UNIVERSITY OF CALIFORNIA SAN DIEGO

A Spatial Exploration of Mu Opioid Receptor Transcripts in the Locus Coeruleus

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

Jean Chi-Yin Yung

Committee in charge

Professor Matthew Banghart, Chair
Professor Dhananjay Bambah-Mukku
Professor Cory Root

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University of California San Diego

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DEDICATION

To my parents, for their sacrifices, endless support, and unconditional love, which have allowed me to complete this work.

To my siblings, nieces, and nephews for inspiring me to become the best version of myself.

To my friends for their encouragement, friendship, and love.

EPIGRAPH

Looking out over the expanse ahead I saw not an empty wasteland but something simpler:

a blank page on which I would go on.

Paul Kalanithi

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ABSTRACT OF THE THESIS

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Professor Matthew Banghart, Chair

The Locus Coeruleus (LC) is the major source of noradrenaline in the brain and sends distinct projections to supraspinal brain structures and the spinal cord. While ascending projections from the dorsal LC are implicated in anxiogenic and aversive behavior, descending projections from the ventral LC to the dorsal horn of the spinal cord mediate noradrenaline-induced analgesia. In the LC, administration of opioid analgesics increases potassium channel conductance, leading to membrane hyperpolarization and decreased neuronal firing. Yet, why opioid analgesics

paradoxically inhibit a major center for noradrenaline-mediated analgesia is unclear. The answer to this may lie in differential transcription of the mu opioid receptor (MOR) along the dorsal-ventral axis of the LC. We hypothesized that the dorsal portion of the LC, responsible for anxiety-generating behavior, may display higher quantities of MOR RNA transcripts compared to the analgesia-mediating ventral portion of the LC. To elucidate if MOR transcription in the LC follows a dorsal-ventral gradient, we performed RNA *in situ* hybridization on the LC. However, quantification of MOR transcripts in the LC showed no obvious dorsal-ventral gradient of MOR. This suggests that the answer to the paradoxical effect of opioids in the LC does not lie in inhomogeneous MOR transcription along its dorsal-ventral axis.

INTRODUCTION

Chronic pain is a highly prevalent medical condition that afflicts a broad population of U.S. adults (Dahlhamer et al., 2018). Persistent physical pain can impede a person's ability to carry out daily functions, impacting both physical and mental health. To ameliorate pain, opioids are commonly prescribed, but misuse and overprescription can lead to addiction and lethal overdose. Despite public health initiatives to decrease prescription rates, opioid-related addiction rates continue to increase each year (Zelaya, 2020). Understanding how exogenous opioids interact with the body's endogenous opioid system may be key in untangling the desired analgesic effects of prescription opioids from their adverse side effects. However, the specific mechanisms through which opioids interact with certain pain modulatory systems in the brain has yet to be fully elucidated.

Opioid drugs exert their analgesic function by exploiting the body's native endogenous opioid system. Endogenous opioids, like enkephalin and endorphins, are naturally synthesized within the body and allow the body to innately regulate pain sensation. Exogenous opioids, like morphine and fentanyl, exploit the existing endogenous opioid system by binding to opioid receptors (Corder et al., 2018). Several endogenous opioid receptors exist within the nervous system, such as the mu-, delta-, and kappa-opioid receptors. While kappa and delta receptors are known to mediate stress, anxiety, and pain, commonly used analgesics like morphine primarily target mu receptors (Valentino & Volkow, 2018; Vo et al., 2021). Despite their unique functions, all endogenous opioid receptors are coupled with the $G_{i/o}$ inhibitory G protein which impacts a downstream signaling cascade, leading to overall neuronal depression (Corder et al., 2018).

The ability to depress neuronal firing is central to the analgesic character of opioids. Opioids reduce nociception by mediating descending pathways in the central nervous system

(CNS), as well as by inhibiting peripheral nociceptive afferents. In the descending pain pathway, activation of opioid receptors on GABAergic interneurons in the periaqueductal gray (PAG) disinhibits excitatory projections to the rostral ventral medulla (RVM). The RVM sends projections to the dorsal horn of the spinal cord, impeding incoming pain signaling from the peripheral nervous system (PNS) before it is relayed to the thalamus (Lueptow et al., 2018). In addition to acting through the descending pain pathway, opioids can directly inhibit nociceptive afferents in the PNS and obstruct pain sensory information before it is transmitted to the CNS (Corder et al., 2018). Studies have implicated an additional brain structure in the descending pain pathway, the Locus Coeruleus (LC), though its role in the pathway has not yet been fully elucidated (Bajic & Proudfit, 1999; Kim et al., 2018).

The Locus Coeruleus (LC) is the largest source of noradrenaline production within the brain (Poe et al., 2020). Anatomically, the LC sends both ascending projections to various supraspinal structures and descending projections to the spinal cord. Within the LC, these projections are topographically distinct. The dorsal region of the LC sends projections towards areas such as the prefrontal cortex (PFC), while the ventral region sends projections to the dorsal horn of the lumbar spinal cord (Li et al., 2016).

