UNIVERSITIY OF CALIFORNIA SAN DIEGO

The effects of opioid-induced hyperalgesia on neural activity in the ventrolateral periaqueductal gray

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Biology

by

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The Thesis of Emilya Ventriglia is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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University of California San Diego

2020

DEDICATIONS

To my Mother, my Grandmother, and my Grandfather.

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ABBREVIATIONS

CREB	cAMP response element-binding protein;
DH	Dorsal Horn;
DLF	dorsolateral funiculus;
ERK	Extracellular signal-regulated kinase;
GABA	gamma aminobutyric acid
IEG	immediate early gene
MAPK	mitogen activated protein kinase
MEK	mitogen activated protein kinase kinase
MOR	Mu opioid receptor
NMDA-R	N-methyl-D-aspartate receptor
OIH	opioid-induced hyperalgesia
PKC	protein kinase C
RVM	Rostral ventromedial medulla
vlPAG	ventrolateral PAG

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ABSTRACT OF THE THESIS

The effects of opioid-induced hyperalgesia on neural activity in the ventrolateral periaqueductal gray

by

Emilya Ventriglia

Master of Science in Biology

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Professor Matthew Banghart, Chair

Opioids are among the most widely used medication classes globally and are frequently prescribed in the treatment of pain conditions. Paradoxically, some individuals taking opioids might develop a condition of increased pain sensitivity referred to as opioid-induced hyperalgesia (OIH). Understanding the underlying biological mechanisms of OIH will improve treatment outcomes. Current proposed mechanisms of OIH include neuroplastic changes to the descending pain modulatory pathway between the midbrain periaqueductal gray, brainstem rostral ventromedial

medulla, and spinal cord Dorsal Horn. In order to extend current knowledge of activity-dependent changes in the descending pain modulatory pathway in OIH, we used immunohistology techniques to examine the activity of three well-studied activity markers– Fos, NPAS4, and pERK1/2– in the ventrolateral periaqueductal gray in a mouse model of OIH. Furthermore, we evaluated changes in these markers as a result of persistent Formalin-evoked pain during OIH. It was found that basal Fos expression was reduced in OIH mice compared to controls, but this difference was abolished when assessing formalin-evoked activity. Together, these data support a model of reduced descending antinociceptive control from the vlPAG to the RVM that may enhance concomitant RVM-mediated pain facilitation in OIH.

INTRODUCTION

Opioid-induced Hyperalgesia

Opioid drugs are capable of producing lasting pain relief and continue to be prescribed in high rates across America today. In spite of their pivotal use in the treatment of chronic and severe pain, there is an increasing amount of evidence that suggests opioid therapies can exacerbate pain or occlude recovery (Sjøgren et al., 2015). It is becoming increasingly recognized that opioids can paradoxically increase pain sensitivity in a phenomenon known as opioid-induced hyperalgesia (OIH) (Lee et al., 2011).

OIH is characterized as an increase in pain sensitivity in individuals taking opioids, often alongside the development of a novel, generalized, and/or antinomically distinct pain (Conno et al. 1991; Devulder 1997; Nabil M. K. Ali, 1986). OIH appears to be modality specific and has been separately demonstrated to differentially impact thermal and mechanical pain sensitivities (Angst et al., 2003; Chu, Clark and Angst, 2006; Compton et al. 2001). OIH has been observed in patients exposed to opioids in both long-term and continuous treatments, as well as in acute settings such as perioperative or from a single exposure (Kim et al., 2014; Yang et al., 2019; Chu, Clark and Angst, 2006; Doverty et al., 2001; Compton et al. 2001; Guignard et al. 2000; Angst et al., 2003). Accordingly, OIH poses a threat to achieving adequate pain relief in individuals taking opioid drugs across numerous contexts by enhancing existing pain as well as generating novel pains.

Despite instances of OIH being recognized in Western medicine as far back as 1870, identifying OIH in the clinic has been an ongoing challenge (Albutt, 1870). There are no official diagnostics criteria for OIH, though clinical criteria have been proposed in recent years (Eisenberg et al., 2015). Although OIH is considered a distinct phenomenon, it is challenging to distinguish it clinically from opioid analgesic tolerance or withdrawal, namely because all three result in increases in reported pain. A decreased efficacy of opioids from repeated use over time

(i.e. analgesic tolerance) can be overcome by increasing dosage, while OIH is thought to be exacerbated by the same dose-escalation and pain then worsened. However, the current evidence suggesting dose escalation in OIH results in worsened condition is indirect and anecdotal, which may promote misdiagnoses or incorrect assumptions about the relationship between tolerance and OIH mechanisms (Kim et al., 2014; Yang et al., 2019). It should be noted that many studies have inextricably linked the development of tolerance to OIH, particularly where animal models of OIH show a rightward shift in opioid dose-response curve (i.e. analgesic tolerance) (Mao, Price, and Mayer, 1994; Laulin et al., 1999; Vanderah et al., 2001; Célèrier et al., 200). This has caused some of the leading OIH researchers to posit a direct relationship between the two phenomena (Ossipov et al. 2003). Alternatively, OIH can be distinguished from tolerance based on the nature of the pain complaint, where tolerance results in the return of old pain whereas OIH results in an exacerbation of existing pain *in conjunction* with novel pain phenomena.

Similarly, the delineation of OIH from withdrawal is sometimes controversial and challenging. Opioid withdrawal can be alleviated by opioid administration whereas OIH would theoretically be exacerbated (Vera-Portocarrero et al., 2011). For instance, it is not clear whether paradoxical pain in a perioperative setting reflects OIH rather than acute opioid withdrawal, particularly with short-acting opioids like remifentanil. In order to differentiate OIH from withdrawal, it was determined that OIH develops despite continuous opioid administration in rats receiving opioids continuously through pellet implantation (Vanderah et al., 2001). Alternatively, it may be that the current clinical terms are insufficient to adequately describe the underlying mechanisms and their impact on behavior.

Information of the clinical prevalence of OIH is currently weak. Clinical evidence for OIH has largely been derived from studies looking at long-term opioid use, in a perioperative setting, during opioid-cessation, and in volunteer studies (Kim et al., 2014; Yang et al., 2019; Chu, Clark and Angst, 2006; Doverty et al., 2001; Compton et al. 2001; Guignard et al. 2000;

Angst et al., 2003). For example, all patients in a study undergoing treatment for chronic low-back pain that received one month of oral morphine treatment displayed significantly decreased pain threshold and tolerance in the cold pressor assay, indicating the development of OIH (Chu, Clark and Angst, 2006). In a preclinical setting, rodent models have demonstrated acute and long-term opioid exposure can result in hyperalgesia across multiple opioid drugs and routes of administration (Woolf, 1981; Célèrier et al., 2001; Larcher et al., 1998; Yaksh, Harty, and Onofrio 1986; Vanderah et al., 2001; Lu et al. 2015).

Several mechanisms for OIH have been proposed, namely involving increased excitability of peripheral nociceptors, upregulation of pronociceptive spinal neuromodulators, and potentiation of supraspinal sites that mediate descending facilitation (Lee et al., 2011). Neural connections and synaptic machinery are dynamic and readily modify in response to relevant stimuli (i.e. plasticity), resulting in short- and long-term changes in circuitry that are capable of exerting changes in behavior; as such, repeated exposure to powerful drugs such as opioids leads to neuroplastic changes that underlie tolerance, withdrawal, and likely OIH (Cohen and Greenberg, 2008; Ji et al., 2003). Plasticity occurring spinally and supraspinally are thought to involve changes in the expression and activity of the glutamatergic/NMDA system and with neuromodulators like dynorphin, ultimately leading to alterations in synaptic excitability (Angst et al., 2003; Célèrier et al., 2001; Larcher et al., 1998). A current conceptual framework for the pathogenesis of OIH that incorporates these multifactorial mechanisms consists of an opponent-process theory between the on-target opioid effect of analgesia and its opponent process of hyperalgesia, whereby opioid-induced analgesia is impaired through repeated opioid exposure and pronociceptive effects are concurrently enhanced (Célèrier et al., 2001). The ventrolateral region of the midbrain periaqueductal gray (PAG) and its projections to the rostral ventromedial medulla (RVM) are canonically considered the supraspinal locus of not only opioid-induced analgesia but also opioid-related pain sensitization, making the connections between these structures an ideal candidate to investigate the pathology of OIH. We will discuss

previous studies that implicate the RVM and its spinal projections in the maintenance of OIH; however, it is not yet understood what role, if any, the PAG performs. Here, we investigate the plausibility of PAG involvement in RVM-mediated pain facilitation and assess this region's activity in the context of pain.

