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Title page

Pain, Motivation, Migraine and the Microbiome: New Frontiers for Opioid Systems and Disease

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Running Title Page

Running title: New Frontiers for Opioid Systems and Disease

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Non-Standard Abbreviations:

BNST Bed nucleus of stria terminalis

CGRP Calcitonin gene related peptide

CeA central nucleus of the amygdala

CFA Complete Freud's Adjuvant

CPP Conditioned place preference

CSD Cortical spreading depression

DA Dopamine

DOR Delta opioid receptor

DREADDS Designers receptors exclusively activated by designer drugs

HPA Hypothalamic pituitary axis

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KCl Potassium Chloride

KOR Kappa opioid receptor

LPS Lipopolysaccharide

MDD Major Depressive Disorder

MAPK mitogen-activated protein kinase

M3G Morphine-3-glucuronide

M6G Morphine-6-glucuronide

MOR Mu opioid receptor

NAc Nucleus Accumbens

N/OFQ Nociceptin

NOP Nociceptin opioid receptor

NTG Nitroglycerin

OIH opioid induced hyperalgesia

PACAP Pituitary adenylate cyclase-activating polypeptide

PNI Peripheral nerve injury

pnVTA Paranigral nucleus of the VTA

PR Progressive ratio

SCG Secretogranin

TBI Traumatic brain injury

TH Tyrosine hydroxylase

TLR2 Toll-like receptor 2

VIP Vasoactive intestinal peptide

vNAcSh Ventral NAc shell

VTA Ventral tegmental area

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Abstract

For decades the broad role of opioids in addiction, neuropsychiatric disorders and pain states has been somewhat well established. However in recent years, with the rise of technological advances, the existing dogma is not only being challenged but we are identifying new disease areas in which opioids play a critical role. This review highlights four new areas of exploration in the opioid field. The most recent addition to the opioid family, the nociceptin receptor system shows promise as the missing link in understanding the neurocircuitry of motivation. It is well known that activation of the kappa opioid receptor system modulates negative affect and dysphoria but recent studies now implicate the kappa opioid system in the modulation of negative affect associated with pain. Opioids are critical in pain management, however the often forgotten delta opioid receptor system has been identified as a novel therapeutic target for headache disorders and migraine. Lastly, changes to the gut microbiome have been shown to directly contribute to many of the symptoms of chronic opioid use and opioid related behaviors. This review summarizes the findings from each of these areas with an emphasis on identifying new therapeutic targets.

Significance Statement

The focus of this mini review is to highlight new disease areas or new aspects of disease in which opioids have been implicated, this includes pain, motivation, migraine and the microbiome. In some cases this has resulted in the pursuit of a novel therapeutic target and resultant clinical trial. We believe this is very timely and will be a refreshing take on reading about opioids and disease.

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Introduction

Traditionally, reviews of the opioid system in behavior have focused on the role of the mu, delta and kappa opioid receptors in addiction, pain and more recently stress. In celebration of INRC's 50th anniversary we have chosen to honor the pioneering research on the diverse functions of the opioid system in disease by highlighting the most recent and more novel research actively ongoing. This includes the nociceptin system, which has gained traction in recent years as a key player in modulation of motivated states. It is well known that the kappa opioid system drives negative affective states, however recent findings show that negative affect is a critical component in pain processing. The delta opioid system, which has had a turbulent journey through behavioral research shows promise in the regulation of headache disorders and migraine. Finally, the most widely studied opioid receptor, the mu opioid receptor greatly impacts gut microbiota composition and contributes to symptoms of chronic opioid use disorder. The fact that the behaviors highlighted in this review are somewhat independent, diverse and involve opioid receptors expressed both peripherally and centrally adds strength to the ever growing field of opioid receptor function in behavior and disease.

Nociceptin Neurocircuitry and Motivation

Motivation and reward-related neurocircuitry serve a critical role in regulating the internal states and responses to environmental conditions that enable an organism to adapt and survive. Particularly, dysfunction within these motivational processes can lead to reward-related behaviors that manifest into severe behavioral phenotypes including mood, substance use, and eating disorders (Volkow *et al.*, 2011; Russo and Nestler, 2013). Additionally, environmental stressors can have an enormous impact on the output of this neurocircuitry, resulting in adaptive changes that can have long term effects on reward-seeking behaviors (Koob and Volkow, 2016). Efforts to understand the anatomical specificity of reward motivation and its regulation by neuropeptides have provided some converging evidence that supports the nociceptin system as a candidate to modulate stress responsivity and reward-seeking behaviors (Toll *et al.*, 2016). The endogenous neuropeptide, nociceptin (N/OFQ), and its receptor, nociceptin opioid peptide receptor (NOP) are widely distributed throughout the brain (Darland *et al.*, 1998). Not only has this system been implicated in modulating normal appetitive behaviors, it is also implicated in a variety of psychiatric illnesses related to depression, substance abuse, binge eating, and

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anxiety (Mollereau *et al.*, 1994; Mollereau and Mouledous, 2000; Norton *et al.*, 2002; Zheng *et al.*, 2002; Ozawa *et al.*, 2015; Toll *et al.*, 2016; Der-Avakian *et al.*, 2017). Importantly, many of the underlying negative affective states integral to these illnesses may be modulated by a wide and diverse network of N/OFQ-NOP neurocircuitry. It is well known that N/OFQ and NOP mRNA expression dramatically overlap with multiple key feeding, reward, and stress-related brain regions. Specifically, anatomical studies highlight this neuromodulator system's network of peptide and receptor interactions spanning multiple stress- and reward-related brain nuclei including the nucleus accumbens (NAc), striatum, bed nucleus of stria terminalis (BNST), the central nucleus of the amygdala (CeA), hippocampus, ventral tegmental area (VTA), and several hypothalamic areas (Anton *et al.*, 1996; Darland *et al.*, 1998; Mollereau and Mouledous, 2000). This system has been suggested to have a natural role in regulating an animal's food-seeking behavior and as a result has modulatory influence in reward-seeking behaviors, generally. Additionally, its presence within the hypothalamic pituitary axis (HPA) circuitry places it as a prominent candidate to influence stress responsivity and affective state (Devine *et al.*, 2001). Yet, how, where, and what specific neurocircuitry coordinates this intersection of stress, reward, and motivation remains unresolved and an important avenue for research. As such, recent investigations have pursued NOP agonists and antagonists as alternative therapeutics for mood disorders, such as depression and anxiety, as well as binge-eating and substance use disorders.