In addition to being spatially distinct, the ascending and descending projections from the LC also differ in function. The ascending projections to the prefrontal cortex (PFC) are involved in anxiogenic and aversive behavior, while the descending projections from the LC to the spinal cord (SC) have been implicated in pain modulation (Hickey et al., 2014; Hirschberg et al., 2017). Chemogenetic activation of the LC to SC projections in rats suffering from neuropathic pain resulted in antinociception as evidenced by increased withdrawal thresholds in the Von Frey test for mechanical allodynia. This effect was abolished with an intrathecal injection of a noradrenaline

receptor antagonist, suggesting that the LC to SC projections mediate analgesia through the release of noradrenaline into the spinal cord (Hirschberg et al., 2017).

How opioids impact the function of the LC has been the interest of various studies. *In vitro* administration of opioids onto rat brain slices has been shown to exert an overall inhibitory effect on LC neurons. This concentration-dependent inhibition is caused by increased intracellular potassium conductance, resulting in hyperpolarization of the LC neurons (Williams et al., 1982). However, additional *in vivo* studies have shown that morphine analgesia in the PAG relies on noradrenaline release in the spinal cord. Local morphine injections into the periaqueductal gray (PAG) increased response latencies in rats during hot plate and tail flick assays. In contrast, injection of morphine into the PAG paired with an intrathecal injection of phentolamine, an alpha-adrenergic receptor antagonist, blocked the increase in response latencies. Blocking noradrenaline action in the spinal cord decreases the analgesic potency of morphine injected into the PAG, suggesting that spinal noradrenaline is necessary for morphine analgesia (Yaksh, 1979). Additionally, recent data from the Banghart Lab has shown similar results in mice *in vivo*. Systemic injection of morphine in mice resulted in analgesia, subsequently increasing withdrawal latencies during a hotplate assay. However, intrathecal injection of phentolamine fully prevented the expression of systemic morphine analgesia at lower doses and decreased the analgesia at higher systemic morphine doses. This further corroborates the requirement of noradrenaline release in the spinal cord for complete display of systemic morphine analgesia (Lubejko & Banghart, unpublished, 2022).

The source of spinal noradrenaline release in response to morphine analgesia has been purported through additional studies. Immunohistochemical staining revealed an increased quantity of cells positive for c-fos, an immediate early gene, in the LC of mice that received

systemic morphine injections compared to mice that received saline injections (Lubejko & Banghart, unpublished, 2022). The presence of c-fos in a cell indicates recent neuronal activity, suggesting that the LC is activated upon systemic injection of morphine and could be the source of spinal noradrenaline release. However, this presents a puzzling paradox. The inhibitory role of opioids on LC cells *in vitro* seemingly contradicts the necessity of spinal noradrenaline release in systemic morphine analgesia and LC activation in the presence of morphine. How is it that noradrenaline release from the LC is implicated in pain alleviation, but evidence has shown that the administration of morphine, a known analgesic, hyperpolarizes its cells?

Perhaps the answer to this paradox lies in the quantity of mu-opioid receptors (MORs) present on the ascending and descending projections from the LC. While previous work has established that the administration of opioids hyperpolarizes LC neurons, few studies have acknowledged the dichotomous function between the dorsal versus ventral LC when evaluating its response to opioids. For these reasons, an examination of the dorsal-ventral transcription of MOR in the LC may elucidate the seemingly contradictory effect of opioids on the LC. We hypothesized that dorsal regions of the LC may contain higher quantities of MOR RNA transcripts, while ventral regions of the LC may contain smaller quantities. Thus, the ascending projections, mediating anxiogenic behavior, are expected to be more inhibited in the presence of opioids, compared to the descending projections implicated in analgesia. To further investigate the role of MOR on LC neurons in opioid analgesia, we used fluorescent *in situ* hybridization to probe for MOR RNA transcripts throughout the mouse locus coeruleus. First, we sought to confirm the knockout of MOR in dopamine hydroxylase-positive cells in a Dbh-cre;$Oprm1^{fl/fl}$ mouse line for subsequent use in behavioral experiments investigating the role of MOR in morphine analgesia. In addition, we compared the presence of MOR RNA transcripts in the knockout mouse line to an $Oprm1^{fl/fl}$

mouse line lacking cre recombinase to evaluate effective thresholding values used to discriminate between MOR-positive and MOR-negative cells. Furthermore, to investigate the existence of a MOR transcript gradient in the LC that may explain the paradoxical role of opioids, we quantified the spatial distribution of MOR transcripts in *Oprm1*$^{fl/fl}$ mice. Ultimately, understanding MOR transcription within the LC may contribute to our understanding of how opioids interact with the LC, bringing us closer to better understanding the impact of opioids on the brain.

