to many pain matrix structures, such as amygdala and PAG (Shi et al., 2022). Therefore, it is possible that the analgesic effects of ACC-DBS are due to postsynaptic DPMS engagement. MORs are present both on local ACC cells and afferents (particularly from the thalamus) terminating in the ACC (Vogt et al., 1995). Furthermore, it is understood that terminating afferents are highly excitable near DBS electrodes (Bower and McIntyre, 2020). This suggests that local opioid release could occur during ACC-DBS to either engage the DPMS or suppress thalamocortical relay of noxious sensory information. Preclinical and clinical data are needed to test these hypotheses.

Motor cortex stimulation

For more superficial brain targets, some researchers and physicians have opted for intracortical or epidural stimulation. Using this method, a craniotomy is performed, and electrodes are placed on the surface of the brain in the epidural space. Intracortical stimulation (ICS) is used for patients with chronic

neuropathic pain that cannot be treated by medication and does not respond to other forms of stimulation, such as post-stroke pain (Moisset et al., 2020). For chronic pain patients, ICS is mostly performed on the surface of the motor cortex in a procedure called intracortical motor cortex stimulation (iMCS). iMCS is typically applied at stimulus frequencies between 30 and 90 Hz and requires constant, continuous stimulation *via* an implanted device for patients to continue the therapy at all times (Fontaine et al., 2009; Lefaucheur et al., 2009). The stimulus pulse amplitude is set at 80% of the amplitude necessary to elicit a motor response, but is generally imperceptible to the patient (Moisset et al., 2020).

Primary motor cortex (M1) is not particularly rich in endogenous opioid peptides or receptors. Rat M1 exhibits radiolabeled ligand binding at MORs at intermediate levels in layers I and VI, but the level of MOR expression is much less than in nearby limbic cortical areas. Ligand binding to DORs is also very low (Lewis et al., 1983). Similarly, M1 dynorphin and enkephalin immunoreactivity reveals extremely sparse expression of these endogenous opioids (Fallon and Leslie, 1986). However, because M1 stimulation is thought to activate the DPMS, endogenous opioid signaling in downstream circuits could still be an important mechanism of action. In the rat, iMCS has been shown to effectively activate M1 layer V output neurons *via* transsynaptic mechanisms, underscoring a mechanism by which superficial electrodes can affect motor cortex output (Hussin et al., 2015). In rodents, iMCS activates PAG and decreases activity in the DH, as assessed by recordings of neuronal activity and immunohistochemistry for immediate early genes, such as cFOS (Pagano et al., 2012; França et al., 2013). Some of the strongest evidence implicating endogenous opioid signaling in M1 stimulation-driven analgesia arises from the finding in rats that the resulting analgesia is consistently blocked by systemic naloxone (Fonoff et al., 2009). Further, PAG naloxone pretreatment in rats blocked the inhibition of sensory evoked potentials in the somatosensory cortex induced by M1 stimulation (Chiou et al., 2013). These preclinical data suggest that release of endogenous opioids may be a key component of iMCS-induced analgesia.

Exactly how M1 stimulation activates the DPMS remains unclear. In rats, iMCS activates striatum, cerebellum and some thalamic areas, while responses to noxious stimuli in VPL, S1, and PFC are inhibited (Jiang et al., 2014; Kim et al., 2016). In humans, functional imaging and electrophysiological studies have revealed that iMCS rapidly activates lateral thalamus. Hours later, activation of medial thalamus, ACC, orbitofrontal cortex (OFC), and PAG is observed. The PAG receives input from ACC and OFC, and functional connectivity between ACC and PAG in particular is associated with pain suppression in the contexts of opioid analgesia, placebo analgesia, and attentional analgesia (García-Larrea et al., 1999; Peyron et al., 2007). It is plausible to hypothesize that the prefrontal pain modulatory network engages the PAG, yet it remains unclear precisely how M1 stimulation recruits the prefrontal cortex and how this unfolds on such a slow timescale. The precentral gyrus in the macaque, which contains M1, additionally sends projections to PAG, suggesting a possible direct route for DPMS activation *via* iMCS (von Monakow et al., 1979).