N/OFQ-NOP in Motivation and Major Depressive Disorder

One in six Americans suffer from Major Depressive Disorder (MDD) and an estimated one-third of these patients treated with current therapies are resistant to treatment (Rush *et al.*, 2006; Greenberg *et al.*, 2015). Mood disorders, including MDD and bipolar disorder, have been linked to reward-processing deficiencies, that contribute to the functional impairments that define these disorders(American Psychiatric Association, 2013; Whitton *et al.*, 2015). These reward processing and motivational deficits are generally classified as anhedonia, a loss of interest or pleasure, and avolition, a lack of motivation to perform tasks. Recent evidence proposes that different psychiatric disorders may present subtle differences in particular reward processes, such as consummatory pleasure, motivation, and reward learning (Treadway and Zald, 2011; Barch *et al.*, 2016). Additionally, studies have found that MDD patients with anhedonia have poorer prognoses (Vrieze *et al.*, 2014) and have a higher prevalence of treatment failure (McMakin *et al.*, 2012). This prevalence of anhedonia and

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avolition suggests that aberrant reward processing is a core feature underlying depression pathophysiology. Moreover, stressful conditions can precipitate these conditions, or the expression of related symptoms in healthy individuals (Berenbaum and Connelly, 1993; Kendler *et al.*, 1999; Charney and Manji, 2004), as well as deficits in brain reward system function in laboratory animals (Der-Avakian *et al.*, 2014; Donahue *et al.*, 2014). Although pharmacotherapies are widely prescribed, significant limitations such as intolerable side effects, delayed antidepressant action, and low efficacy following treatment remain as consistent barriers to successful treatment (Berton and Nestler, 2006). As such, there is an urgent need for a better understanding of the pharmacological, behavioral, neuroanatomical aspects of neuropeptidergic systems that could present more effective therapeutic approaches in the development of successful antidepressants (Werner and Coveñas, 2010)

Early preclinical studies unveiled the N/OFQ-NOP receptor system as a potential candidate to modulate mood-related disorders as NOP antagonist administration was demonstrated to have antidepressant-like effects in rodent models of depression. Specifically, NOP receptor antagonists reduced immobility time in the forced swimming test in mice, a measure considered indicative of antidepressant-like behavior (Redrobe *et al.*, 2002). Additionally, converging evidence from genetic knockout studies demonstrated that NOP knockout mice and rats display reduced immobility time in forced swimming and tail suspension tests compared with wild-type controls (Gavioli *et al.*, 2003, 2004). Further, investigators at Eli Lilly developed the potent and selective NOP receptor antagonist, LY294009448, now named BTRX-246040 (Toledo *et al.*, 2014) and found antidepressant-like behavioral effects in the forced swimming test in mice, which was absent in NOP knockout animals (Witkin *et al.*, 2016). Other recent studies have corroborated these previous findings by demonstrating NOP antagonists effect to reduce depressive-like behaviors induced by lipopolysaccharide (LPS) administration (Medeiros *et al.*, 2015), repeated, uncontrollable foot-shock (Holanda *et al.*, 2016), and unpredictable chronic mild stress (CMS) (Vitale *et al.*, 2009) which all can be reversed by classical antidepressants. Additionally, Der-Avakian and colleagues (2017) found that repeated social defeat stress induced reward learning deficits in rats that resulted in increased N/OFQ mRNA expression in the nucleus accumbens shell as well as increased NOP mRNA in the VTA. This study also found that repeated social defeat stress reduced Fos mRNA expression in the VTA, indicating a reduction in neuronal activity.

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Given the previously described data, it is critical to better understand how changes in reward neurocircuitry could manifest as depressive-like symptoms in humans. Early clinical investigation presented evidence of higher plasma levels of N/OFQ across different patient populations with depression with a reduction of these peptide levels following treatment with antidepressants (Gu *et al.*, 2003; Zhang *et al.*, 2009). Although these data suggest that elevated N/OFQ levels are associated with depression states, how these affective states are specifically driven by N/OFQ action is not completely understood. Complimentary studies examining different means of N/OFQ detection in real-time are warranted. Given these findings, recent clinical investigation of the NOP antagonist BTRX-246040 as a novel oral treatment for MDD has demonstrated possible clinical potential (Toledo *et al.*, 2014; Post *et al.*, 2016). This double-blind, parallel-group, fixed-dose, placebo-controlled, 8-week proof-of-concept study randomized 136 patients to receive BTRX-246040 (N=70) or placebo (N=66) at 11 different sites in the US. Patients who met criteria for MDD without psychotic features (as defined by the Diagnostic and Statistical Manual of Mental Disorders 4th Edition Test Revision, DSM-IV-TR) were evaluated using the GRID-Hamilton-17 Depression Rating Scale, 17 items (GRID-HAMD-17) (Hamilton, 1959; Williams *et al.*, 2008). Once daily oral dosing (40 mg) of BTRX-246040 in these patients was evaluated for 8 weeks and with efficacy of treatment based on a change from baseline to 8 weeks when compared to placebo treatment. The Least-squares (LS) mean differences from placebo was −1.5 (95% CI −4.7, 1.7) and the probability that BTRX-246040 was better than placebo was 82.9%. Although this did not meet the pre-defined, proof-of-concept criterion, when analyses included the post-study 9-10 week follow-up data, this LS mean change was −2.9 and the probability that treatment with BTRX-246040 had a greater reduction in GRID-HAMD-17 total score than placebo was 97.4%. These findings established the first human data that provided evidence of NOP receptor antagonism as a potential strategy for the treatment of MDD. BTRX-246040 is currently under investigation in a double-blind, placebo-controlled Phase 2a study for MDD treatment.

These previous studies have laid the foundational work that presents the N/OFQ-NOP receptor system as a promising target for the treatment of MDD. However, how this activity is modulated pharmacologically and what specific neurocircuitry facilitate depressive-like behaviors has not yet been fully elucidated. In particular, understanding differences in particular reward processes, such as affect, motivation, and reward-learning could provide clinically relevant information to guide treatment. One major component of MDD is avolition, or the lack of motivation to perform tasks. Motivation involved in reward-seeking behavior is thought to be mediated via