CHAPTER 1: Dbh-cre;*Oprm1*$^{fl/fl}$ Mouse Line Validation

1.1 Results

A primary identifier of LC cells is their noradrenergic character, which can be confirmed by the presence of dopamine beta hydroxylase (DBH) or tyrosine hydroxylase (TH). TH synthesizes L-dihydroxyphenylalanine from L-tyrosine, upstream of noradrenaline synthesis. Likewise, DBH synthesizes noradrenaline from dopamine. Both enzymes can be used to positively identify a noradrenergic cell. Thus, neurons within the LC can be targeted in mice using transgenic mouse lines with DBH or TH promoters.

This is exemplified by the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line in which the cre recombinase enzyme is exclusively expressed in cells with DBH. The cre enzyme is responsible for catalyzing DNA recombination at loxP sites present on the gene, resulting in excision. In the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line, loxP sites surround exons 2 and 3 of the MOR gene, *Oprm1*. Therefore, in theory, all DBH positive cells in Dbh-cre;*Oprm1*$^{fl/fl}$ mice lack MOR (Sauer, 2002).

However, cre-catalyzed recombination within transgenic mouse lines can be imperfect, resulting in residual expression of floxed genes. Therefore, we first sought to validate the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line and confirm the knockout of mu opioid receptors (MOR) within cells of the Locus Coeruleus (LC). To address this, we performed RNA *in situ* hybridization on the LC cells of Dbh-cre;*Oprm1*$^{fl/fl}$ mice. The knockout of MOR within the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line can be achieved by excising select exons within the *Oprm1* gene. The excision of some, but not all exons, is sufficient in preventing the production of functional protein products. However, the remaining exons can still be detected by a conventional *Oprm1* probe, falsely conflating the quantity of functional transcripts identified. To circumvent this, we used an *Oprm1-O4* probe, which specifically targets the exons 2 and 3, excised in the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line. While

Dbh-cre;*Oprm1*$^{fl/fl}$ mice are expected to lack the MOR in DBH-positive cells, *Oprm1*$^{fl/fl}$ mice contain the loxP sites surrounding the *Oprm1* gene, but are deficient in the cre-recombinase needed for the loxP excision. Therefore, they retain the functioning MOR gene and closely model wild type mice. As a result, the quantity of *Oprm1-O4* puncta present in Dbh-cre;*Oprm1*$^{fl/fl}$ LC cells can be compared to *Oprm1*$^{fl/fl}$ LC cells to evaluate effectiveness of the knockout.

To confirm the identity of LC neurons, we quantified the number of *Oprm1-O4* puncta in cells also displaying tyrosine hydroxylase (TH) transcripts using a pipeline created with the program, CellProfiler (Figure 1). To compare probability distributions of *Oprm1-O4* puncta present in LC cells of Dbh-cre;*Oprm1*$^{fl/fl}$ mice compared to *Oprm1*$^{fl/fl}$ mice, we found the cumulative probability of *Oprm1-O4* puncta per cell for each mouse line. Compared to the *Oprm1*$^{fl/fl}$ curve, the Dbh-cre;*Oprm1*$^{fl/fl}$ curve is shifted to the left (Figure 2A). Further analysis shows that a given LC cell in the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line has a 50% probability of containing 7 or less *Oprm1-O4* puncta, while a LC cell in the *Oprm1*$^{fl/fl}$ mouse line has a 50% chance of containing 12 or less *Oprm1-O4* puncta. This suggests that overall, the LC cells in the *Oprm1*$^{fl/fl}$ mice contained more puncta on average compared to the Dbh-cre;*Oprm1*$^{fl/fl}$ mice.