The Descending Pain Modulatory Pathway

It is widely recognized that supraspinal brain regions are capable of exerting modulatory control over ascending nociceptive transmission from the spinal cord. In particular, the vlPAG and its projections to the RVM constitute a powerful descending pain modulatory circuit (Fields and Basbaum, 1978; Heinricher and Ingram, 2008). Subsequently, the RVM sends outputs (i.e. the dorsolateral funiculus (DLF)) that are capable of suppressing or enhancing ascending pain transmission at the level of the spinal cord Dorsal Horn (DH), which receive inputs from primary afferent nociceptors (Basbaum and Fields, 1979, Fields et al., 1977).

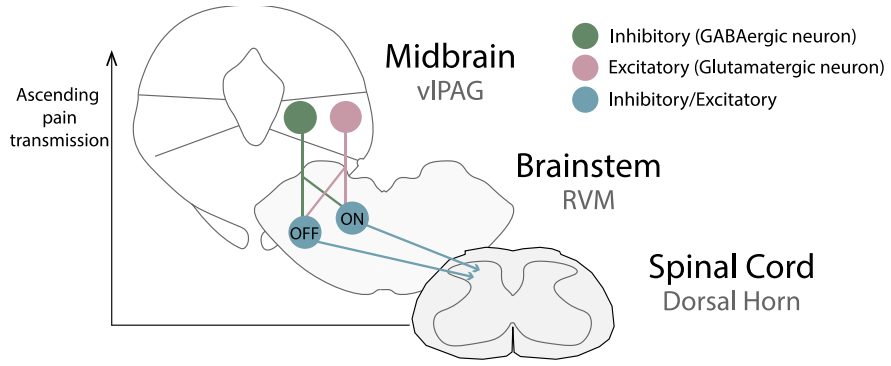

Figure 1. The Descending pain modulatory pathway
Schematic representation of the three loci of the descending pain modulatory pathway. The vlPAG sends both inhibitory and excitatory projections to the RVM, which sends its own inhibitory and excitatory projections to the DH. Ascending pain transmission is bidirectionally modulated through differential activation and/or suppression of these subpopulations. Adapted from Lau and Vaughan (2014).

The pain modulatory control that the PAG-RVM-Spinal pathway exerts is foremost understood to be mediated by the activity of two subpopulations of spinally-projecting neurons within the RVM, known as ON and OFF cells (Fields, Malick, and Burstein, 1995). These RVM subpopulations have been characterized based upon their intrinsic firing response to noxious stimuli (Heinricher, Cheng, and Fields, 1987; Barbaro, Heinricher, Fields, 2009; Vanegas, Barbaro, and Fields, 1984). When a noxious thermal or mechanical stimulation is delivered to awake or an anesthetized animal, a nocifensive withdrawal reflex engages, e.g. rapid hind paw retraction to local pinprick. RVM OFF-cells are tonically active but rapidly cease activity prior to nocifensive behaviors (Vanegas, Barbaro, and Fields, 1984). Selectively activating OFF-cells greatly diminishes nocifensive responses to noxious stimuli, suggesting that OFF-cell activity is antinociceptive (Heinricher, Barbaro, Fields, 2009; Babaro, Heinricher, Fields, 1986; Cheng, Fields, Heinricher, 1986). Conversely, ON-cells respond to noxious stimuli with bursts of activity and produce behavioral hyperalgesia when stimulated (Neubert, Kincaid, Heinricher, 2004; Heinricher and Neubert, 2004). Taken together, the reciprocal activity patterns that RVM ON- and OFF-cells display underlie a powerful and dynamic pain-tuning system (Barbaro, Heinricher, and Fields, 1989).

Upstream, the PAG exerts descending bidirectional control over RVM ON- and OFF-cells through a combination of excitatory and inhibitory projections (Morgan et. al, 2008; Tortorici and Morgan, 2002; Aimone and Gebhart, 1986). Analgesia produced through the vlPAG is thought to be the activation of excitatory glutamatergic projection neurons that elicit an increase in activity of RVM OFF-cells that is secondarily accompanied by a suppression of ON-cell bursting (Behbehani and Fields, 1979; Aimone and Gebhart, 1986; Tortorici and Morgan, 2002; Morgan et. al, 2008). Direct and indirect chemogenetic and pharmacologic activation of glutamatergic neurons in the vlPAG is analgesic (Aimone and Gebhart, 1986; Samineni et al., 2017; Depaulis, Morgan, and Liebeskind, 1987). The majority of vlPAG projections to the RVM are thought to be glutamatergic, suggesting that the predominant action

of the vlPAG exerted onto the RVM is antinociceptive (Aimone and Gebhart, 1986). Indeed, non-specific electrical or chemical stimulation of the PAG produces robust analgesia mediated through the RVM (Hosobuchi, Adam, and Linchitz, 1977; Sandkühler and Gebhart, 1984; Zorman et al., 1981; Vanegas, Barbaro, and Fields, 1984). Previous work has demonstrated that suppressing glutamatergic neurons in the vlPAG produces a hypersensitivity to thermal and mechanical stimuli (Samineni et al., 2017). This indicates that the antinociceptive input to the RVM alone may be capable of bidirectionally influencing pain perception. Taken together, a model for OIH pathology can be constructed, whereby a tonically active antinociceptive vlPAG-RVM-Spinal cord pathway is downregulated in a state of OIH, resulting in a loss of inhibition of ascending pain transmission. Similar to Samineni et al.'s work, this would result in an increase in pain sensitivity like that of OIH (2017; Fig. 2B).

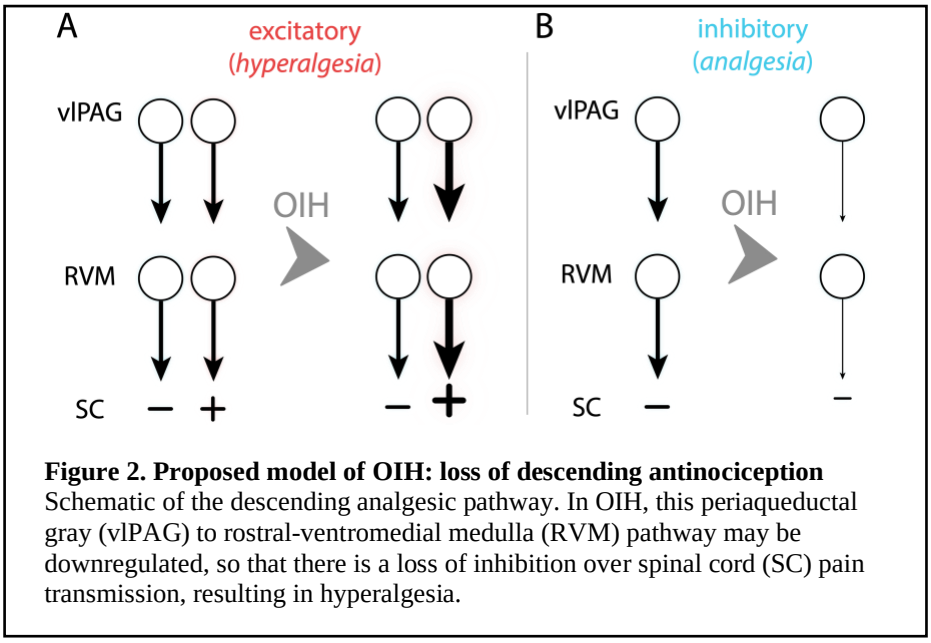

Figure 2. Proposed model of OIH: loss of descending antinociception
Schematic of the descending analgesic pathway. In OIH, this periaqueductal gray (vlPAG) to rostral-ventromedial medulla (RVM) pathway may be downregulated, so that there is a loss of inhibition over spinal cord (SC) pain transmission, resulting in hyperalgesia.