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dynamic activity and neuroplastic alterations of VTA dopamine (DA) neurons and this activity is coordinated through a plethora of inputs, including nociceptin neurocircuitry. Notably, NOP is broadly expressed on VTA neurons positive for tyrosine hydroxylase (TH), the rate limiting enzyme for dopamine, and electrophysiological studies have reported that slice application of N/OFQ can inhibit dopamine neuron activity (Norton *et al.*, 2002; Zheng *et al.*, 2002). Further, converging behavioral pharmacology evidence has demonstrated that systemic activation of the NOP receptor can reduce reward-related behaviors, including food consumption or preference for drugs of abuse (Ciccocioppo *et al.*, 2000; Kotlińska *et al.*, 2002; Zhao *et al.*, 2003; Zaveri, 2011; Witkin *et al.*, 2014; Kallupi *et al.*, 2017). In particular, disruptions in activity of the mesolimbic dopaminergic system, specifically within the ventral tegmental area (VTA), have been implicated in regulating reward consumption, effort, and motivational drive (Stauffer *et al.*, 2016; Morales and Margolis, 2017). Recently, there has been important progress in identifying and characterizing neurocircuits that impact motivational states through regulation of VTA dopamine activity (Juarez and Han, 2016); however, how neuromodulators and specific endogenous neuropeptides engage and regulate motivation through the VTA and other limbic areas remains mostly unexplored. Recent evidence has suggested that there is remarkable heterogeneity of neuronal subtypes and anatomical localization within the VTA, as well as transmitter and neuropeptide systems that engage dopaminergic outputs (Jhou *et al.*, 2009; Tan *et al.*, 2012; van Zessen *et al.*, 2012; Morales and Margolis, 2017). Given this framework, new research has sought to understand the mechanisms by which the N/OFQ-NOP receptor system is interconnected to modulate reward-seeking behavior through circuit-based investigation on endogenous VTA regulation and how mesolimbic NOP activation impacts motivation and depressive-like states.

Although previous studies have paired pharmacological and behavioral techniques to interrogate the N/OFQ-NOP receptor system, relatively new preclinical tools such as optogenetics and chemogenetics have given investigators the opportunity to examine this system with cell-specific precision. Our recent work (Parker *et al.*, 2019) used optogenetics, chemogenetics, and fiber photometry (alongside genetic knock studies) to interrogate the role NOP and nociceptin neurocircuitry might play in the VTA, specifically, in the modulation of dopamine cell activity and its relation to reward motivation. Although several studies have demonstrated a role for NOP activation to inhibit DA activity, few studies have evaluated the behavioral consequence of NOP stimulation in VTA dopamine neurons. Here, we sought to reveal the nociceptinergic neurocircuitry that had

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functional connectivity with the VTA. In this study, we uncovered a unique subpopulation of prepronociceptin (Pnoc) neurons located within the paranigral nucleus of the VTA (pnVTA) that are engaged during reward-seeking behavior. These studies used a progressive ratio (PR) operant conditioning task in which the effort required to receive a sucrose reward exponentially increases following each subsequent reward presentation. This task is designed to directly measure the effort an animal is willing to expend to receive a reward. Eventually, the animal will reach a point in which it will no longer seek the reward called the "breakpoint" (Richardson and Roberts, 1996). Our data demonstrated that chemogenetic and optogenetic stimulation of these pnVTA^{Pnoc} neurons suppress the motivation to seek sucrose in the PR test, resulting in lower breakpoint and fewer rewards presented, while inhibition or ablation of these neurons increased animals' performance. Using fiber photometry, we also discovered that these pnVTA neurons are especially engaged during low-yield reward-seeking behavior and most active during the final nosepoke immediately preceding the animal's breakpoint. Additionally, this study found that pnVTA^{Pnoc} neuron stimulation was aversive, both in real-time and conditioned place preference tests. Further, we demonstrated that global NOP knockout, conditional NOP knockout within the VTA, and specific deletion of NOP from VTA dopamine cells dramatically enhanced reward-seeking behaviors in a manner similar to pnVTA^{Pnoc} neuron ablation and inhibition. We also demonstrated that the conditional rescue and stimulation of NOP in VTA dopamine neurons greatly diminishes motivation to seek sucrose rewards.

Collectively, these data demonstrate a uniquely specific role of intra-VTA nociceptin neuropeptide release and NOP activation that acts to constrain dopamine neuron activity during reward-seeking behavior. In our case, an absent VTA nociceptin or NOP system allowed excessive reward-seeking under conditions in which a normally functioning NOP system would typically engage and promote appropriate operant responding for sucrose through dopamine neuron inhibition. Although this investigation provides insights into the coordination of motivated behavior, these data likely represent only a small portion of the neurocircuitry involved. Taken together our study supports the conclusion that VTA NOP expression is critical for maintaining natural reward-seeking behavior and that functional changes in expression could manifest into altered reward seeking. It is possible that aberrant changes in this nociceptinergic neurocircuitry activity could manifest as the depressive-like symptomology present in individuals with MDD. Avolition represents only a portion of MDD symptomology, yet these behavioral differences could also reveal insights into the comorbidity of MDD and motivational

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dysregulation disorders such as substance use disorder. Other considerations include the role sex and gender have in the prevalence in MDD in patient populations. Although our data did not reveal any sex differences in the role of pnVTA nociceptinergic circuitry in reward motivation in our study, there is considerable evidence supporting a robust difference in MDD, with almost twice as many women suffering from MDD than men (Hyde *et al.*, 2008; Salk *et al.*, 2017; Hyde and Mezulis, 2020). Importantly, there is limited investigation into whether nociceptin neurocircuitry may be differentially affected among these distinct MDD populations and new research should explicitly interrogate these avenues as to provide better insights into appropriate therapeutic strategies for the treatment of MDD.

Conclusions

It is well known that depression and other mood disorders are complicated disease states, driven by a confluence of physiological and environmental factors that often require several therapeutic approaches. Currently, preclinical studies are seeking to better understand the pharmacological and neuroanatomical means by which the nociceptin/NOP system and NOP ligands affect emotional states and subsequent motivated behaviors. The identification of successful small molecule candidates like BTRX-246040 offer therapeutic potential, yet our understanding of how this system is engaged during these treatments is still not clear. However, given the preliminary success of these ligands preclinically and clinically, researchers need only to resolutely identify any side effects of chronic treatment and provide insight into the therapeutic advantages of NOP ligands in ameliorating depressive-like states in patients.

The Kappa Opioid System in the regulation of the negative affective component of pain

Negative affect refers to the experience of a negative emotional (affective) state. This is often described as a aversive/dysphoria-like or depressive-like state. Negative affect is manifested in a number of neuropsychiatric diseases such as addiction and pain. Here we will focus on negative affect in pain processing and mechanisms involved.

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It has been well-established that activation of kappa opioid receptors (KOR) induces dysphoria and aversive-like states in rodent models (Mucha and Herz, 1985; Shippenberg *et al.*, 1993; Knoll and Carlezon, 2010). In fact, it was a study in humans in which activation of KOR was first described to elicit 'dysphoric and psychotomimetic effects' (Pfeiffer *et al.*, 1986). The mechanisms underlying negative affective states are not entirely clear, but we do know that the nucleus accumbens (NAc) region, seems to be highly and consistently implicated. Interestingly, clinical studies show reduced NAc activity, alteration in reward evaluation, decision making and motivation in pain patients (Oluigbo *et al.*, 2012; Baliki and Apkarian, 2015). The NAc is part of the mesolimbic pathway, which is the reward-mediating pathway in the mammalian brain, composed of dopaminergic neurons projecting from the ventral tegmental area (VTA) of the midbrain to the NAc in the forebrain. Dopamine from these VTA dopaminergic neurons is released in the NAc in response to reinforcers, such as food, social interaction or drugs of abuse.