In a meta-analysis of 14 studies that used iMCS in 210 chronic pain patients, subjective classification of outcomes yielded a positive response to iMCS in ∼55% of patients, which dropped to 45% in patients that were able to be assessed more than 1 year later. For the patients that provided visual analog scale scores of pain, their pain ratings improved by 56% after receiving the intracortical stimulation. Importantly, however, in the two studies that had internal controls for stimulation by cycling through "on" and "off" stimulation periods, patients did not show significant differences in pain outcomes between the two (Fontaine et al., 2009), suggesting the possibility that at least some aspects of iMCS pain relief result from placebo effects. Alternatively, "wash-out" effects of stimulation or induction of plasticity may also contribute to persistently reduced pain outcomes during the "off" stimulation periods. Future experiments are required to parse the contribution of these factors.

As assessed by PET imaging using [11C]diprenorphine, iMCS leads to endogenous opioid release in patients with refractory neuropathic pain in anterior midcingulate cortex (aMCC), PAG, PFC, and cerebellum, with aMCC and PAG changes correlating with pain relief (Maarrawi et al., 2007). Additionally, high opioid receptor availability in insula, thalamus, PAG, ACC, and OFC were positively correlated with later MCS pain relief efficacy (Maarrawi et al., 2013). However, another study appears to challenge the evidence pointing to endogenous opioid recruitment of the DPMS by iMCS. Although M1 stimulation increased discharge rates in LC neurons in rats experiencing neuropathic pain, lidocaine block of LC or intrathecal alpha2-adrenergic antagonists did not attenuate M1 stimulation-induced antinociception in neuropathic pain or control rats (Viisanen and Pertovaara, 2010). Continued study is needed to elucidate the exact mechanisms of endogenous opioid release during iMCS, and how it may correlate with resultant analgesia.

Repetitive transcranial magnetic stimulation

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive neurostimulation method during which an electromagnetic coil is placed against the scalp in alignment with a target brain region. A current is passed through the coil to produce pulsatile changes in the magnetic field surrounding the coil. This magnetic field passes through the skull and into the brain, where it induces electrical currents which modulate the activity of neurons in target regions. rTMS is

most commonly used to treat depression in patients who are unresponsive to or unable to tolerate medications (Speer et al., 2000). However, a systematic review of the literature concluded that rTMS is effective for central pain, peripheral nerve disorders, fibromyalgia, and migraine, and that studies using rTMS for orofacial pain, phantom limb pain, lower back pain, and complex regional pain syndrome were promising but inconclusive (Yang and Chang, 2020). Importantly, when targeted to the appropriate brain regions, the reported rTMS effects are pain-specific (Nahmias et al., 2009).

Repetitive transcranial magnetic stimulation (rTMS) treatment paradigms are widely used in the clinic and are therefore highly standardized. Typically, a patient receives rTMS for several min per session, undergoing 10s of sessions over several months. rTMS frequencies for pain treatment range between ∼0.5 and 10 Hz, with the consensus being that frequencies greater than 5 Hz are most effective (Lefaucheur et al., 2006; Moisset et al., 2015). rTMS has been extensively studied at two sites: the dlPFC, based on its accessibility and role in pain processing, and primary motor cortex (M1). M1 rTMS has been consistently reported to provide pain relief in both chronic pain patients and experimental models of pain (Lefaucheur et al., 2006; Nahmias et al., 2009; de Andrade et al., 2011; Moisset et al., 2015). Although there is some disagreement in the literature (Yoo et al., 2006), there is a general consensus that dlPFC rTMS also provides pain relief in models of experimental pain in healthy subjects (Graff-Guerrero et al., 2005; Borckardt et al., 2007; Nahmias et al., 2009; Valmunen et al., 2009; de Andrade et al., 2011; Taylor et al., 2012). While rTMS is performed contralateral to the painful site, bilateral analgesia can be evoked in humans (Nahmias et al., 2009). M1 rTMS produces bilateral analgesia in healthy patients that does not affect thermal detection thresholds, which points toward a role for diffuse descending pain modulation (Nahmias et al., 2009). rTMS provides both short-term pain relief immediately after the stimulation session, which may take 2–3 days to reach its peak, as well as long term relief that lasts for weeks to months after the end of session in contrast with the previously introduced stimulation techniques (Lefaucheur et al., 2001, 2006). Interestingly, the impact on pain affect lasts longer than on the sensory component of pain (Passard et al., 2007).