Interestingly, non-specific electrical stimulation of the vlPAG not only increases the activity of OFF-cells, but excites ON-cells, pointing to a latent pronociceptive vlPAG-to-RVM pathway that is not accounted for in the aforementioned model (Vanegas, Barbaro, and Fields, 1984). Thus, we may alternatively suggest that OIH is the product of enhanced pronociception (Fig. 2A). Hyperalgesia in rodent models of neuropathy, inflammatory pain, and opioid-

withdrawal can be reversed by inactivation of the PAG or RVM via locally administered lidocaine or electrolytic lesion (Petrovaara, Wei, & Hamalaien, 1996; Pertovaara, 1998; Kaplan and Fields, 1991; Bederson, Fields, and Barbaro, 1990; Porreca et al., 2001; Khasabov et al., 2017, Kovelowski et al., 2000; Neubert, Kincaid, Heinricher, 2004; Kincaid et al., 2006; Salas et al., 2018). Likewise, behavioral expression of OIH is attenuated by lidocaine inactivation of the RVM or lesioning of the DLF without altering normal pain thresholds or response to systemic morphine (Vanderah et al., 2001; Rivat et al., 2009; Li et al., 2017). While it has not yet been shown whether inactivation of the PAG also abolishes OIH, this region has been demonstrated to have enhanced excitability in a rat model of OIH; when this hyperexcitability was resolved with CaMKII inhibitors, hyperalgesia was reversed (Li et al., 2017). In essence, these findings demonstrate the necessity of descending facilitation that is recognized from the RVM-to-Spinal cord, but also hints at a pronociceptive input from the vlPAG-to-RVM. On the other hand, we do not know the molecular identity of the vlPAG subpopulations that was found to be hyperexcitable. In the proposed model of enhanced antinociceptive vlPAG output, these upregulated cells would be a distinct population of RVM projection cells, distinct from the canonical opioid-sensitive antinociceptive pathway. On the other hand, it is possible that the hyperexcitability observed may indicate an upregulation of GABAergic interneurons, resulting in reduced antinociceptive output from the vlPAG onto the RVM, equally leading to a hyperalgesic state. In light of the extensive data linking OIH mechanisms to the RVM and parallel similar pathologies that already implicate the PAG, investigation into the vlPAG pertaining to OIH is warranted.

Morphine Action on Descending Modulation

Morphine acts on μ-opioid receptors (MOR), which are inhibitory G-protein coupled receptors. MOR are particularly enriched in the vlPAG–in both GABAergic and glutamatergic populations– as well as on RVM ON-cells (Commons et al 1999, Commons et al 2000, Wang

and Wessendorf 2002; Yaksh, Yeung, and Rudy, 1976; Marinelli et al., 2002). The classical model of opioid-induced analgesia suggests that opioids work through disinhibition in the vlPAG. In the presence of opioids, MORs expressed on local GABAergic interneurons in the vlPAG are activated and rapidly suppress activity. This disrupts their tonic inhibitory control over glutamatergic outputs, thus mediating analgesia through excitation of OFF cells that is paired to inhibition of ON-cell activity (Liebeskind, Guilbaud, Besson, & Oliveras, 1973; Sandkühler and Gebhart, 1984; Zorman et al., 1981; Sharpe, Garnett, & Cicero, 1974; Tortorici and Morgan, 2002; Cheng, Fields, Heinricher, 1986; Fang et al., 1989; Tortorici and Morgan, 2002; Bernal, Morgan, and Craft, 2007).

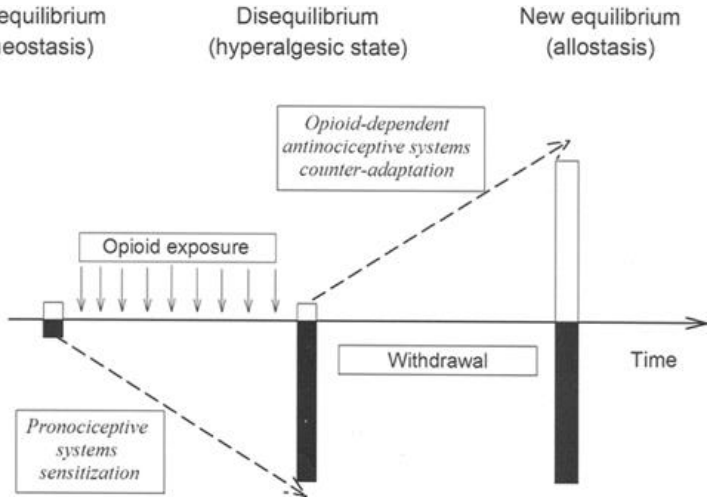

Figure 3. Opponent-process theory of OIH
A schematic model that depicts the neuroadaptive changes as a result of opioid exposure. Initially, opioid exposure elicits a compensatory sensitization of pronociceptive systems that is observed as the development analgesic tolerance. When opioid exposure ceases or analgesic tolerance, the disparity between enhanced pronociception and a baseline antinociceptive system leads to a hyperalgesic state. The time course of this hyperalgesia (i.e. withdrawal period) continues until a counter-adaption develops in opioid-dependent antinociceptive systems and a new equilibrium is achieved. The allostasis is associated with long-term pain vulnerability, which is attributed to a high-level equilibrium between the opioid-dependent antinociceptive systems and pronociceptive systems. Adapted from Célèrier et al. (2001).

The mechanism by which morphine evokes hyperalgesia is not yet fully understood, but in the descending pain pathway it appears to involve similar mechanisms to morphine-induced

analgesia. In fact, MOR expression is necessary for the development of OIH, whereby global knock-out of MOR expression blocked the development of OIH without perturbing normal pain thresholds (Roeckel et al., 2017). Similarly, ultra-low dose naloxone, a non-selective opioid antagonist, alleviated the expression of OIH in rat without altering concomitant analgesic tolerance (Aguado et al., 2013). It is postulated that repeated or high-dose morphine exposure elicits neuroplastic changes in sites that mediate morphine analgesia, particularly within the descending pain pathway, that function to oppose morphine's analgesic action and enhance an opposing anti-analgesic effect. For instance, an increased ratio of ON- to OFF-cells in the RVM has been demonstrated with repeated morphine exposure in rats (Meng and Harasawa, 2007). In the PAG, there is evidence of neuroplastic changes involving β-arrestin2, which were found necessary for the expression of OIH and that involve pERK1/2 and JNK signaling pathways (Aberoumandi et al., 2019). We therefore hypothesize that such neuroplastic changes induced in a state of OIH may be evidenced in expression of select activity-dependent genes.

Immediate Early Genes

Immediate early genes (IEG) are genes whose expression is modulated by neuronal activation, allowing their expression levels to serve as activity markers. IEGs codify for transcription factors that perform crucial post-translational operations on neuron structure, function, and survival and have been implicated in activity-dependent changes in synaptic strength (Fleischmann et al. 2003; Chandramohan et al. 2008; Deisseroth et al. 2003; Wu, Deisseroth, and Tsien, 2001).