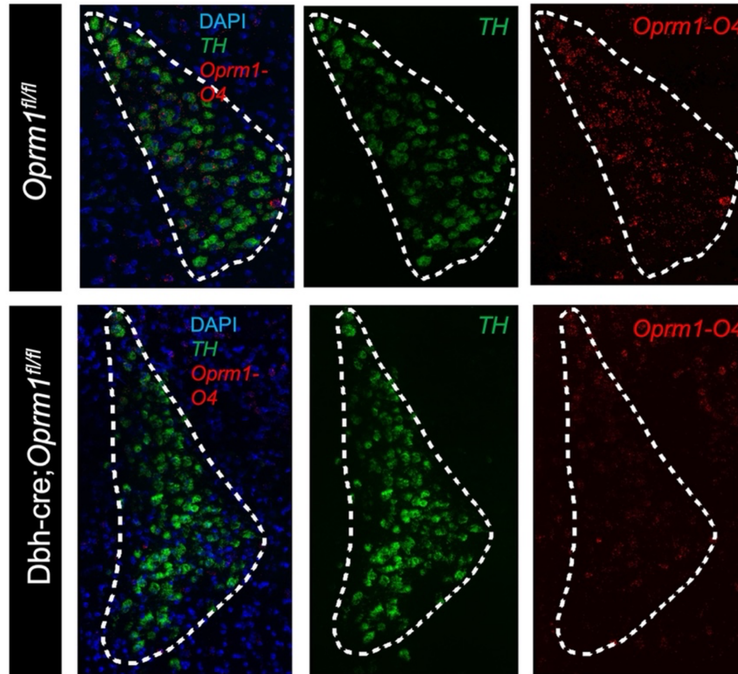

Figure 1: RNA *in situ* hybridization of the Locus Coeruleus (LC). Representative images of *in situ* hybridization in the LC of an *Oprm1*$^{fl/fl}$ and Dbh-cre;*Oprm1*$^{fl/fl}$ mouse. DAPI stains nuclei and is shown in blue. Tyrosine hydroxylase (*TH*) is shown in green. Mu opioid receptor is (*Oprm1-O4*) shown in red.

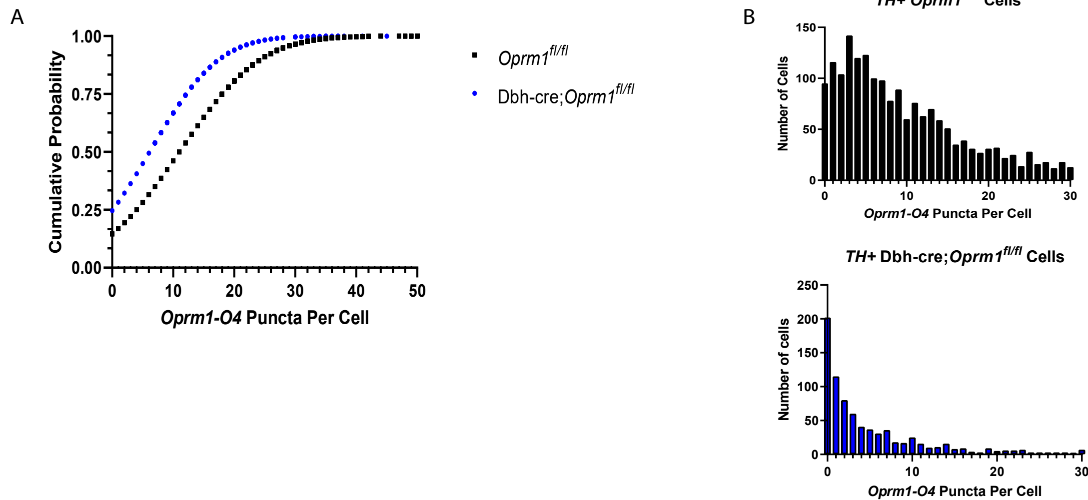

Figure 2: Differing quantities of *Oprm1-O4* RNA transcripts in *Oprm1*$^{fl/fl}$ and Dbh-cre;*Oprm1*$^{fl/fl}$ Mouse Locus Coeruleus (LC). A, Cumulative probability graph of Mu opioid receptor transcripts (*Oprm1-O4*) in *Oprm1*$^{fl/fl}$ and Dbh-cre;*Oprm1*$^{fl/fl}$ mice. **B,** Distribution histograms of *Oprm1-O4* puncta quantities in Oprm1$^{fl/fl}$ mice and Dbh-cre;*Oprm1*$^{fl/fl}$ mice.

Next, to determine the puncta quantity necessary to consider a neuron MOR positive, we created histograms visualizing the number of LC cells displaying a given quantity of *Oprm1-O4* puncta in both mouse lines. The peak of the *Oprm1*$^{fl/fl}$ histogram suggests that the greatest number of LC cells contain 4 *Oprm1-O4* puncta (Figure 2B). This coincides with a drop-off in the number of puncta in the Dbh-cre;*Oprm1*$^{fl/fl}$ mice (Figure 2C). Therefore, we designated a cell as MOR-positive if it contained 4 or more *Oprm1-O4* puncta.

The 4 puncta threshold was then applied to all Dbh-cre;*Oprm1*$^{fl/fl}$ and *Oprm1*$^{fl/fl}$ LC cells to quantify the number of MOR-positive and MOR-negative LC cells present in each mouse line (Figure 3). Of the 1895 cells collected from 2 *Oprm1*$^{fl/fl}$ mice, 75.88% were positive for MOR, while 24.12% were negative. In contrast, out of the 815 LC cells collected from 2 Dbh-cre;*Oprm1*$^{fl/fl}$ mice, 56.07% were positive for MOR, while 43.93% were negative. Overall, the *Oprm1*$^{fl/fl}$ mice displayed 32% more MOR-positive cells compared to the Dbh-cre;*Oprm1*$^{fl/fl}$ mice. Though the number of MOR-positive cells were decreased in Dbh-cre;*Oprm1*$^{fl/fl}$ mice, a substantial amount of positive cells still remained. This suggests an apparent incomplete knockout of MOR within the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line.

