

Muscarinic acetylcholine receptor 5 (M5) expression in and regulation of the habenula.

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ABSTRACT

The prevalence of neuropsychiatric disorders have been increasing at alarming rates. Moreover, only about half of patients are responsive to first-line antidepressant medications (i.e. selective serotonin reuptake inhibitors), making identification of new therapeutic targets for these disorders critical. The M5 receptor's unique restricted expression in the brain makes it an attractive therapeutic target as there may be reduced unwanted side effects. It is the only muscarinic acetylcholine receptor localized to dopamine neurons. As a result, the majority of studies thus far have focused on M5 regulation of dopamine transmission, but the M5 receptor is not solely on dopamine neurons, leaving a large field of M5 regulation research unstudied. Our laboratory has previously published that constitutive M5 receptor deletion leads to anxiogenic effects. Yet, recently collected preliminary data from our lab indicates that selective deletion of M5 from dopamine neurons does not mediate these effects. PACAP is a stress-associated neuropeptide, and PACAP neurons in the lateral habenula are important for emotional processing. Traditionally, elevations in LHb activity produce aversion and dysphoria, but a recent study demonstrated that chemogenetic activation of PACAP LHb neurons led to appetitive behaviors and reduced fear expression. This project aims to characterize the expression profile of M5 receptors in the LHb and determine if M5 receptors are localized to PACAP neurons. It was hypothesized that deletion of the M5 from the habenula will lead to increased anxiety-like behavior. For the first time, it was discovered that M5 receptors were colocalized with PACAP neurons in both the medial and lateral habenula, with greater expression in the lateral habenula. Yet, to our surprise, M5 region-specific knockouts in the lateral habenula produced anxiolytic behaviors in open field testing. This initial behavior study needs to be followed up with PACAP-specific deletion of M5 receptors to more directly align with previous literature.

INTRODUCTION

Current State of Neuropsychiatric Disorders

Major depression is a significant health issue affecting a large amount of the population globally, and its prevalence has been increasing significantly in the last couple of decades. Most recently, Goodwin et al., (2022) highlights that from 2015-2019, there have been widespread increases in the amount of the United States population experiencing major depression. Supporting the findings in Goodwin et al. (2022), an increase in depressive symptoms has been present across age groups, ranging from increases in depressive symptoms in adolescents since 2010, to significant increases among older adults (Twenge et al., 2017; Yang et al., 2022). The same findings have also been replicated globally, from in Germany to in China (Steffen et al., 2020; Ren et al., 2020). However, research also indicates that there has not been an equivalent increase in treatment for major depression alongside the increase in depression prevalence (Goodwin et al., 2022). In addition, depression and other neuropsychiatric disorders such as anxiety are extremely comorbid, and comorbidity of depression or anxiety is the rule rather than the exception (ter Meulen et al., 2021). Research surrounding neuropsychiatric disorders such as major depression is becoming increasingly important in order to adequately support this increasing proportion of the population struggling. This paper focuses on muscarinic acetylcholine receptor 5 (M5), and discovering its role in the regulation of habenula circuits that are directly applicable to neuropsychiatric disorders.

Depression is not the only major neuropsychiatric disorder that is having an increasing prevalence among the global population in the last couple of decades. In addition to the trend of increased levels of depressive symptoms appearing, increased levels of anxiety have also been prevalent across the population. Anxiety is especially important to understand, as it is the most

prevalent mental disorder across the globe (Stein et al., 2017). Goodwin et al., (2020) indicates that from 2008-2018, there have been significant increases in the level of anxiety among adults in the United States population. Additionally, the treatment gap for anxiety disorders is global and increasing (Alonso et al., 2018). For similar reasons as depression, research surrounding the neuropsychiatric disorder of anxiety is becoming increasingly important.

Interestingly, in the wake of the COVID-19 pandemic, the population struggling with neuropsychiatric disorders has spiked at alarming rates. According to the World Health Organization, the COVID-19 pandemic triggered a 25% increase in the prevalence of both depression, as well as anxiety, across the globe (World Health Organization, 2022). The increase found by the World Health Organization has also been replicated by other research, showing anxiety levels are elevated across the globe after the COVID-19 pandemic, as well as depressive symptoms that increased threefold during the pandemic in comparison to pre-pandemic levels (Kupcova et al., 2023; Ettman et al., 2020). Even more alarming, there is emerging discussion that long COVID-19 symptoms are displaying comorbidity with those struggling with low quality of life due to long COVID symptoms, posing a major issue as a result of the COVID-19 pandemic (Efsthathiou et al., 2022). Although some research investigating how depression arises as a result of COVID-19, possibly a consequence of the nervous system's inflammatory response, an answer is not yet discovered (Shetty et al., 2023). Finding therapeutic targets for neuropsychiatric disorders to support this rapidly increasing population is an increasingly important field of research this work hopes to expand on.