KORs are expressed in the NAc on presynaptic terminals of dopaminergic neurons (Werling *et al.*, 1988; Ebner *et al.*, 2010; Al-Hasani and Bruchas, 2011) and activation of KORs decreases dopamine release (Spanagel *et al.*, 1992; Margolis *et al.*, 2003), which is known to drive aversive and negative emotional states (Wadenberg, 2003; Cahill *et al.*, 2014; Wise and Koob, 2014). This dopaminergic dysfunction is thought to contribute to development of chronic pain (Cahill *et al.*, 2014; Taylor *et al.*, 2014, 2016; Yalcin and Barrot, 2014; Cahill and Taylor, 2017; Liu *et al.*, 2019). This is somewhat validated by many whom report reduced motivation for goal-directed behaviors during pain and has become a well-known feature of pain-induced negative affect (Narita *et al.*, 2005; Michael D. Leitl *et al.*, 2014; Schwartz *et al.*, 2014; Hipólito *et al.*, 2015; Liu *et al.*, 2019; Massaly *et al.*, 2019). Furthermore, intra-VTA opioid reward and evoked dopamine release within the NAc are reduced in chronic pain states (Taylor *et al.*, 2015). It has also been shown that pain states alter reward processing, for example animals exposed to chronic pain developed a preference for the morphine-paired side, in a conditioned place preference (CPP) model when the dose of morphine was increased, known reinforcing doses of morphine do not induce a place preference under painful conditions (Wu *et al.*, 2014). It was not until the last few years that laboratories began studying the kappa opioid receptor system in the context of negative affect and pain processing.

A recent study found that the morphine CPP described above is blocked upon administration of KOR antagonist JDTic, implicating a crucial role for KORs in the modulation of the aversive/negative affective component of pain in a chronic peripheral nerve injury model (PNI) (Liu *et al.*, 2019). The same group also showed that blockade of KOR restores the blunted CPP following intra-VTA activation of μ opioid receptors (MOR) in neuropathic pain states. This suggests that KORs contribute to the loss of MOR reward-related behaviors in neuropathic pain (Liu *et al.*, 2019). This was further supported by measuring evoked dopamine release, demonstrating that KOR modulation of dopamine release contributes to the tonic aversive component of pain. In addition, it has been shown that KORs are also necessary for the decrease in motivated behaviors characteristic in pain states. Blocking KORs specifically in the contralateral and ventral NAc shell (vNAcSh) in rats and mice, respectively, prevents inflammatory pain-induced decrease in motivation, as measured in an operant task (Massaly *et al.*, 2019). Furthermore, a decrease in sucrose self -administration is observed following local administration of the KOR agonist U50488 into the NAc shell. Together these studies confirm both the necessity and sufficiency of KOR in pain-induced negative affect.

Further studies to better understand the mechanisms by which KOR modulates pain-induced negative affect show that both KOR mRNA in male but not female mice, and dynorphin mRNA in both male and female mice, are increased in the NAc following PNI (Liu *et al.*, 2019). An increase in GTPδS binding was also observed in the NAc following PNI in males but not females (Liu *et al.*, 2019) and in an inflammatory pain model (Massaly *et al.*, 2019) . These findings suggest that there may be an increase in KOR receptor expression and function in chronic pain states, which appears to also be sex-dependent.

It is well known that a large proportion of medium spiny neurons (MSNs) in the NAcSh contain dynorphin and locally control presynaptic neurotransmitter release (Nestler and Carlezon, 2006; Al-Hasani *et al.*, 2015), however, until recently little was known about their role in modulation of pain-induced negative affect. Interestingly, photo-stimulation of dynorphin-containing neurons in the vNAcSh decreases motivation in a sucrose self-administration paradigm but this is not potentiated in an inflammatory pain model (Massaly *et al.*, 2019), suggesting that pain does not potentiate dynorphin-mediated aversion but, based on findings summarized above, KORs are required to modulate negative affect.

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Though it appears that dynorphin in not directly involved in modulation of affect, as shown in behavioral paradigms, evidence suggests that dynorphin-containing neurons may in fact be involved in the described KOR compensatory changes (Liu *et al.*, 2019; Massaly *et al.*, 2019). In a rat complete Freud's adjuvant (CFA) model expression of dynorphin A is increased, as measured by immunohistochemistry, as well as an observed enhancement in excitability in dynorphin-containing neurons, as measured in whole cell patch clamp recordings, together suggesting as increase in dynorphin tone during inflammatory pain (Massaly *et al.*, 2019). Further investigation shows that both frequency and amplitude of spontaneous inhibitory postsynaptic current is decreased in dynorphin-containing neurons, suggesting that disinhibition of these neurons during inflammatory pain increases excitability in the vNAcSh (Massaly *et al.*, 2019). When dynorphin-containing neurons are silenced using inhibitory designer receptors exclusively activated by designer drugs (DREADDS), CFA treated rats no longer show the characteristic decrease in motivation associated with pain, implicating a critical role for dynorphin, as well as KOR, in the NAc in processing of pain-induced negative affect. This is further confirmed by PET-imagining studies, showing a decrease in radioligand binding at KORs in the NAc following CFA suggesting an elevation in dynorphin.

In summary, it is known that KOR-mediated aversion and negative affect occurs in the NAc by altered dopamine transmission from the VTA, but the studies described here are the first to directly show that KOR also mediates negative affect in pain states and explores the mechanisms. Evidence suggests that compensatory changes occur in the KOR system during pains states, as shown by increased function and expression. It seems likely that dynorphin expressing MSNs modulate these KOR effects in turn with other neuropeptide systems yet to be explored. It also worth noting that there is evidence to suggest that stress together with pain may dysregulate KOR signaling (Massaly *et al.*, 2016). It has been shown that stress causes activation of both KOR and p38 mitogen-activated protein kinase (MAPK), co-expressed in GABAergic neurons, in the nucleus accumbens, cortex, and hippocampus (Bruchas *et al.*, 2007). Studies from this same group went on to show that p38α MAPK in serotonergic neurons play a critical role in the modulation of affective behavioral responses (Bruchas *et al.*, 2011).