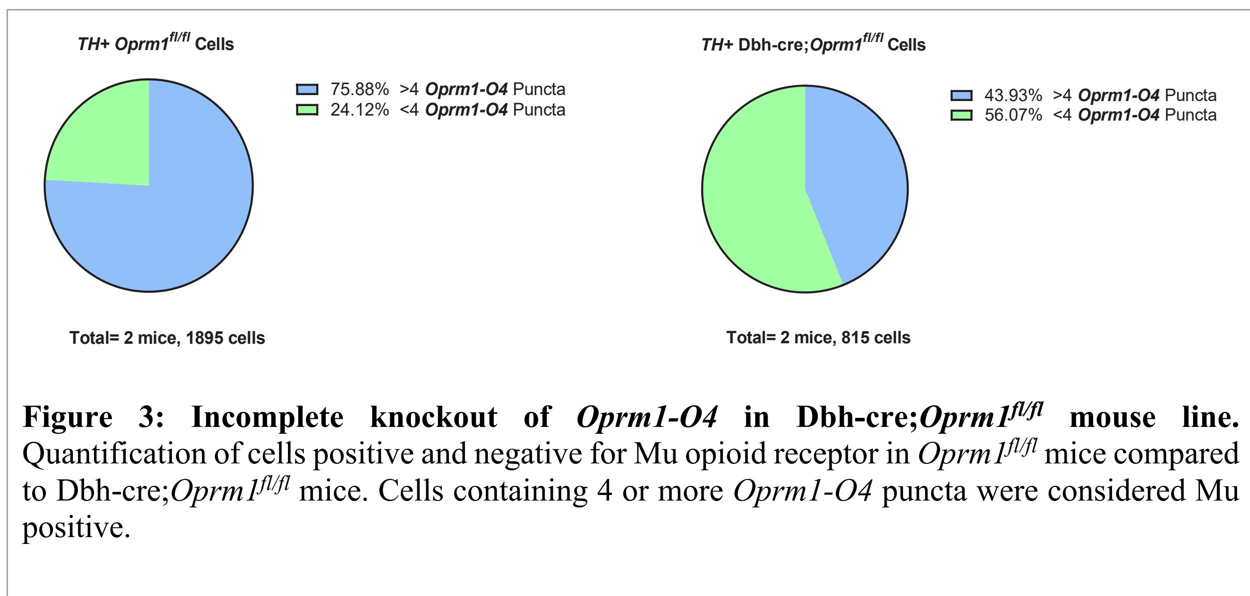

Figure 3: Incomplete knockout of *Oprm1-O4* in Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line. Quantification of cells positive and negative for Mu opioid receptor in *Oprm1*$^{fl/fl}$ mice compared to Dbh-cre;*Oprm1*$^{fl/fl}$ mice. Cells containing 4 or more *Oprm1-O4* puncta were considered Mu positive.

CHAPTER 2: Quantification of Mu Opioid Receptor Transcripts Along the Dorsal-Ventral Axis of the LC

2.1 Results

To understand if a dorsal-ventral gradient of MOR transcripts exists in the LC, we compared the coordinate location of LC cells in *Oprm1*$^{fl/fl}$ mice to the quantity of *Oprm1-O4* puncta they contained. Quantification of puncta was done through a pipeline constructed in CellProfiler, which recognized the number of LC cells present in an image and the number of *Oprm1-O4* puncta contained within each cell. The pixel coordinate location of each LC cell within an image was converted to millimeter (mm) values, using a conversion value. These mm values were then assigned to coordinate locations within the mouse brain, according to the Allen Brain Atlas. From these coordinate locations, representative concentration graphs were created to visualize the quantity of *Oprm1-O4* puncta in each cell in relation to its dorsal-ventral and medial-lateral coordinates in the mouse brain (Figure 4). However, LC projections differ in function according to their position in the dorsal-ventral axis. Thus, we evaluated the quantity of Oprm1-O4 puncta in a cell solely in regard to its dorsal-ventral location (Figure 5). A linear regression line modeled over the data resulted in a Pearson's coefficient of -0.1521, suggesting a weak negative association between dorsal-ventral location of *Oprm1*$^{fl/fl}$ LC cells and quantity of *Oprm1-O4* puncta in each cell.