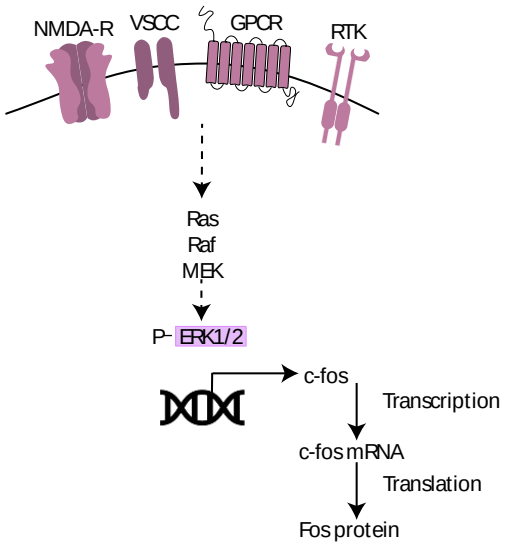

Figure 4. Overview of Fos and pERK1/2 signaling pathway
Schematic represents the general pathway of intracellular signaling cascades that leads to phosphorylation of ERK1 and ERK2 kinases that can activate c-fos promoters, ultimately leading to Fos expression. Schematic is adapted from (Cruz, Rubio, and Hope, 2015).

The first widely used and most studied IEG is *c-fos*, a proto-oncogene that is widely expressed in many neuronal populations. Fos expression remains relatively low in a basal state (Lyons and West, 2011) when the intracellular calcium levels are low and transiently increase in response to robust or persistent extracellular stimuli (Morgan and Curran, 1986; Curran and Morgan, 1995). Typically, Fos signaling is transduced via a cascade originating with the activation of glutamate receptors (NMDA-R) and L-type voltage-sensitive calcium channels (VSCC) (Dudek and Fields, 2001; Berretta et al., 1997; Cohen and Greenberg, 2008; Vanhoutte et al., 1999; Murphy, Worley, and Baraban, 1991). Strong, persistent activation of these receptors mediates a slow, strong increases in calcium that has the potential to activate kinase pathways, namely through mitogen-activated protein kinases (MAPK) –PKC pathways (Lyons and West, 2011; Murphy et al. 2002; Deisseroth and Tsien, 2002; Stachowiak et al., 1989). These kinases in turn exert regulatory control over Fos levels by phosphorylation and activation

of Fos promoters in the nucleus, such as CREB (Lyons and West, 2015). Within minutes of relevant external stimuli, *c-fos* transcription is activated, leading to an increase in detectable mRNA and subsequent translation of Fos protein with a half-life of approximately 2 hours (Muller et al., 1984). This transient rise in Fos protein is detectable in neurons between 20min to 4-16 hours after, with a peak at around 1hr (Gao and Ji, 2009).

Functionally, Fos protein is often a constituent of the Activator Protein-1 (AP-1) complex via its dimerization with Jun proteins, which regulates gene expression through interactions at specific promoter and enhancer motifs (Chiu et al, 1988). Through its ability to activate AP-1, Fos is involved in signaling that regulates the expression of "late genes" and effector proteins, some of which appear to be involved in synaptic plasticity (Tuvikene et al. 2016). In the context of pain, Fos activation patterns have been correlated with neuroplasticity related to prolonged exposure to opioids as well as hyperalgesic states, particularly in the vlPAG. Acute persistent pain, i.e. formalin hind paw injections, is capable of evoking robust Fos activity in the forebrain of rodents, including the PAG (Senba et al. 1993; Wei et al. 2001). Interestingly, the increased PAG Fos expression associated with diabetic neuropathy pain was attenuated with successful treatment of hyperalgesia with gabapentin, a drug similarly observed to treat OIH in rats (Morgado, Terra, and Tavares, 2010; Stoicea et al., 2015).

Although not classified as an immediate early gene, the activity-dependent expression of phosphorylated extracellular signal-regulated protein kinase (pERK) is immediately upstream of IEGs and can serve as a valuable neural marker for activity. ERK1 and 2 are two serine/threonine kinases of the MAPK family that are capable of driving IEG expression through phosphorylation of specific promoter motifs (Roskoski et al. 2012). As such, ERK1/2 regulates Fos-mediated gene expression as well as numerous other regulatory functions and neuroplastic changes in neurons (Murphy et al. 2002; Gilley, March, and Cook, 2009; Mazzucchelli et al., 2002; Roskoski et al. 2012). Importantly, ERK signaling is associated with pain hypersensitivity (Ji et al. 1999; Ji and Woolf, 2001; Ji et al., 2003). Compared to Fos,

pERK1/2 signal is detected in an earlier, more narrow time window. Similar to Fos expression, pERK1/2 expression requires persistent, high-threshold noxious stimuli (Sasaguri et al., 2005; Gioia, Moscheni, and Gagliano, 2005).

Lastly, NPAS4 (neuronal PAS domain protein 4) is another IEG that has been utilized as a neural activity marker, notably in the context of synaptogenesis and in contextual memory formation (for review see: Sun and Lin, 2016). Unlike Fos and pERK1/2, NPAS4 is insensitive to paracrine signaling, making it more specific to neuronal depolarization than Fos and pERK1/2 (Lin et al., 2008; Sun and Lin, 2016).To date, very few studies have investigated NPAS4 expression in the PAG and it is not yet known whether it is viable to measure activity-related changes in the PAG.

The present study

The goal of the present study was to investigate a potential role of the vlPAG in mediating OIH. It was hypothesized that neuroplastic changes occur within the vlPAG following sustained morphine exposure correlated to the expression of a hyperalgesic state. First, we tested for the presence of hyperalgesia by measuring mouse mechanical pain thresholds, determined using the von Frey reflexive pain assay. Next, in order to identify the presence of neuroplastic changes, we employed immunohistochemistry techniques to quantify the expression of select IEGs in the rostro-caudal extent of the vlPAG in both saline and morphine-treated animals. On the basis of Aberoumandi et al.'s findings, we expected to detect a significant rise in the pERK1/2 and Fos expression in the vlPAG of morphine-treated mice (2019).

RESULTS

To assess changes in pain thresholds as a result of chronic morphine exposure, von Frey mechanical thresholds were collected before and after four days of morphine or saline administration. It has previously been shown that four days of high-dose morphine is sufficient to produce the behavioral sensitization to potentially noxious stimuli, characteristic of opioid-induced hyperalgesia (Johnson et al., 2014). Mechanical hypersensitivity was observed in morphine-treated mice (n=5), as shown by the reduced paw withdrawal threshold in morphine treated mice compared to saline controls, suggesting induction of OIH (Mann-Whitney test, *p=0.0079; Fig 7A).

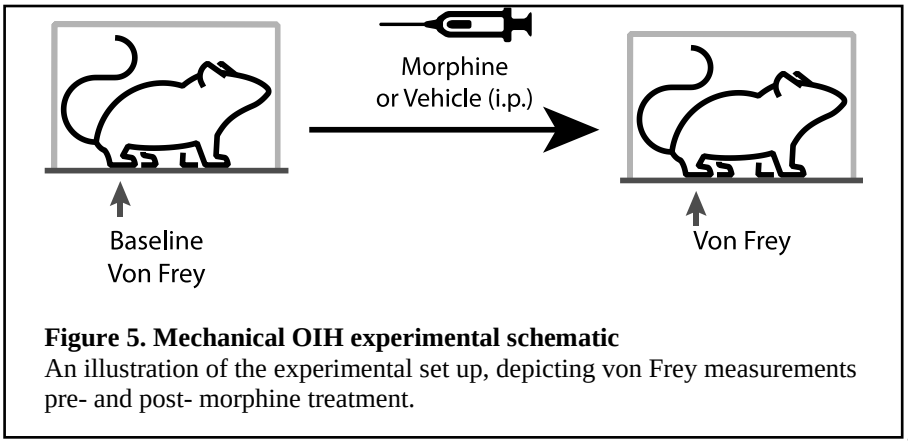

Figure 5. Mechanical OIH experimental schematic
An illustration of the experimental set up, depicting von Frey measurements pre- and post- morphine treatment.