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KORs are attractive targets as they are antinociceptive without the unwanted side effects that are commonly associated with activation of MOR (respiratory depression and constipation) (Porreca and Burks, 1983; Porreca *et al.*, 1984; Unterwald *et al.*, 1987; Shippenberg *et al.*, 1988; Di Chiara, 1998; Field *et al.*, 1999; Kivell and Prisinzano, 2010). Antinociception is achieved by activation of central or peripheral KOR (Porreca *et al.*, 1987; Millan *et al.*, 1988; Stein *et al.*, 1989, 1990; Stein, 1991; Horan and Porreca, 1993; Vanderah *et al.*, 2008), however, as described above, activation of central KOR drives a negative affective state, hence the push towards the development of peripherally restricted drugs (Aldrich and Vigil-Cruz, 2003; Beck *et al.*, 2019). Most recently, a KOR agonist JT09, shows promise as it is peripherally restricted, matches morphine's therapeutic properties and is orally active (Beck *et al.*, 2019). It is not yet known how targeting KORs peripherally might, if at all, alter central pain processing and negative affect. An important consideration is that the effects KOR depend on the pain model. For example, KOR is not involved in the aversion induced in acute pain states (Michael D. Leitl *et al.*, 2014; Michael D Leitl *et al.*, 2014; Bagdas *et al.*, 2016) but is involved in modulation of negative affect in chronic pain states (Liu *et al.*, 2019; Massaly *et al.*, 2019). One final, important consideration is that the effects of KOR modulation in pain are sex dependent. This has been reported both in preclinical animal models as well as in human imaging studies. PET studies in humans show that KOR binding is greater in men than women in many brain regions, including the anterior cingulate cortex, which has been associated with pain affect (Vijay *et al.*, 2016). These findings hold promise for the continued development of KOR targets in the treatment of pain but the mechanisms are clearly complex and dependent on a number of factors that need to be considered throughout the drug development process.

Opioids and Headache Disorders

Headache disorders are highly disabling conditions that are widespread throughout the world. According to the most recent Global Burden of Disease Study, headache was ranked as third most prevalent and a common type of headache, migraine, was ranked as sixth most prevalent. The greatest burden for headache is carried by women in their reproductive years, and globally in this population migraine produced 20.3 million years lost due to disability in 2016 alone (GBD 2016 Headache Collaborators, 2018). There is still a limited understanding of the mechanisms underlying headache and as with other pain conditions, opioid receptors appear to play an

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important role in the regulation of headache; with all four opioid receptors being implicated in migraine pathophysiology.

Clinically available opioids are often prescribed for migraine (Bigal and Lipton, 2009), and these analgesics act primarily through MOR (Borsodi *et al.*, 2019). Chronic opioid use can result in a paradoxical increase in pain, known as opioid induced hyperalgesia (OIH) (Hayhurst and Durieux, 2016). This phenomenon is clearly observed in headache patients, and is classified as mediation overuse headache (Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition, 2018). In this case opioids initially provide relief but long term can result in increased frequency and severity of headache symptoms (Bigal and Lipton, 2009; Buse *et al.*, 2012). Not only is medication overuse headache difficult to treat, but can result in opioid dependence and abuse, which has contributed to the opioid epidemic (Reid *et al.*, 2002; Colás *et al.*, 2004; Loder, 2006; Diener *et al.*, 2016).

Epidemiologically, evidence clearly shows that the use of opioids can facilitate the transition from episodic to chronic migraine (Bigal and Lipton, 2009) and increase associated disability (Buse *et al.*, 2012). The mechanisms underlying this facilitation were recently examined preclinically in a massive exploratory peptidomic study (Anapindi *et al.*, 2019). This investigation determined whether there were shared mechanisms between OIH and chronic migraine. Both conditions are characterized by dysregulation in neuropeptides, and it was hypothesized that there may be overlapping alterations that could bridge these two conditions. Mice underwent a classical OIH paradigm in which they received escalating doses of morphine over 4 days. Another group of mice were treated according to a chronic migraine model (Pradhan, Smith, McGuire, *et al.*, 2014), in which they received chronic intermittent injection of nitroglycerin (NTG), a known human migraine trigger, (Figure 2). NTG is used as a human experimental model of migraine (Ashina et al., 2017), and its use in rodents has been shown to produce migraine-associated effects such as allodynia, altered meningeal blood flow, and photophobia (Demartini et al., 2019). Both models resulted in severe cephalic allodynia, and multiple brain and peripheral regions were collected for label-free, non-biased liquid chromatography-mass spectrometry. Although there were significant changes in neuropeptide expression and levels in either paradigm alone, there were very few overlapping peptides that were affected in both OIH and chronic migraine. Of these shared peptides, pituitary

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adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP) and secretogranin (SCG) consistently showed significant changes in chronic migraine and OIH. As a confirmation of these findings, the role of PACAP was re-examined in both OIH and chronic migraine models. PACAP can bind to three different receptors (VPAC1, VPAC2, and PAC1), but PAC1 is most commonly associated with migraine-related effects. The PAC1 antagonist, M65, was found to inhibit cephalic allodynia induced by both OIH and chronic NTG treatment, thus confirming the role of this peptide in regulating both disorders (Anapindi *et al.*, 2019). Previous studies have identified PACAP as a target for migraine, but it has not been considered as treatment for OIH, and this work indicates that it may be a mechanistic link through which opioids facilitate migraine chronicity.

In contrast to the pro-migraine effects of MOR activation, delta opioid receptor (DOR) agonists appear to mitigate migraine-related symptoms and DOR has been identified as a novel therapeutic target for headache disorders (Charles and Pradhan, 2016). While DOR agonists are not highly effective in models of acute pain, they have shown efficacy in a number of models of chronic pain, including inflammatory and neuropathic pain (Vicente-Sanchez *et al.*, 2016). Increased evidence indicates that DORs are functionally upregulated during chronic pain states (Cahill *et al.*, 2007; Pradhan *et al.*, 2013; Gendron *et al.*, 2016; Vicente-Sanchez *et al.*, 2016), and may serve as protective mechanism in the face of increased pain processing, including in multiple headache models. An initial report showed that three different selective DOR agonists: SNC80, ARM390, and JNJ20788560, inhibited migraine-associated mechanical allodynia induced by NTG (Pradhan, Smith, Zyuzin, *et al.*, 2014). Another group confirmed that SNC80 could block the effects of NTG, in this case using thermal hyperalgesia as an endpoint (Dripps *et al.*, 2018). It is important to note that agonists that promote both high- (SNC80) and low- (ARM390) internalization of DOR were active in this migraine model, indicating that this is not a ligand-biased effect (Vicente-Sanchez and Pradhan, 2018). These studies indicate that DOR agonists may be effective as acute migraine therapies.