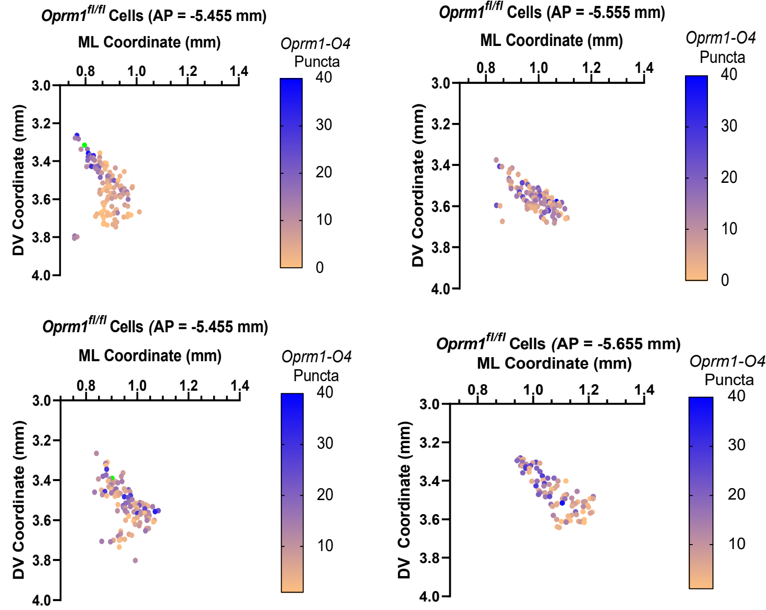

Figure 4: *Oprm1-O4* transcripts in the LC of *Oprm1*$^{fl/fl}$ mice. Representative concentration graphs of mu opioid receptor (*Oprm1-O4*) transcription *Oprm1*$^{fl/fl}$ mice. Each point represents a single tyrosine hydroxylase-positive cell in the Locus Coeruleus and is plotted according to its dorsal-ventral and medial-lateral location in the mouse brain. Cells expressing more than 40 *Oprm1-O4* puncta are shown in green.

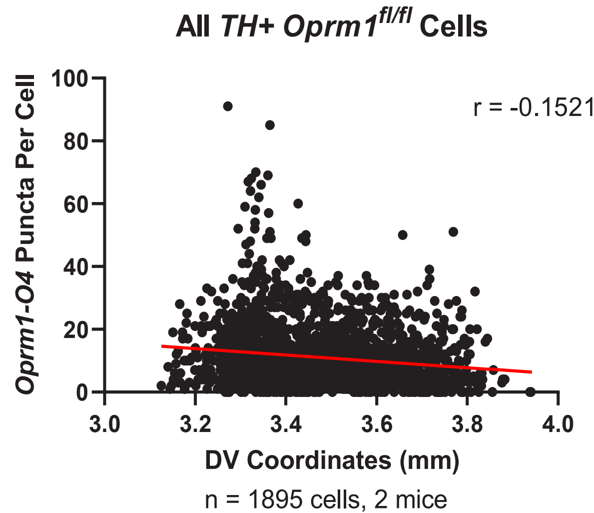

Figure 5: Scatter plot *of Oprm1-O4* transcripts in the LC of *Oprm1*$^{fl/fl}$ mice. Each value represents a single tyrosine hydroxylase-positive cell. The number of *Oprm1-O4* puncta contained in each cell is plotted against the cell's dorsal-ventral coordinate.

It is important to note that the shape of the LC is asymmetric along the anterior-posterior axis of the brain. As the structure of the LC progresses from anterior to posterior, the shape of the LC also shifts in the dorsal-ventral plane. This presents a challenge, as posterior regions of the LC do not share the exact dorsal-ventral coordinates as anterior portions. Therefore, visualizing cells from all segments of the LC collectively may occlude any dorsal-ventral gradient of MOR transcripts. To address this, all 1895 quantified *Oprm1$^{fl/fl}$* LC cells were subdivided into three groups according to their positions along the anterior-posterior axis (Figure 6). Cells from anterior LC slices (AP = -5.38 mm to -5.455 mm), medial LC slices (AP = -5.555 mm to -5.655 mm) and posterior slices (AP = -5.78 mm to -5.855 mm) were grouped together. Linear regression lines plotted over each group resulted in a Pearson coefficient of -0.2211 for anterior slices, -0.2294 for medial slices and -0.1251 for posterior slices. These values suggest a weak strength of association between dorsal-ventral location of the cell and *Oprm1-O4* puncta within it.