To investigate whether OIH is associated with changes in basal activity within the vlPAG, NPAS4, Fos, and pERK1/2 immunostaining was performed in coronal vlPAG sections (Fig. 6, 7, 8). Foremost, NPAS4 staining was determined not viable. No NPAS4 puncta were detectable in the vlPAG; however, cortical and hippocampal regions displayed staining, indicating the IHC

protocol and tissue were viable (Figure 6).

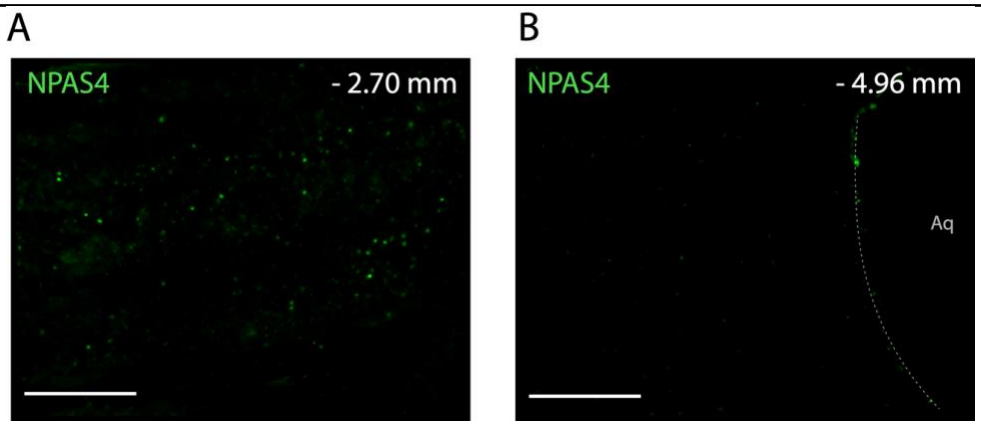

Figure 6. Acute formalin phase I pain-evoked NPAS4-IR in opioid-induced hyperalgesia
Representative images from a mouse showing (A) cortical NPAS4-IR and (B) vlPAG lack of staining.

Along the rostro-caudal axis, morphine-treated mice displayed reduced Fos-IR compared to controls at -4.16mm (***$p<0.05$), -4.24 (**$p<0.01$), -4.48mm (*$p<0.1$), and -4.72mm(*p) (Mann-Whitney test, Fig. 7C). Unlike the differences between specific bregma levels, the mean estimated Fos-IR cell count for the full rostro-caudal extent of the vlPAG was not significantly reduced in morphine treated mice (2246 ± 197.9) compared to controls (3338 ± 516.2) ($p=0.0079$; Fig.7D, E; mean ± SEM). Similarly, basal-state mean pERK1/2-IR cell totals for morphine treated mice (871.0 ± 63.5) were not significantly different from that of controls (576.0 ± 230.5) (Mann-Whitney test, $p>0.05$; Fig. 8C). While we note a general weak trend of morphine-treated mice having greater caudal pERK1/2-IR than controls, we were unable to statistically test this comparison (Fig. 8B).

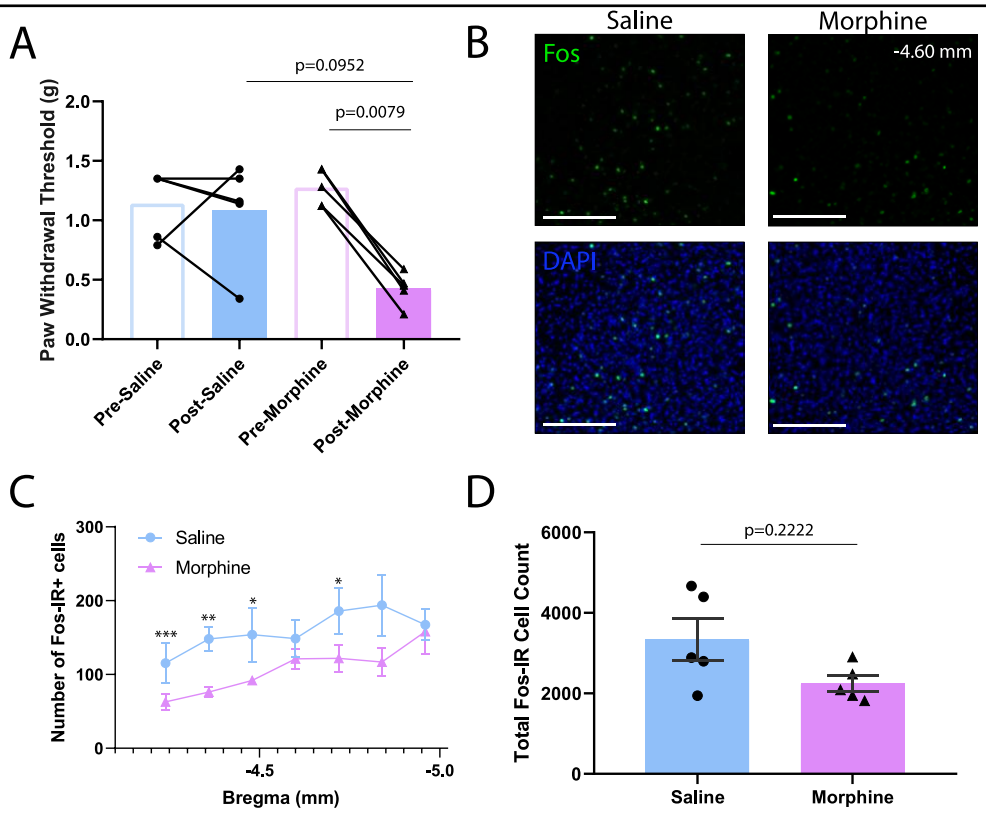

Figure 7. Basal state Fos-IR in opioid-induced hyperalgesia mouse model
(A) Mechanical pain sensitivity pre- and post-treatment comparing saline controls to morphine receiving mice. Data are presented as the mean withdrawal threshold in grams ± SEM. A significant difference was determined between pre- and post-morphine mechanical thresholds as well as comparing post-treatment saline and morphine animals (p=0.0087); Mann-Whitney test). (B) Representative images from coronal brain slices containing the vlPAG and immunostained for Fos (green) and DAPI (blue). Images were selected from one control (saline column) and one experimental (morphine column) mouse at the same bregma level. Top panels display Fos labeling, bottom panels depict Fos and DAPI overlaid. Scale bars represent 200µm. (C) Number of Fos-IR cell bodies in the vlPAG per bregma level along the rostro-caudal axis. Each data point represents mean ± SEM number of cells at each level (n=5, seven slices per mouse). Morphine-treated counts were determined to be significantly different from saline at indicated bregma depths using a Mann-Whitney test (***p=0.05, **p=0.01, *p=0.1). (D) Whole -brain estimate of total number of Fos-IR cell bodies in the vlPAG. Each data point represents the total Fos-IR from seven slices for one mouse multiplied by three (n=5 saline, n=6 morphine). No significant difference was observed (Mann-Whitney, p >0.05).