DOR has also shown promise in other types of headache disorders. Chronic migraine is defined as >15 headache days per month over a 3-month period (Headache Classification Committee of the International Headache Society (IHS) The International Classification of Headache Disorders, 3rd edition, 2018), and affects

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~1-3% of the population. These patients are often refractory to treatment and suffer from overwhelming disability. SNC80 was shown to inhibit established cephalic allodynia in a NTG model of chronic migraine-associated pain (Moye, Tipton, *et al.*, 2019). Acute injection of this DOR agonist was also effective in a related model of chronic migraine induced by mild traumatic brain injury (TBI), otherwise known as post-traumatic migraine (Moye and Pradhan, 2017; Moye, Tipton, *et al.*, 2019). Importantly, long term treatment with SNC80 also prevented the development of chronic migraine in this same model of post-traumatic headache (Moye, Novack, *et al.*, 2019). These data suggest that DOR may be effective for both episodic headache and could also protect from the induction of headache chronicity. In addition, SNC80 was also shown to effectively block cephalic and peripheral allodynia in the model of OIH described above (Moye, Tipton, *et al.*, 2019). These findings particularly stress that MOR and DORs distinctly regulate head pain processing, as chronic treatment with the mu agonist, morphine, induced pain which was still blocked by DOR activation. Furthermore, medication overuse headache can also be induced by triptans, a class of drugs specifically developed for the treatment of migraine; and SNC80 also inhibited allodynia induced by chronic sumatriptan treatment (Moye, Tipton, *et al.*, 2019). These studies suggest that DOR may be an effective target not just for migraine but for headache disorders more broadly. Importantly, in contrast to morphine and sumatriptan, chronic DOR agonist treatment produced a limited form of medication overuse headache/opioid induced hyperalgesia (Moye, Tipton, *et al.*, 2019), further supporting the development of DOR as a target for headache.

DOR activation may also modulate headache symptoms beyond pain. In a conditioning paradigm, SNC80 prevented the development of a conditioned place aversion to NTG (Pradhan, Smith, Zyuzin, *et al.*, 2014). This study would suggest that DOR may also regulate negative affective state induced by migraine. Especially considering that DOR agonists have anti-depressant and anxiolytic properties they may be ideal candidates for addressing the high co-morbidity between headache and emotional disorders (Silberstein *et al.*, 2007). In addition, DOR activation was also found to beneficially modulate migraine aura-related symptoms. Cortical spreading depression (CSD) is thought to be the physiological correlate of migraine aura, the visual disturbances experienced by ~30% of migraine patients (Charles and Baca, 2013). CSDs are slowly propagated waves of depolarization followed by inhibition of brain activity. They can be induced experimentally by dripping potassium chloride (KCl) onto the dura and measuring changes in optical reflectance and corresponding DC shifts in local

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potentials (Brennan *et al.*, 2007). Migraine preventives have been shown to decrease CSD events (Ayata *et al.*, 2006; Bogdanov *et al.*, 2011), and is used as a screening tool for migraine prophylactics. SNC80 significantly decreased the number of CSD events evoked by KCl (Pradhan, Smith, Zyuzin, *et al.*, 2014) showing that DORs can regulate the cortical excitability associated with migraine, and again suggests that DOR agonists could also prove to be migraine preventives.

DOR is highly expressed in brain regions that are involved in head pain processing further supporting its role in headache pathophysiology. In dorsal root ganglia, DOR are found on a small subset of neurons that express the pro-migraine peptide calcitonin gene related peptide (CGRP) (Bardoni *et al.*, 2014). There is also evidence from rodent and human post-mortem tissue studies showing the expression of DOR in trigeminal ganglia – first order neurons innervating the head (Mansour *et al.*, 1988; Mennicken *et al.*, 2003; Pradhan *et al.*, 2011; Rice *et al.*, 2017), as well as the dura (Rice *et al.*, 2017). Furthermore, DOR is also expressed in central regions modulating migraine, including the trigeminal nucleus caudalis and cortex (Mansour *et al.*, 1988; Peckys and Landwehrmeyer, 1999; Mennicken *et al.*, 2003), as well as in regions more broadly regulating pain perception (Pradhan and Clarke, 2005; Gendron *et al.*, 2016). The expression of DOR in limbic regions such as the hippocampus, striatum, and amygdala highlight its role in emotional regulation (Lutz and Kieffer, 2013), which could be beneficial in light of the high co-morbidity between headache disorders and anxiety and depression. Considering the finding that PACAP facilitates migraine and OIH, while DOR agonists are inhibitory in these models, future studies will focus on the relationship between the PACAPergic system and DOR. There is also high anatomical co-expression between PACAP/PAC1 and DOR in many CNS regions (Anapindi *et al.*, 2019), thus further supporting an interplay between these two systems. Unlike MOR activation, DOR agonists have low abuse liability and do not appear to cause physical dependence (Negus *et al.*, 1998; Brandt *et al.*, 2001; Stevenson *et al.*, 2005; Do Carmo *et al.*, 2009). In addition, DOR agonists cause less respiratory depression and fewer effects on gastrointestinal transit (May *et al.*, 1989; Gallantine and Meert, 2005; Poole *et al.*, 2011) relative to MOR agonists. One caveat to the development of DOR agonists is that some of them are pro-convulsant. However, this appears to be due to ligand directed signaling, and it may be possible to distinguish this adverse

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behavior from pain-relieving effects through drug development (Pradhan *et al.*, 2012). Figure 3 summarizes all of the headache models in which DOR agonists have been shown to be effective.

DOR agonists are actively being developed for migraine treatment. A clinical trial (phase 1) was recently performed by Trevena Inc. to test the DOR agonist, TRV250, for acute migraine. This agonist was specifically developed with reduced seizure liability, without compromising headache-relieving effects. The data as only been disclosed in a press release from the company, and indicates that in healthy volunteers TRV250 has safety, tolerability and a pharmacokinetic profile that would support moving to Phase II trials for efficacy in migraine. Along with these clinical studies, studies are ongoing to determine the molecular mechanisms through which DOR regulates headache.

The kappa opioid receptor (KOR) has also recently emerged as a therapeutic target for headache disorders, and in this case an antagonist strategy is being proposed. Upregulation of the endogenous KOR ligand, dynorphin, has been identified as a marker of stress (Bruchas *et al.*, 2010; Carlezon and Krystal, 2016) and this may be one way in which stress mechanistically results in migraine. Stress is commonly identified as a migraine trigger (Maleki *et al.*, 2012) and in support of this idea, KOR antagonists were recently shown to be effective in a stress-induced headache model (Xie *et al.*, 2017). Rats treated with chronic sumatriptan to induce medication overuse headache, concurrently resulted in hypersensitivity to a bright light stress cue. Subsequent exposure to this cue produced an increase in plasma CGRP, a pro-migraine peptide, as well as induction of cephalic and peripheral allodynia. Systemic injection of either a long (nor-BNI) and short (CYM51317) acting KOR antagonist blocked both the augmented CGRP and allodynia. Furthermore, chronic treatment with sumatriptan increased dynorphin and phosphorylated KOR in the amygdala, and intra-amygdala injection of nor-BNI was also pain-relieving. These results open the possibility of targeting KOR antagonists for the treatment of migraine, especially in patients with increased sensitivity to stress.