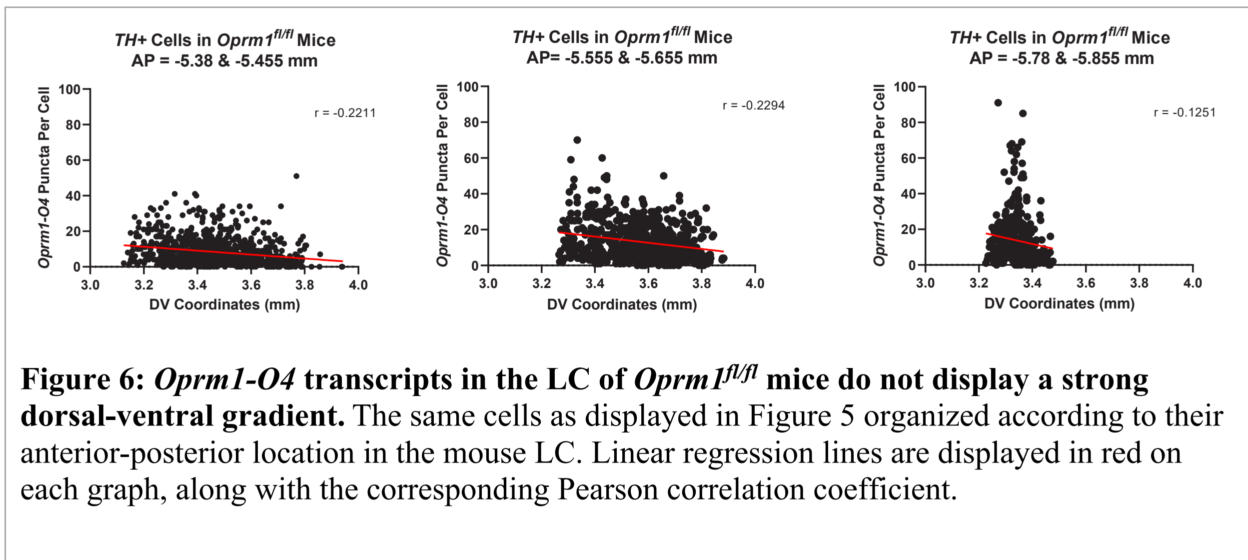

Figure 6: *Oprm1-O4* transcripts in the LC of *Oprm1$^{fl/fl}$* mice do not display a strong dorsal-ventral gradient. The same cells as displayed in Figure 5 organized according to their anterior-posterior location in the mouse LC. Linear regression lines are displayed in red on each graph, along with the corresponding Pearson correlation coefficient.

DISCUSSION

Previous studies have shown that *in vitro* administration of opioids in rat brain slices results in hyperpolarization and decreased neuronal firing rates in LC cells (Williams et al., 1982). However, recent studies from the Banghart Lab have shown that *in vivo* administration of morphine requires release of noradrenaline in the spinal cord for mice to display complete analgesia (Lubejko & Banghart, unpublished, 2022). Additional immunohistochemical data from the Banghart lab confirms the activation of the LC in response to systemic morphine injection, implicating it as a potential source of noradrenaline release in the spinal cord (Lubejko & Banghart, unpublished, 2022). How opioids inhibit LC neuronal firing and therefore likely decrease noradrenaline release from the LC, while also requiring noradrenaline release in the spinal cord to realize its full analgesic capability, is unclear.

To address the paradoxical role of opioids in the LC, we first sought to validate the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line for use in behavioral experiments to begin to determine a role for MOR expression in noradrenaline-producing neurons during painful stimuli and opioid analgesia. Additionally, we sought to use this mouse line to gauge background fluorescence and delineate thresholds for discriminating between MOR-positive and MOR-negative cells. The cumulative probability graph comparing the two mice lines suggests that LC cells from Dbh-cre;*Oprm1*$^{fl/fl}$ mice have a higher probability of containing less puncta compared to the *Oprm1*$^{fl/fl}$ mice (Figure 2). An overview of the two mice lines showed that while the Dbh-cre;*Oprm1*$^{fl/fl}$ knockout mice did display a decreased percentage of MOR-positive cells compared to *Oprm1*$^{fl/fl}$ mice, a large percentage of MOR-positive cells remained (Figure 3). From these data, we concluded that the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line contained an incomplete knockout of MOR in dopamine hydroxylase-positive cells.

However, while RNA *in situ* hybridization can act as a proxy for the presence of MOR in each cell, it does not fully represent the amount of protein translated from the RNA transcripts. It is possible that the amount of RNA transcripts present in each mouse line is incongruent with the amount of functional protein translated. Future studies investigating the amount of functional MOR protein produced in the LC of each mouse line include isolating LC cells, then performing an enzyme-linked immunosorbent assay (ELISA) to quantify amounts of MOR protein present in each line. This information in conjunction with our *in situ* hybridization data can further clarify the effectiveness of the MOR knockout in the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line.

Nevertheless, the finding that the Dbh-cre;*Oprm1*$^{fl/fl}$ mouse line contains an incomplete knockout has significant implications in regards to behavioral findings obtained from the Banghart Lab. Previous data from the Banghart Lab has confirmed that complete systemic morphine analgesia requires noradrenaline release in the spinal cord, and that the LC is activated in the presence of systemic morphine (Lubejko & Banghart, unpublished, 2022). However, *in vitro* administration of morphine decreases LC neuronal firing (Williams et al., 1982). This suggests that opioids may directly inhibit LC neurons, but also simultaneously drive activity in the LC through other synaptic circuits. Therefore, knockout of MOR in the LC would remove the direct inhibition of opioids on LC neurons, allowing the activation of LC neurons through synaptic mechanisms to prevail. It would then be expected that Dbh-cre;*Oprm1*$^{fl/fl}$ mice lacking the MOR in DBH+ cells display increased analgesia or perhaps a faster onset to full morphine analgesia compared to controls. However, Dbh-cre;*Oprm1*$^{fl/fl}$ mice given a systemic injection of morphine had similar withdrawal latencies and latency to peak analgesia during a time course of hotplate exposures, compared to *Oprm1*$^{fl/fl}$ mice injected with systemic morphine. It is possible that the incomplete knockout of MOR in the Dbh-cre;*Oprm1*$^{fl/fl}$ mice line resulted in residual MOR

sufficient in detecting systemic morphine, hyperpolarizing the LC cells. This could explain why Dbh-cre;*Oprm1*$^{fl/fl}$ mice in the hotplate assay experienced similar levels of analgesia compared to *Oprm1*$^{fl/fl}$ mice contrary to predicted results.