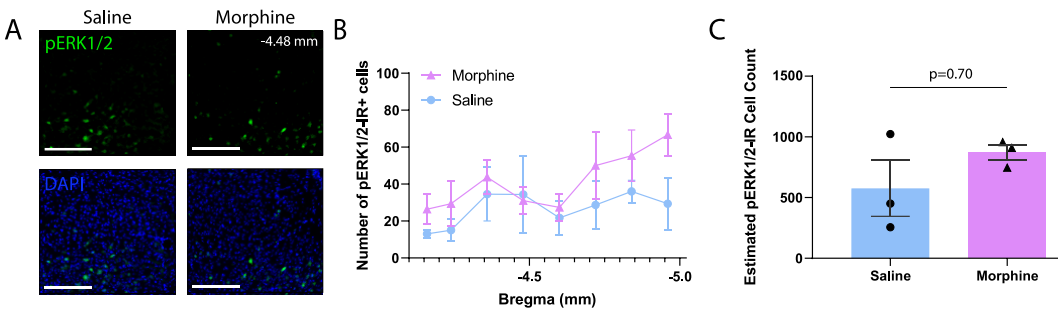

Figure 8. Basal state pERK1/2-IR in opioid-induced hyperalgesia mouse model
(A) Representative images from coronal brain slices containing the vlPAG and immunostained for pERK1/2 (green) and DAPI (blue). Images were selected from one control (saline column) and one experimental (morphine column) mouse at the same bregma depth. Top panels display pERK1/2 labeling, bottom panels depict pERK1/2 and DAPI overlaid. Scale bars represent 200µm. (B) Number of pERK1/2-IR cell bodies in the vlPAG per bregma level along the rostro-caudal axis. Each data point represents mean ± SEM number of cells at each level (n=3, seven slices per mouse). Treatment versus saline was not determined to be significantly different using a Mann-Whitney test due to small sample size ($p < 0.05$). (C) Whole-brain estimate of total number of pERK1/2-IR cell bodies in the vlPAG. Each data point represents the total pERK1/2-IR from seven slices for one mouse multiplied by three (n=3). No significant difference was observed due to sample size in a Mann-Whitney test ($p < 0.05$).

In order to determine whether pain-evoked activity was altered in a state of OIH, Fos and pERK1/2 immunostaining was performed on tissue collected following acute persistent inflammatory pain by formalin injection into the animal's hindpaw. Formalin-induced inflammatory pain proceeds in three stages that are characterized by differential nocifensive response and possible mechanisms of action (Dubuisson and Dennis, 1977; Fig. 9B).

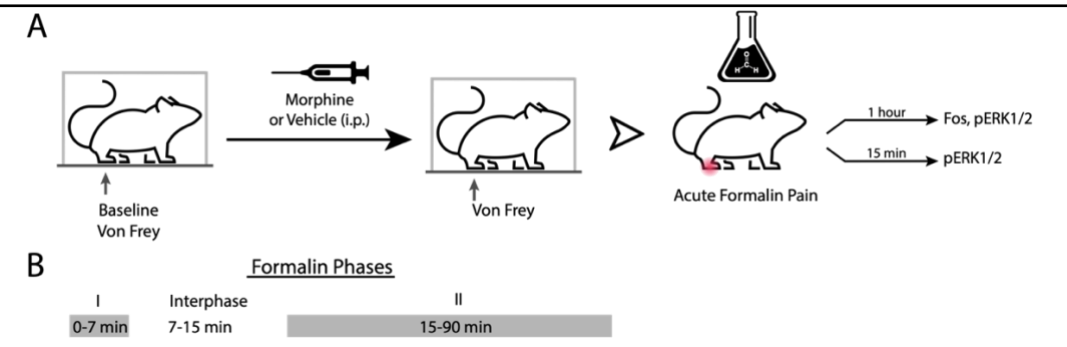

Figure 9. Schematic of Formalin-evoked inflammatory pain behavioral paradigm
(A) Timeline for experiments that includes schematic of the von Frey chamber and formalin-induced inflammatory pain. (B) Schematic depicting the formalin-induced inflammatory pain phases I and II, as described by Dubuisson and Dennis (1977).

In order to compare expression patterns between phases while maximizing the amount of tissue utilized from a single animal, we employed two time frames of expression capture for pERK1/2 designated tissue and one previously validated time frame for Fos designated tissue. Phase I ('acute') pain-evoked neural activity was detected in tissue harvested 1 hour after induction using Fos antibody and in tissue harvested 15 minutes after induction using pERK1/2 antibody (Fig. 9A; Fig. 10, 11). There was no significant difference observed between the number of Fos-IR cells in the vlPAG of OIH animals compared to controls ($p>0.05$; Fig. 10C, D).

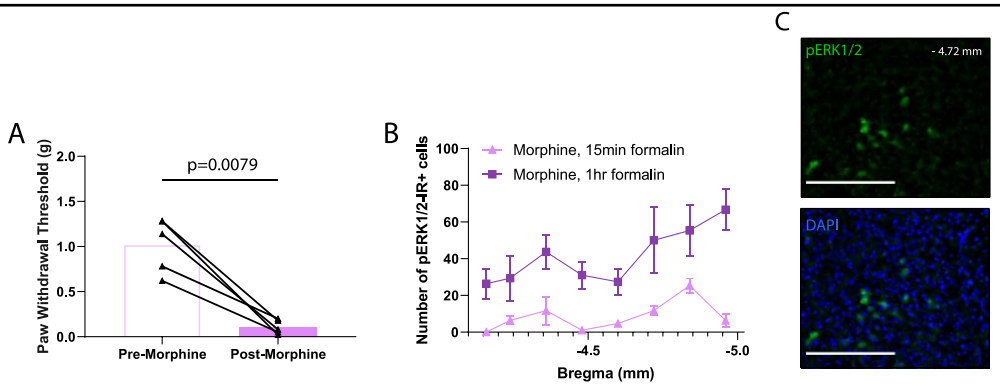

Figure 10. Acute formalin phase I pain-evoked pERK1/2-IR in opioid-induced hyperalgesia

(A) Mechanical pain sensitivity pre- and post-treatment in morphine receiving mice. Data are presented as the mean withdrawal threshold in grams ± SEM. A significant difference was determined between pre- and post-morphine mechanical thresholds (p=0.0079; Mann-Whitney test). (B) Representative image from coronal brain slices containing the vlPAG and immunostained for pERK1/2 (green) and DAPI (blue). Top panels display pERK1/2 labeling, bottom panels depict pERK1/2 and DAPI overlaid. Scale bars represent 200µm. (C) Number of pERK1/2-IR cell bodies in the vlPAG per bregma level along the rostro-caudal axis in morphine-treated mice either 15-min or 1-hr post-injection. Each data point represents mean ± SEM number of cells at each level (n=3, six or seven slices per mouse). No statistically significant difference was determined due to small samples size (Mann-Whitney $P > 0.05$).

However, it is interesting to note that formalin-induced Fos-IR cell counts were significantly greater in both morphine (p=0.0011) and saline (p=0.0281) treated mice than those observed in the basal state, indicating the activation of pain-responsive subpopulations within the vlPAG (Fig. 11C, Fig.7C). Using pERK1/2 immunolabeling for Phase I, weak to modest labeling was detected in morphine-treated mice (Fig.10A, B, C). Labeling was primarily concentrated in the caudal