Finally, NOP receptor has also been implicated in headache pathophysiology. Nociceptin in highly expressed in human trigeminal ganglia (Hou *et al.*, 2003), with up to 70% of neurons being nociceptin positive. In addition, in an electrophysiological model of migraine, nociceptin significantly inhibited neurogenic dural vasodilation .

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Nociceptin levels have also been examined in a migraine patient population. Interictally, the levels of the endogenous NOP ligand, N/OFQ, were significantly lower in the plasma of migraine patients compared to healthy controls; and N/OFQ levels further dropped during a migraine attack (Ertsey *et al.*, 2005). Together, these studies promote further investigation into the role of NOP and its receptor in headache disorders.

Opioid-related behaviors and the gut microbiome

The effects of opioids – particularly mu opioid agonists – in the gut is well known. The slowing of gut motility is a desirable effect of over-the-counter opioid agonists, such as loperamide, for the treatment of diarrhea. On the other hand, severe constipation and abdominal cramping is a common and often dose-limiting side effect of chronic opioid use. More recently, however, opioids have been shown to affect the gastrointestinal environment beyond their influence on gut motility.

The gut microbiome refers to the collection of micro-organisms that reside in the gut and is increasingly acknowledged to contribute to brain development and behavior (Mayer *et al.*, 2014) It is now well-established that mu-opioid agonists significantly change the composition of gut bacteria (Acharya *et al.*, 2017; Xu *et al.*, 2017; Wang *et al.*, 2018). In fact, the influence of mu opioid agonists on gut bacteria composition is incredibly labile, with changes to the gut microbiome detected after only one day of morphine treatment (Wang *et al.*, 2018). The gut microbiome also fluctuates significantly following intermittent morphine exposure as the gut cycles through phases of drug onset and withdrawal (Lee *et al.*, 2018). Importantly, we now know that changes to the gut microbiome directly contribute to many of the symptoms of chronic opioid use. For example, depleting the gut microbiome with oral antibiotics inhibits the development of morphine tolerance (Kang *et al.*, 2017) although this relationship seems to depend on the type and length of antibiotics used (Lee *et al.*, 2018). Moreover, fecal microbiota transfer from morphine withdrawn to drug naïve mice was sufficient to induce symptoms of withdrawal including hyperalgesia, negative affect, and inflammation (Lee *et al.*, 2018). Although more research is needed, these early studies suggest the gut microbiome is an important contributor to the full expression of behaviors associated with chronic opioid use.

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A key question is what is the mechanism by which gut bacteria influence opioid-related behaviors? Compiled analysis of the multitude of studies describing the gut microbiota following opioid exposure reveal some common themes. While opioid treatment does not alter overall species richness (how many different types of bacteria), it does alter the abundance of specific key bacterial taxa. For example, chronic opioid treatment leads to a relative *reduction* in Gram-negative bacteria in the Bacteroidetes phyla and a relative *increase* in Gram-positive bacteria in the Firmicutes phyla (Feng *et al.*, 2006; Babrowski *et al.*, 2012; Banerjee *et al.*, 2016; Wang and Roy, 2017; Lee *et al.*, 2018; Wang *et al.*, 2018). The reduction in the Bacteroidetes/Firmicutes ratio has been correlated with inflammation in several diseases, including obesity and irritable bowel syndrome (Power *et al.*, 2014) and may contribute to the inflammation associated with chronic opioid use (Cahill and Taylor, 2017).

Gut bacteria perform many functions within the gut including fiber fermentation, carbohydrate digestion, bile acid formation, and drug metabolism. Interestingly, a role for gut bacteria in morphine metabolism has been described. Certain gut bacteria have beta-glucuronidase activity, an enzyme responsible for hydrolyzing morphine metabolites (morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) back into morphine (Walsh and Levine, 1975; Koster *et al.*, 1985). Therefore, transformation of M3G and M6G into morphine by gut bacteria may influence the pharmacokinetic profile of morphine by reversing drug metabolism and prolonging morphine effects. Reductions in bacteria with beta-glucuronidase activity has been proposed to contribute to opioid tolerance (Wang *et al.*, 2018) although further research is necessary to further confirm these ideas.

Another important area of opioid-induced dysbiosis is bile acid formation. Chronic opioid treatment leads to reductions in both primary and secondary bile acid transformation (Banerjee *et al.*, 2016; Wang *et al.*, 2018; Sindberg *et al.*, 2019). Bile acids are not only important for nutrient absorption and metabolism but also inhibit the expansion and translocation of certain pathogenic bacteria (Yoon *et al.*, 2017). To this point, reduction of bile acid following chronic morphine treatment is associated with the expansion of pathogenic bacteria *Enterococcus faecalis* (Banerjee *et al.*, 2016). Treatment of drug naïve mice with *E. faecalis* was sufficient to increase morphine tolerance, suggesting it is a key bacterial species driving opioid dependence (Wang *et al.*, 2018).

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Treatment with chronic opioids also leads to a significant loss in gut barrier integrity and bacterial migration (Koster *et al.*, 1985; Banerjee *et al.*, 2016). Both Gram negative and Gram positive bacteria have been shown to colonize the liver, spleen and lymph nodes following chronic opioid treatment (Hilburger *et al.*, 1997; Meng *et al.*, 2015). This process has been shown to be dependent on toll-like receptor 2 (TLR2), given that TLR2 knock-out mice do not show morphine-induced gut epithelial barrier dysfunction (Meng *et al.*, 2013). The loss of gut barrier integrity following opioid treatment can exacerbate viral infection and sepsis, which has serious clinical implications given the propensity to use opioids in clinical emergency settings (Meng *et al.*, 2015; Banerjee *et al.*, 2016; Shakhsheer *et al.*, 2016; Zhang *et al.*, 2018).

Interventions that restore the gut microbiome are an obvious target to improve opioid efficacy and minimize the effects of withdrawal. Fecal microbiota transfer has provided initial proof-of-principle that manipulating the gut microbiome can influence opioid dependent behaviors (Lee et al., 2018). Moreover, dietary supplementation with omega 3 polyunsaturated fatty acids restored the gut microbiome, reduced inflammation, and opioid-seeking behaviors in a contingent opioid dependence model (Hakimian et al., 2019). While these initial studies are promising, much more research is needed to fully explore the therapeutic potential of targeting the gut microbiome in mitigating the effects of chronic opioids in clinical populations. In particular, studies examining how the gut microbiome contributes to opioid function in both males and females is urgently needed. To date, the vast majority of preclinical research cited above was performed in males only. Studies examining these effects in both males and females is required to fully capture the relationship of the gut microbiome and opioids as it relates to human clinical populations.