Next, we sought to analyze the presence of a dorsal-ventral gradient of MOR transcripts in the LC by quantifying MOR puncta in *Oprm1*$^{fl/fl}$ mice. While some representative concentration graphs displaying individual slices of LC indicated the possibility of a MOR transcript gradient (Figure 4), further inspection of all LC cells compiled into a scatter plot showed a weak relationship between the dorsal-ventral location of a cell and the quantity of puncta contained within it (Figure 5). Due to the low Pearson's coefficient calculated from the linear regression line mapped over the scatter plot, we concluded that there was no considerable gradient apparent when considering all cells from a range of anterior-posterior locations.

We then further segregated the *Oprm1*$^{fl/fl}$ LC cells into three groups according to their anterior-posterior locations to better capture the dynamic morphology of the LC (Figure 6). Linear regression lines fitted over each plot produced small negative Pearson coefficient values, again suggesting only a weak decrease in puncta transcripts as the location of the cells moves from dorsal to ventral LC. Overall, this data conveys a lack of a MOR transcript gradient along the dorsal-ventral axis of the LC. This suggests that the paradoxical role of opioids in the LC, namely, how opioids decrease neuronal firing in the LC while also requiring the release of noradrenaline to display their full analgesic capabilities, cannot be explained by differing MOR RNA transcripts along the dorsal-ventral axis of the LC. An additional experiment that would further clarify the differential transcription of MOR on the descending and ascending projections from the LC, would be to perform a similar RNA *in situ* hybridization experiment in the presence of distinctly colored retrograde tracers injected into the LC targets within the prefrontal cortex and spinal cord. These

retrograde tracers would be expected to label cells in the LC according to their projection terminals. In this case, the destination of the LC cell, whether it sends projections to supraspinal structures or to the spinal cord, would be definitively known. Thus, the number of *Oprm1-O4* puncta present on each type of projection can be quantified and compared directly.

While the lack of a MOR transcript gradient in the LC does not explain the opioid paradox, it suggests that opioids may be interacting with pain modulatory circuits through different mechanisms. It is possible that excitatory inputs into the LC are dominant to the opioid-driven hyperpolarization of LC cells, allowing LC neurons to remain active in the presence of opioids when their circuitry remains intact *in vivo*. This is supported by data from the Banghart Lab showing systemic injection of morphine into mice increases the number of c-fos positive cells in the LC (Lubejko, unpublished data, 2022). Potential brain areas of interest that send inputs to the LC include, the rostral ventral medulla (RVM) and ventral lateral periaqueductal gray (PAG) (Bajic & Proudfit, 1999; Clark & Proudfit, 1991). A small number of studies have investigated the synaptic connections and opioid sensitivity of PAG to LC circuit elements, but the strength and nature of these projections, as well as how opioids influence this circuit has yet to be determined (Kim et al., 2018). Future studies examining how opioids affect inputs into the LC will further elucidate this. Ultimately, investigation into these questions will provide more insight into how opioids engage the LC in analgesia, bringing us closer to understanding the biological impact of opioids on the brain and body.

.

MATERIALS AND METHODS

All animals were handled according to UC San Diego Institutional Animal Care and Use Committee guidelines. P56 female and male mice on Dbh-cre;*Oprm1*$^{fl/fl}$ and *Oprm1*$^{fl/fl}$ backgrounds were anesthetized using isoflurane and decapitated. Brains were immediately removed and frozen in blocks of Tissue-Tek O.C.T. Compound (Sakura) on dry ice. They were then sliced into 8 µm sections at -20°C using a cryostat (Leica CM1950) and adhered onto Superfrost slides (Fisher Scientific). Slides were stored at -80°C until processed.

Selected slides were processed according to the ACD RNAscope Multiplex Fluorescent V2 Assay manual. They were then mounted using ProLong Gold Antifade Mountant (Molecular Probes) and covered with 1.5 thickness coverslips (Fischer Scientific). Sections were then imaged with a Keyence (BZ-X710 series) at x60 magnification. All images were processed using ImageJ and the number of fluorescent puncta was quantified using CellProfiler. A conversion value of 0.00012581 mm/pixel was used to convert pixels to mm values, while the Allen Brain Atlas was used to assign coordinates to the acquired images.

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