In humans, evidence for the involvement of endogenous opioids in M1 rTMS-induced analgesia has emerged from studies in healthy subjects in which naloxone blocked the rTMS-induced short-term analgesia. However, dlPFC studies by different groups reached different conclusions. A landmark study found that naloxone attenuated the analgesic effect of M1 stimulation but not dlPFC or sham rTMS (de Andrade et al., 2011), whereas another study found that naloxone blocked the analgesic effect of dlPFC rTMS (Taylor et al., 2012). A PET study using the radioligand [11C]carfentanil administered several hours after rTMS treatment of a diffuse area containing M1 and primary somatosensory cortex in healthy subjects revealed endogenous opioid release in the ipsilateral ventral striatum, mOFC, PFC, ACC, contralateral insula, superior temporal gyrus, dlPFC, and precentral gyrus, without impacting striatal D2 receptor availability (Lamusuo et al., 2017).

Transcranial direct current stimulation

Transcranial direct current stimulation (tDCS) applies low levels of electrical current *via* small battery powered electrodes placed on the head. Although it is not currently approved by the Federal Drug Administration in the United States as its regulatory status is only "investigational," studies on small cohorts have shown promising results for the use of tDCS in patients with fibromyalgia, spinal cord injury, and migraine (Fregni et al., 2006a,b; Dasilva et al., 2012). In other studies, however, tDCS was not effective for chronic low back pain or in spinal cord injury (O'Connell et al., 2013; Wrigley et al., 2013). Similar to iMCS and rTMS, tDCS appears most effective when applied over the motor cortex. Interestingly, PET imaging for radiolabeled opioids revealed motor cortex tDCS-driven endogenous opioid release, which reveals a possible mechanism for the measured improvements in thermal pain thresholds (DosSantos et al., 2014). Although both tDCS and placebo stimulation caused endogenous opioid release in PAG and precuneus, tDCS alone produced analgesia and additional opioid release in left PFC. Though naloxone was not administered to determine the causality of opioid signaling in the observed analgesia, these studies suggest opioidergic signaling is responsible at least in part for the tDCS-induced pain relief.

Future outlook

Technological innovation

Stimulus pulse paradigms

In recent years, there have been several innovations regarding the electrical stimulus waveforms applied by neurostimulation therapies for chronic pain. With SCS, many of these innovations apply tonic SCS at frequencies not typically utilized by conventional (i.e., 40 to 60 Hz) SCS. Kilohertz frequency SCS (KHFSCS), ultra-low frequency SCS (ULFSCS), and burst SCS all provide pain relief without producing paresthesias. KHFSCS utilizes frequencies greater than 1,000 Hz (Kapural et al., 2015), while ULFSCS applies frequencies below 0.1 Hz (Jones et al., 2021). Burst SCS employs bursts of SCS pulses at ∼40 Hz with an intraburst frequency of 500 Hz (de Ridder et al., 2013). Similar to conventional SCS, the physiological mechanisms of analgesia for each of these novel forms of SCS are unknown, presenting the same challenges to improving their design and implementation. However, these

paresthesia-free SCS waveforms allow for placebo-controlled clinical studies, providing exciting new opportunities to systematically examine the effects of these new therapies in the patient's pain experience.

In addition to new tonic SCS waveforms, new stimulus paradigms are emerging in clinical neuromodulation. Differential targeted multiplexed SCS (DTMSCS) applies two simultaneous SCS waveforms: a lower frequency 50 Hz waveform, and a higher frequency 1,200 Hz waveform (Vallejo et al., 2020). It is hypothesized that in addition to inducing conventional segmental pain inhibition, DTMSCS also affects properties of spinal glial cells (Vallejo et al., 2020). A recent innovation in DBS, Coordinated Reset DBS (CRDBS), applies precisely timed, spatially distributed stimuli to desynchronize pathological brain activity, possibly by rectifying aberrant synapses which were remodeled by disease conditions (Tass, 2003). Interestingly, CRDBS may produce long-lasting therapeutic benefit, even after the stimulus pulse is switched off (Wang J. et al., 2016). These stimulus paradigms suggest that it is critical to consider the effects of neuromodulation therapies on pre-and post-synaptic terminals and on non-neuronal cells, and that improving our scientific understanding of how the timing of exogenous electrical stimuli is integrated by neurons and synapses may allow for the evidence-based design of novel stimulus protocols which directly target the synaptic basis of pathological neural activity.