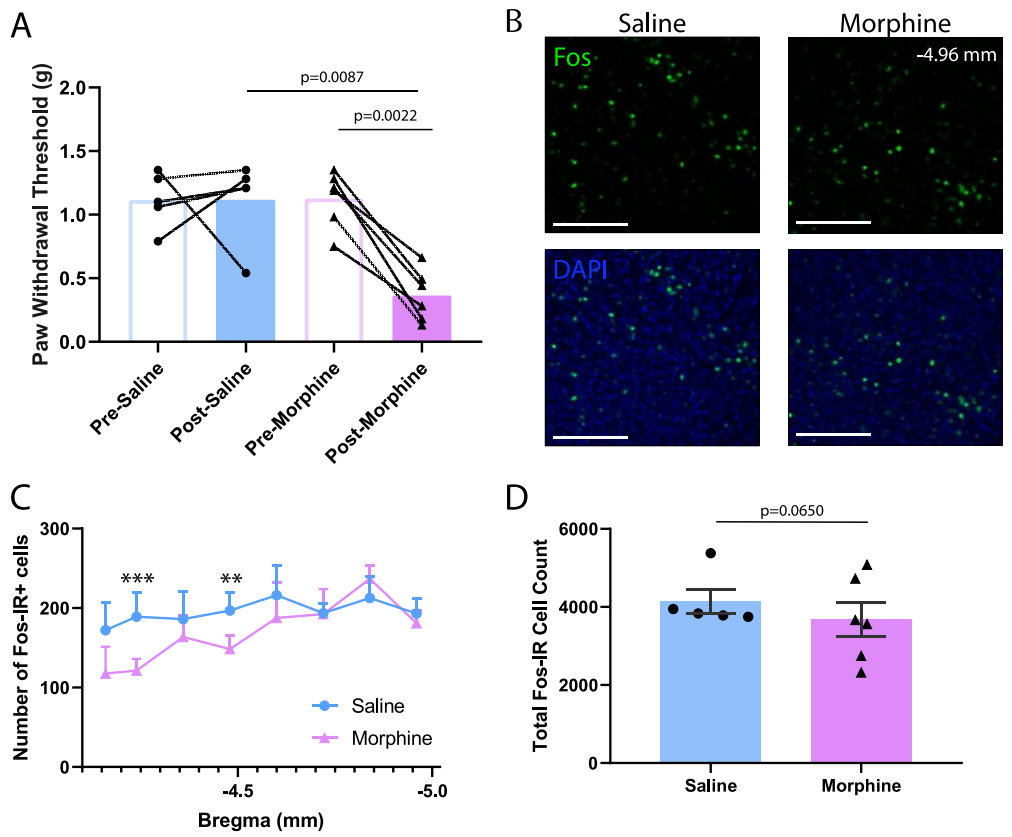

Figure 11. Acute formalin phase I pain-evoked Fos-IR in opioid-induced hyperalgesia
(B) Mechanical pain sensitivity pre- and post-treatment comparing saline controls to morphine receiving mice. Data are presented as the mean withdrawal threshold in grams ± SEM. A significant difference was determined between pre- and post-morphine mechanical thresholds as well as comparing post-treatment saline and morphine animals (*$p < 0.05$; Mann-Whitney test). (C) Representative images from coronal brain slices containing the vlPAG and immunostained for Fos (green) and DAPI (blue). Images were selected from one control (saline column) and one experimental (morphine column) mouse at the same bregma depth. Top panels display Fos labeling, bottom panels depict Fos and DAPI overlaid. Scale bars represent 200µm. (D) Number of Fos-IR cell bodies in the vlPAG per bregma level along the rostro-caudal axis. Each data point represents mean ± SEM number of cells at each level (n=5 saline, n=6 morphine, seven slices per mouse). Morphine-treated counts were determined to be significantly different from saline at indicated bregma depths using a Mann-Whitney test (***p=0.05, **p=0.01). (E) Whole -brain estimate of total number of Fos-IR cell bodies in the vlPAG. Each data point represents the total Fos-IR from seven slices for one mouse multiplied by three (n=5 saline, n=6 morphine). No significant difference was observed (Mann-Whitney, $p > 0.05$).

portion of the vlPAG, which is consistent with previous findings that emphasize the predominance of caudal region activity in promoting descending pain modulation (Yaksh, Yeung, and Rudy, 1976; Manning and Franklin, 1998). Phase II (‘tonic’) pain-evoked neural activity was detected in tissue harvested 1 hour after induction using pERK1/2 antibody (Fig.

12). This analysis did not demonstrate any significant difference between morphine and saline treated animals (Mann-Whitney test $p<0.05$, Fig. 12 B, C).

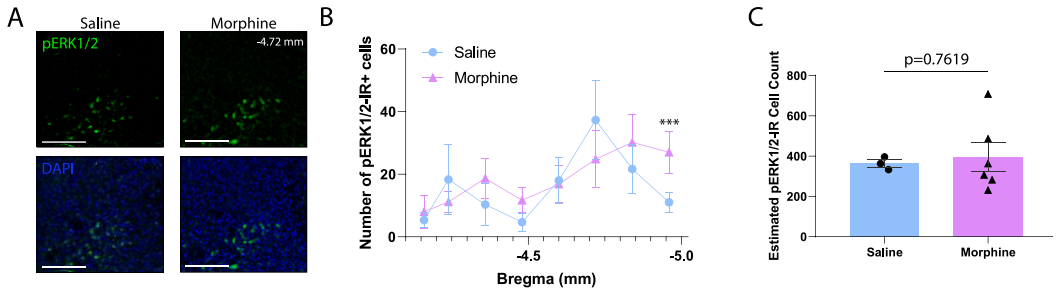

Figure 12. Acute formalin phase II pain-evoked pERK1/2-IR in opioid-induced hyperalgesia (A) Representative images from coronal brain slices containing the vlPAG and immunostained for pERK1/2 (green) and DAPI (blue). Images were selected from one control (saline column) and one experimental (morphine column) mouse at the same bregma depth. Top panels display pERK1/2 labeling, bottom panels depict pERK1/2 and DAPI overlaid. Scale bars represent 200µm. (B) Number of pERK1/2-IR cell bodies in the vlPAG per bregma level along the rostro-caudal axis. Each data point represents mean ± SEM number of cells at each level (n=3 saline, n=6 Morphine, seven slices per mouse). No significant difference was observed between treatments. (C) Whole -brain estimate of total number of pERK1/2-IR cell bodies in the vlPAG. Each data point represents the total pERK1/2-IR from seven slices for one mouse multiplied by three (n=3 saline, n=6 morphine). No significant difference was observed.

DISCUSSION

Opioid-induced hyperalgesia is believed to be at least partially mediated by descending pain facilitation from the RVM, a pain modulatory locus that is under direct control of the vlPAG. We used an immunohistological analysis to demonstrate that Fos protein expression is significantly reduced in male mice that received repeated systemic morphine compared to controls. We also investigated the formalin-evoked activity levels in the vlPAG and found no statistically significant difference between OIH and control animals.

Our data indicate a possible facilitatory relationship between the vlPAG and RVM in the promotion of OIH that is evident in a resting state but not in a state of persistent inflammatory pain. A reduction in Fos in the basal condition of OIH animals may hint to a loss of tonically active descending antinociception between the vlPAG and RVM (Fig. 2A). Previous work has demonstrated that suppressing glutamatergic neurons in the vlPAG produces a hypersensitivity to thermal and mechanical stimuli, though at a markedly smaller magnitude than observed in OIH (Samineni et al., 2017). Therefore, we theorize that a reduction in basal Fos may correspond to a compensatory reduction in glutamatergic output to the RVM following chronic morphine exposure, thereby resulting in a loss of descending antinociceptive signal from the vlPAG, which may act concomitantly with RVM-mediated descending facilitation to enhance pain sensitivity in OIH. However, our data are limited to overall, non-specific Fos expression. Thus, we do not know whether the downregulated neuronal population are projection cells nor their molecular identity. We propose that future studies be undertaken to identify the molecular identities of these Fos cells and distinguish RVM-projections from interneurons, which may reveal a greater understanding of the underlying circuits effected. Alternatively, the discrepancy of the Fos expression levels between conditions was limited to a small sample size of five mice per condition and thus may not be representative of the overall population. Nevertheless, we believe our work could be a starting point for future studies investigating activity-dependent changes of the vlPAG in cases of OIH.