Conclusion:

In this review we have highlighted four distinct emerging fields of opioid research that have exciting translational potential (Figure 4). The fact that these areas of research are somewhat unrelated and do not all overlap implies that perhaps selective drug targeting to specific opioid receptors may be easier. Here we show that the opioid system can be involved in peripheral and/or central regulation in the modulation of certain disease states, which is very interesting, especially in light of the development of many restricted opioid compounds and the fact that

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much of this research has already led to opioid compounds entering clinical trials. Together these findings highlight the dynamic nature and importance of opioids in a number of diseases and remind us that there is still so much to learn. The ongoing development of new therapeutic targets for opioids is very encouraging and the field will likely see significant developments over the next decade.

Author Contributions

Wrote or contributes to the writing of the manuscript: All Authors

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Footnotes

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Figure Legends

Figure 1. N/OFQ-NOP receptor fields of research. Highlighted are promising new research areas discussed within this review.

Figure 2: Mouse models of chronic migraine associated pain and opioid induced hyperalgesia. (A) Mechanical thresholds are determined by von Frey hair stimulation of the periorbital region. (B) To model chronic migraine associated pain mice are injected every other day with vehicle or nitroglycerin (10 mg/kg IP), and tested on days 1,5, and 9. To test the effect of PAC1 inhibition, mice were treated with M65 (0.1 mg/kg IP) or vehicle on day 10. (B) For OIH, mice were injected twice daily with morphine or vehicle. On days 1-3 they received 20 mg/kg, and

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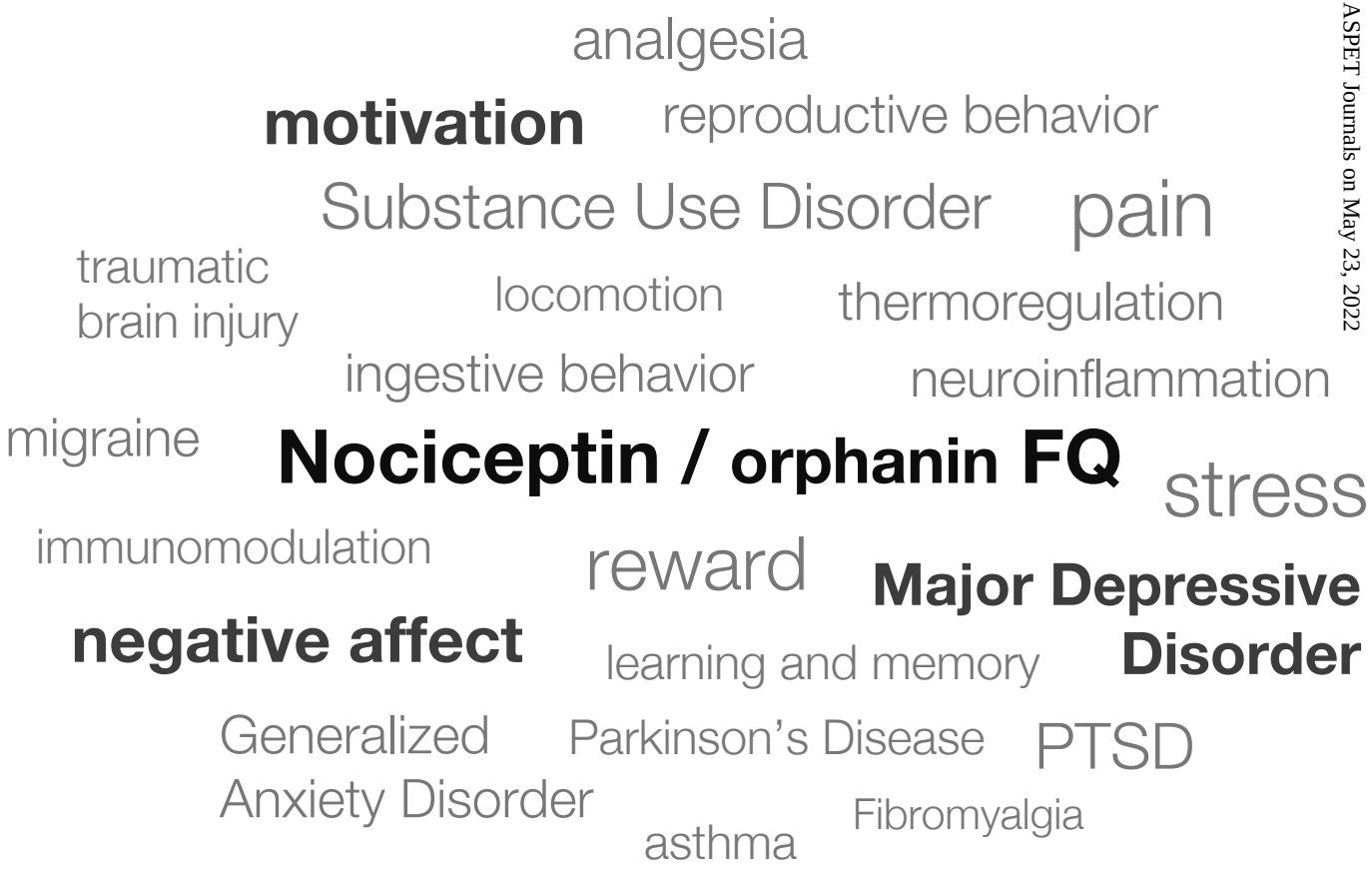
on day 4 40 mg/kg SC morphine/injection; and are tested on days 1 and 3 of treatment. The effect of M65 was determined on day 5, when cephalic OIH had been established.

Figure 3: DOR agonists have been shown to be effective in models of acute and chronic migraine-associate pain (nitroglycerin), negative affect (conditioned place aversion), and aura (cortical spreading depression); as well as in models of post-traumatic headache, and medication overuse headache (MOH) to sumatriptan or morphine (opioid induced hyperalgesia/OIH).

Figure 4: Summary of topics reviewed. All four opioid receptors modulate analgesia, yet they each have unique established roles, which has led to research into more specialized functions. Mu-opioid receptor system has long been known to alter gut motility (prevents diarrhea vs constipation) but more recently its role in gut microbiome composition had implications in chronic opioid use disorder; the established primary role for the kappa opioid system is regulation of negative affect with a more recently discovered role in pain-induced negative affect; the delta opioid receptor system has been identified as promising target for treating headache disorders and migraine, a significant advancement from its more generalized role in chronic pain. Finally, most research thus far has focused on the role of the nociceptin system in depression, here we review recent advances studying its role in motivation as a mechanism to refine treatment approaches.



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