Closed-loop neurostimulation

A major challenge in diagnosis and treatment of chronic pain conditions is that there are no objective biomarkers of the pain experience. Most existing neurostimulation therapies apply stimulation in an "open-loop" fashion, where electrical stimuli are delivered at a constant frequency with no variation in intensity or rate. Given temporal fluctuations in severity of pain symptoms in chronic pain patients, modulating stimulation in response to changes in neural activity or behavioral biomarkers would represent an important treatment advance and may prevent tolerance by delivering stimulation only when needed and limiting unwanted side effects. Closed-loop approaches are beginning to be adopted in neurostimulation for pain, such as monitoring the amplitude of evoked compound action potentials recorded from the dorsal columns to modulate SCS pulse amplitudes. This approach was recently demonstrated to provide superior pain relief compared to open-loop SCS (Mekhail et al., 2020). Improving our understanding of how chronic pain pathogenesis and neurostimulation therapies affect the characteristics and behavior of opioidergic (and other) circuits could reveal new biomarkers with which to design closed-loop stimulation algorithms.

Alternate sites for neurostimulation

Continued study of the complicated matrix of brain areas involved in pain processing has revealed other targets that may provide therapeutic benefit by neurostimulation, including the insular cortex (IC). The IC can be divided along the anterior-posterior axis, with the posterior insula (pI) participating in somatosensory features of pain, whereas the anterior portion (aI) is implicated in encoding pain unpleasantness (Craig, 2002).

Low frequency electrical stimulation of the right pI elicits nociception in humans and primates with some somatotopy (Ostrowsky et al., 2002; Mazzola et al., 2009), while high frequency stimulation of pI and aI reduces pain thresholds with no obvious side effects, consistent with insular inactivation (Denis et al., 2016; Liu et al., 2021). A form of rTMS in IC has been shown to produce bilateral thermal analgesia in humans without affecting the ability to perceive innocuous thermal or vibrotactile sensations (Lenoir et al., 2018). Similarly, pI-rTMS increases thermal pain thresholds in patients with central neuropathic pain, but this did not translate to differences in relief from chronic pain and quality of life (Galhardoni et al., 2019). Although studies have not yet extended ICS to the human insula, one preclinical study in rodents suggests a potential role for low frequency intracortical pI stimulation in relief from chronic neuropathic pain. Importantly for this review, all forms of analgesia examined in this study were blocked by naloxone, clearly implicating endogenous opioid release (Komboz et al., 2022). Although opioid peptides and receptors are prominent in pI, it remains to be determined whether local opioid signaling, activation of afferents from other structures, or projections to the DPMS are involved. Innovation in the brain areas targeted by neurostimulation techniques may elucidate stimulation paradigms that provide pain relief in the absence of adverse side effects.

Innovating clinical paradigms

Pharmacological adjuvants

A key challenge with electrical stimulation of any neural structure is the cellular heterogeneity of the target. Electrical stimulation is inherently non-specific; all neurons in the vicinity of the electrode are subject to modulation, which presents a challenge when the target structure is comprised of diverse neuronal subtypes which may play distinct or even opposing functional roles in neural circuits. In some cases, it may be advantageous to preferentially modulate specific subpopulations of neurons within a target structure. For example, the PAG can be subdivided into populations of glutamatergic and GABAergic neurons with subpopulations of each type projecting to the RVM to drive descending pain modulation. We hypothesize that MOR-expressing PAG-RVM projection neurons may facilitate pain, since they are inhibited by opioid analgesics. Thus, selective recruitment of the MOR-lacking PAG projection neurons using electrical stimulation may produce the most effective pain relief.

We recently demonstrated that pharmacological adjuvants can be combined with DBS to enhance its specificity (Creed et al., 2015; Creed, 2017). Pharmacological adjuvants have also been applied in preclinical (Cui et al., 1996) and clinical (Lind et al., 2004, 2007) studies of SCS, suggesting that co-application of SCS and the $GABA_B$ receptor agonist baclofen may increase analgesia compared to the application of a single therapy alone. Currently, the combined approach of simultaneous electrical and chemical neuromodulation is not widely adopted in the clinical neuromodulation field. However, characterizing differences in ion channel or receptor expression between functional subpopulations of a target structure could identify pharmacological targets to be chemically manipulated during concurrent electrical stimulation. A dual electrical and chemical modulatory approach may allow for greater symptom control in cases where symptoms of a given disease are governed by biochemically distinct neuronal subpopulations. This approach may improve the specificity of a therapy, and thus increase efficacy while limiting off-target effects.