Furthermore, these findings suggest that the vlPAG activity patterns during inflammatory pain is not differentially affected by OIH. It would be interesting to compare these results to similar studies that employ different pain modalities to evoke neural activity in OIH in this region, as it may further expand on the modality-specific nature of OIH. It should also be noted that the magnitude of formalin-evoked Fos-IR was modest compared to previous studies, which have demonstrated multiplicative increases in such a magnitude (Senba et al. 1993; Wei et al. 2001). One possible explanation for this discrepancy of effect lies in the phase in which the peak Fos expression was captured from, depending on the incubation period allotted for formalin-evoked activity. Our protocol allowed one-hour post-formalin hindpaw injection for the transduction of Fos signal. The resulting Fos signal which was believed to primarily capture the neural activation during the acute Phase (I) of inflammatory pain. While both Phase I and II of formalin-induced inflammatory pain recruits midbrain activity, evidence indicates Phase II pain recruits this region to a greater extent, in a mechanism attributed to supraspinal maintenance of spinal sensitization, i.e. a condition where we might expect to see robust IEG expression (Hunskaar and Hole, 1987; Morrow et al., 1998; Vaccarino and Chorney, 1994). We recommend formalin behavioral responses be assessed in future studies to determine whether OIH confers an exaggerated nocifensive response, which might shed light as to the lack of difference between Fos-evoked vlPAG expression in OIH versus control animals.

Three distinct markers for neural activity were investigated in this study that are– or are upstream of– immediate early genes. Not all activity markers are alike and selecting one that yields optimum expression levels for detection and relevance can prove to be a challenge. We found that Fos immunofluorescence provided reasonably consistent, reproducible detection of robust neural activity in the vlPAG. On the other hand, pERK1/2 signal quality and abundance were variable; puncta could be difficult to discern, sometimes completely undetectable despite validation of Fos signal within the same brains. We think this is partially because pERK1/2 is not predominantly nuclear bound, like Fos, and is likely too diffusely expressed to produce a

maximal signal-to-noise ratio in the given set-up. Moreover, NPAS4 successfully stained hippocampal regions of mouse brains but we were unable to produce labeling within the vlPAG in either basal or formalin-pain evoked conditions. NPAS4 may not be viable for the detection of OIH-related activity patterns in the vlPAG, a theory that is substantiated by prior literature that reported a lack of change in HDAC5 expression– the precursor to NPAS4–in a neuropathic pain model for rats, for which we expect to be mechanistically similar (Descalzi et al., 2017). Overall, we found Fos immunolabeling to be the preferred activity marker for use in the vlPAG within the context of OIH and formalin-evoked persistent pain.

The findings of these experiments are confined to male mice and cannot be extrapolated to females. Sexual dimorphisms exist in the nociceptive system in rodents and humans and complicate our current understanding of morphine-evoked analgesia and hyperalgesia. Interestingly, male rats show greater morphine-evoked analgesia while female rats exhibit a greater hyperalgesic shift in OIH conditions (Holtman and Wala, 2005). This is paired with increasing evidence to suggest that morphine antinociception is carried out through different mechanisms than in males, one that evidently does not require vlPAG MOR-expressing cells (Bernal, Morgan, Craft, 2007; Loyd, Wang, and Murphy, 2008; Loyd and Murphy, 2014). In the clinical setting, it is currently not evident what sex differences, if any, exist in OIH, but these differences are integral to targeting pain disparities. Thus, replication of these data is warranted in order determine whether these results are recapitulated in females.

The evidence from this study points towards the idea of the vlPAG undergoing neuroplastic changes in a state of OIH. We found that basal Fos-IR was significantly reduced in mice receiving morphine-treatment compared to controls at particular Bregma levels. These results would seem to suggest a reduction of antinociceptive output from the vlPAG to the RVM, which may promote descending facilitation and ultimately hyperalgesia.

MATERIALS AND METHODS

Animals Experiments were performed on male C57Bl/6J mice between postnatal day 40 and 60. All experimental animals were housed in groups of five or six in cages with TekFresh bedding on a reverse dark/light cycle with food and water *ad libitum*.

Mechanical Hyperalgesia Pain sensitivity in mice was examined using the Von Frey test, which utilizes a series of calibrated nylon filaments ranging from 2.44 to 4.31 g. All Von Frey testing was conducted at the same time each day during the beginning of the animals' dark cycle under red light. Animals were placed in small plexiglass cylinders on top of a wire-mesh floor and allowed to acclimate for 30 minutes. Animals were habituated to the testing chambers for 5 days before baseline testing, and the pre-drug administration baseline was measured across three sessions with one day of rest between each session. The post-drug administration thresholds were compared to the third pre-drug administration baseline threshold. Animals were acclimated on the testing day for 30 minutes on the testing days. The Von Frey filaments were applied to the midplantar surface of the hind paw until slight bending of the fiber occurred. The fiber was allowed to make contact for at least 8 seconds or until a paw withdrawal was observed. The fiber scoring procedure was based on the up-down method and calculated as previously described (Chaplan et al., 1994). A baseline of three pre-drug administration days was collected every other day. A post-drug testing was performed 16hr following the final drug administration.

Chronic Morphine Administration Following baseline nociceptive testing, animals received intraperitoneal (i.p.) injections of either saline or morphine (NIDA) twice daily for four consecutive days, as modified from other protocols for the induction of OIH (Liang et al., 2013; Johnson et al., 2014, Ahmadi et al., 2014). Morphine was administered 20mg/kg with the last

injection on the fourth day increased to 40mg/kg. The experimenter was blinded to the drug from the time point of injections until the completion of the IHC quantifications.

Formalin-induced Pain Male mice were lightly anesthetized using isoflurane and injected with 2.5% formalin in the right hindpaw according to Gong et al., 2014. 10 mice were allowed to recover for 1hr prior to sacrifice for Fos and NPAS4 staining, and 5 mice were allowed to recover for 15min prior to sacrifice for pERK1/2 staining.

Immunohistochemistry Tissue intended for baseline Fos IHC was harvested at least three hours after the behavioral test session ended. All subjects were anesthetized with aerosolized isoflurane and transcardially perfused using ice cold 0.1M PBS and then fixed with 4% paraformaldehyde (PFA) in PBS. Brains were harvested and allowed to post-fix in 4% PFA overnight at 4ºC. Brains were cryoprotected in 30% sucrose for 72hr prior to slicing into 40um slices using a frozen microtome between bregma -4.96 and -4.16 mm. The free-floating sections were rinsed in 0.1M PBS (3 x 10min). Slices were blocked in 10% normal donkey serum (NDS) and 0.3% PBS-Triton (PBS-T) at room temperature for 2hr with gentle agitation. Slices were incubated in respective primary antibodies with 2%NDS and 0.1% PBS-T for 48hr at 4ºC with gentle agitation. Antibodies used were rabbit polyclonal phospho-p44/42 MAPK (p-ERK) (1:250, #9101, Cell Signaling Technology, Danvers, MA, USA); recombinant rabbit monoclonal anti-Npas4 (1:2000, AB18A, Lot NP41-2), Activity Signaling, USA); rabbit monoclonal anti-Fos (1:1000, #2250, Cell Signaling Technology, Danvers, MA, USA). Sections were rinsed in PBS (3 x 10min) and incubated for 2hr at room temperature with secondary antibody Donkey anti-rabbit Alexa-488 (1:500, Jackson ImmunoResearch). Sections were then rinsed in PBS (3 x 10min) and mounted onto glass slides, air-dried, and cover slipped using VECTASHIELD with DAPI (Vector Laboratories). Images were acquired using a Keyence BZ-X710 using a 10x objective.

Immunohistochemistry Quantification Each slice image was quantified for the total number of DAPI nuclei and reporter labels within a hand-drawn vlPAG region of interest based upon the Paxinos brain atlas (Paxinos and Franklin, 2019). For Fos staining, image-based quantification was conducted using CellProfiler to measure positively labeled DAPI nuclei. The pipeline utilized features a rolling-ball background subtraction, Otsu two-class thresholding, and positive puncta counting based on the colocalization of accepted immunolabeling and DAPI-stained nuclei. pERK1/2 labeled cells were hand counted using ImageJ software. The experimenter was blind to the treatments.

Data analysis Behavioral and immunohistology data are presented as means +/- SEM. The development of opioid-induced hyperalgesia and immunofluorescence counts were analyzed by non-parametric Mann-Whitney test (Graphpad Prism).

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