The advent of non-invasive, region-specific drug delivery and devices capable of delivering simultaneous electrical and chemical stimulation (Capogrosso et al., 2018) makes this an even more exciting and tractable possibility. Recently, focused ultrasound has been used to target drug release to specific sites in the brain in a non-invasive manner (Airan and Butts Pauly, 2018; Wang et al., 2018; McMahon et al., 2021). We anticipate that light-driven activation of drugs and neurotransmitters (i.e., photopharmacology) will also emerge as a viable approach that offers improved spatial and temporal precision for *in vivo* drug delivery (Banghart and Sabatini, 2012; Font et al., 2017; Hüll et al., 2018; López-Cano et al., 2021). Photopharmacology may interface particularly well with DBS and iCS, as light sources can be readily incorporated into stimulating electrodes (Royer et al., 2010; Lechasseur et al., 2011).

Early stimulation

Neurostimulation therapies are usually reserved for patients who are treatment refractory to every other standard of care in chronic pain conditions and for other neurological and psychiatric disorders. However, chronic pain, like other neurological and psychiatric disorders, is a disease of neural plasticity, with reorganization of neural pathways involved in pain and affective processing contributing to the persistence of pain symptoms. Recently, it has been proposed that patients receiving stocktickerSCS to manage their chronic pain would benefit from implementing the therapy earlier in disease pathogenesis (Kumar et al., 2014; Taylor et al., 2014; Lad et al., 2016; Campos et al., 2019). Along the same lines, novel DBS protocols have been shown to effectively reverse maladaptive plasticity associated with behavioral symptoms in Parkinson's disease (Wang J. et al., 2016; Mastro et al., 2017; Spix et al., 2021) and addiction (Creed et al., 2015; Lüscher et al., 2015). Because these protocols alter plasticity in neural circuits, their therapeutic effects outlast the duration of stimulation, which is in stark contrast to classically applied tonic ~100 Hz DBS in which motor or psychiatric symptoms reappear nearly immediately after DBS offset (Lüscher et al., 2015). An intriguing prospect would be to apply DBS in patients with pain disorders before nociceptive and affective circuitry undergo pain-induced plasticity that contributes to affective comorbidities or cognitive symptoms of chronic pain (Andrade et al., 2013). Alternatively, designing DBS protocols capable of normalizing chronic pain-induced synaptic adaptations in nociceptive processing pathways would hold enormous therapeutic promise.

Novel pain assessment metrics

Accurate assessment of treatment efficacy is crucial for any therapy. The success of neurostimulation therapies for chronic pain is typically defined as a $\geq$ 50% reduction in a patient's overall pain, measured by the visual analog scale (VAS), verbal rating scale (VRS), or numeric rating scale (NRS). However, subjective measurements made with different scales are not always comparable (Ohnhaus and Adler, 1975; Lund et al., 2005) and may suffer from low reproducibility (van Tubergen et al., 2002). Furthermore, some have shown that the percentage of a patient cohort satisfied with SCS is disproportionately greater than the percentage of the cohort which met the $\geq$ 50% reduction in VAS (Sears et al., 2011). Taken together, these findings suggest that novel, holistic assessments of a patient's pain experience may more accurately capture the efficacy of a neurostimulation therapy than a single pain rating alone. Some have suggested that dynamic pain measures, such as temporal summation and conditioned pain modulation, which are proxy measures for central sensitization and descending inhibitory tone respectively, may hold clinical value in both patient selection and assessing the efficacy of SCS (Yarnitsky et al., 2010; Campbell et al., 2015; Sankarasubramanian et al., 2019, 2021). Others have demonstrated that composite metrics which incorporate measurements of pain intensity, physical functioning, quality of life, and affect more closely represent the patient's impression of therapeutic benefit (Pilitsis et al., 2021). These measures could provide a more accurate and reliable readout of a patient's experience with a therapy for use during stimulator programming and as primary endpoints in clinical trials of neurostimulation therapies.

Improving our mechanistic understanding to improve therapeutic strategies

A key limitation facing all neurostimulation therapies is that we do not understand their therapeutic mechanisms of action. Uncovering these mechanisms may allow for the evidence-based design of targeted therapies which produce robust therapeutic

benefit with minimal side effects. The study of neurostimulation therapies highlights several key knowledge gaps pertaining to understanding the neural substrates of symptom management in neurological disorders.

The rationale for selecting an implant location for a neurostimulation therapy, such as ACC-DBS, is often based on historical lesioning studies (Boccard et al., 2017). However, the selection of stimulus pulse parameters is largely initially arbitrary and empirically adjusted based on subjective patient feedback. The stimulus pulse frequencies which produce therapeutic benefit in the many therapies discussed in this review are quite variable, with some therapies using pulse frequencies greater than 100 Hz, while others use pulse frequencies closer to 20 Hz. A notable finding of the involvement of endogenous opioids in preclinical SCS studies is that opioidergic analgesia during SCS may be dependent on stimulus pulse frequency. SCS applied at 60 Hz required higher doses of naloxone to abolish SCS-induced analgesia and was sensitive to a DOR antagonist, while SCS applied at 4 Hz required lower doses of naloxone to abolish analgesia and was not sensitive to a DOR antagonist (Sato et al., 2013). These data imply that the stimulus pulse frequency, putatively the rate at which axons near the stimulating electrode are conducting artificially generated APs (McIntyre et al., 2004), may affect the characteristics of neurotransmitter release from the presynaptic terminals of stimulated neurons. Future studies should examine how varying stimulus pulse frequency affects neurotransmitter release and pre-and post-synaptic receptor activation.

Many studies of neurostimulation therapies focus on the effects of stimulation on the neurons which are directly responding to the stimulus pulse. However, the resulting effects on postsynaptic networks are likely complex and intricately involved in symptom relief. Novel experimental techniques to study the activity of large networks such as *in vivo* calcium imaging (Göbel and Helmchen, 2007) and high-density electrical recordings (Jun et al., 2017; Juavinett et al., 2019; Steinmetz et al., 2021) provide the opportunity to monitor the behavior and properties of neural networks over time. These techniques could be used to observe the network response to neurostimulation therapies (Trevathan et al., 2021). Crucially, these methods also allow for the characterization of network properties across different behavioral states (Sweeney et al., 2021). Comparing network properties during both pain pathogenesis and intervention could give key insights into the development of neurological disease and reveal novel methods for targeted intervention.

Conclusion

Neurostimulation therapies are important tools in managing intractable chronic pain. Our incomplete understanding of the mechanisms of action of such therapies precludes their improvement to maximize pain relief. In this review, we summarized the evidence that many neurostimulation therapies for pain may provide analgesia in part by modulating opioidergic circuits throughout the neuraxis. Further study is needed to understand the mechanisms by, and extent to which, neurostimulation therapies modulate these circuits. Continued study of the interactions between exogenous electric fields and neuronal and synaptic dynamics will be critical to the evidence-based design of neurostimulation therapies which specifically target mechanisms underlying neurological disease. We believe that a multidisciplinary approach combining basic neurobiological studies, innovation in clinical paradigms, and novel technology development will be key to engineering the next generation of safe and effective therapies for chronic pain.

Author contributions

SL, RG, GL, RS, MB, and MC wrote and edited the manuscript. All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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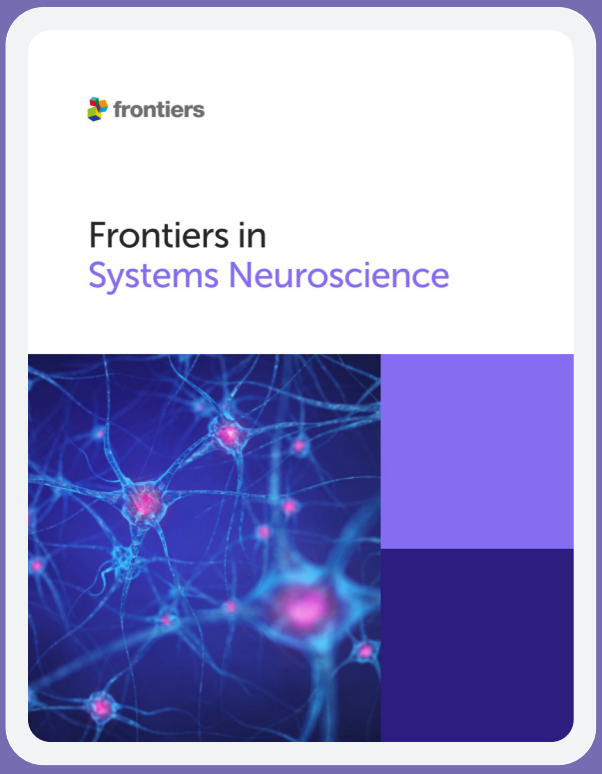

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