Dopaminergic Research and Resulting Emergence of the M5 Receptor

One area of research already explored thoroughly investigates the role of dopamine in these neuropsychiatric disorders. Dopamine is a neurotransmitter that is released from midbrain

neurons in the ventral tegmental area (VTA) into regions of the brain critical for reward and emotional processing as well as cognition. As such, dopamine neurotransmission is important to understand in a variety of therapeutic models for neuropsychiatric disorders. Imbalances in dopamine and alteration of brain circuitry where dopamine plays a crucial role are linked with a wide variety of neurological and neuropsychiatric diseases, including depression, anxiety, addiction, and schizophrenia (Mizuno et al., 2023; Zarrindast and Khakpai, 2015; Franco, Reyes-Resina and Navarro, 2021). Investigation of the neurobiology of dopamine is really essential to the investigation of therapeutic treatments, yet because of its role in reward processing, targeting the dopamine system directly can leave open the possibility for issues with both compliance and abuse liability.

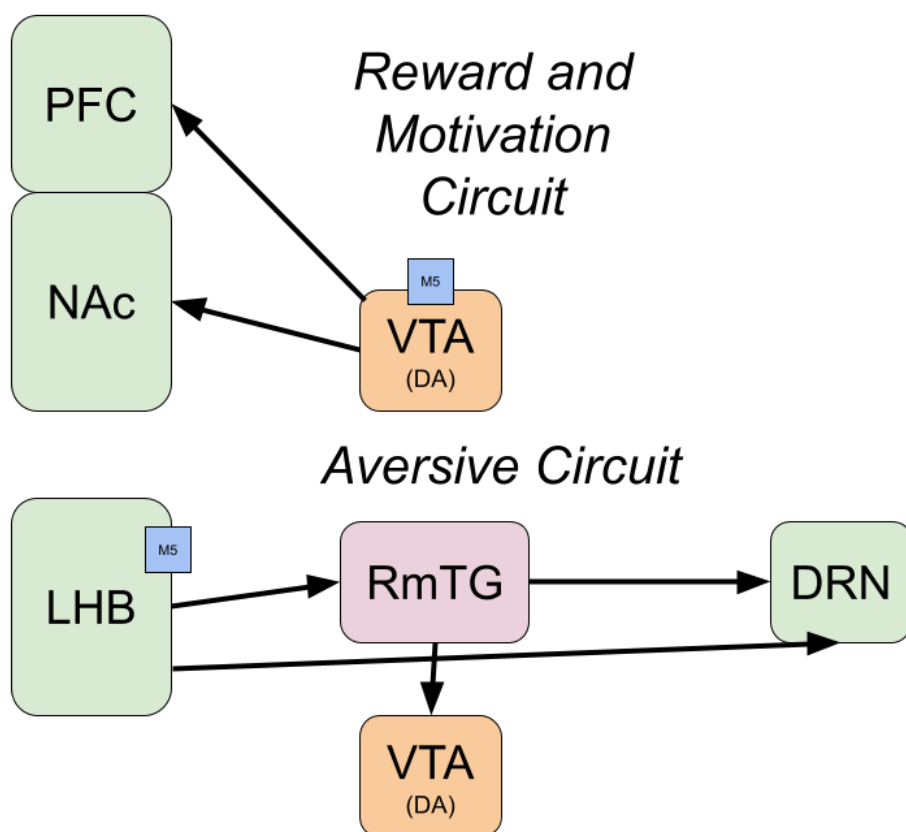


Figure 1. Reward related connections in the brain. This schematic shows some of the dopaminergic connections that are known with the brain circuitry. PFC stands for prefrontal cortex, NAc stands for nucleus accumbens, VTA stands for ventral tegmental area, DA stands for dopamine, DRN stands for dorsal raphe nucleus, RmTG stands for rostromedial tegmental nucleus, LHB stands for lateral habenula, and M5 represents M5 receptors. Arrow direction shows projection of circuitry. See figure 2 for approximate anatomical placement of circuits within the mouse brain.

Recently, the M5 receptor has been emerging as a possible target for therapeutic treatment of neuropsychiatric disorders. Muscarinic acetylcholine receptors are G protein coupled receptors and are mainly found in the parasympathetic nervous system, activated by the neurotransmitter acetylcholine from postsynaptic neurons. Both dopamine and acetylcholine have implications in preclinical models of depression and anxiety, and the M5's unique property of being localized to dopamine neurons, being the only muscarinic acetylcholine receptor to do so has made it an unique and interesting target for research (Morris et al., 2022). The M5 receptor also has incredibly restricted spatial expression. Targeting the M5 receptor, on dopamine neurons or other neurons, is an intriguing area for therapeutic research because of this spatial expression. As it is only expressed in a few areas of the brain related to reward-learning, targeting the M5 receptor as a treatment could limit negative side effects of treatments. Studying the M5 receptor will ideally lead to a better understanding of how this unique receptor can be a target for treatments of neuropsychiatric disorders.

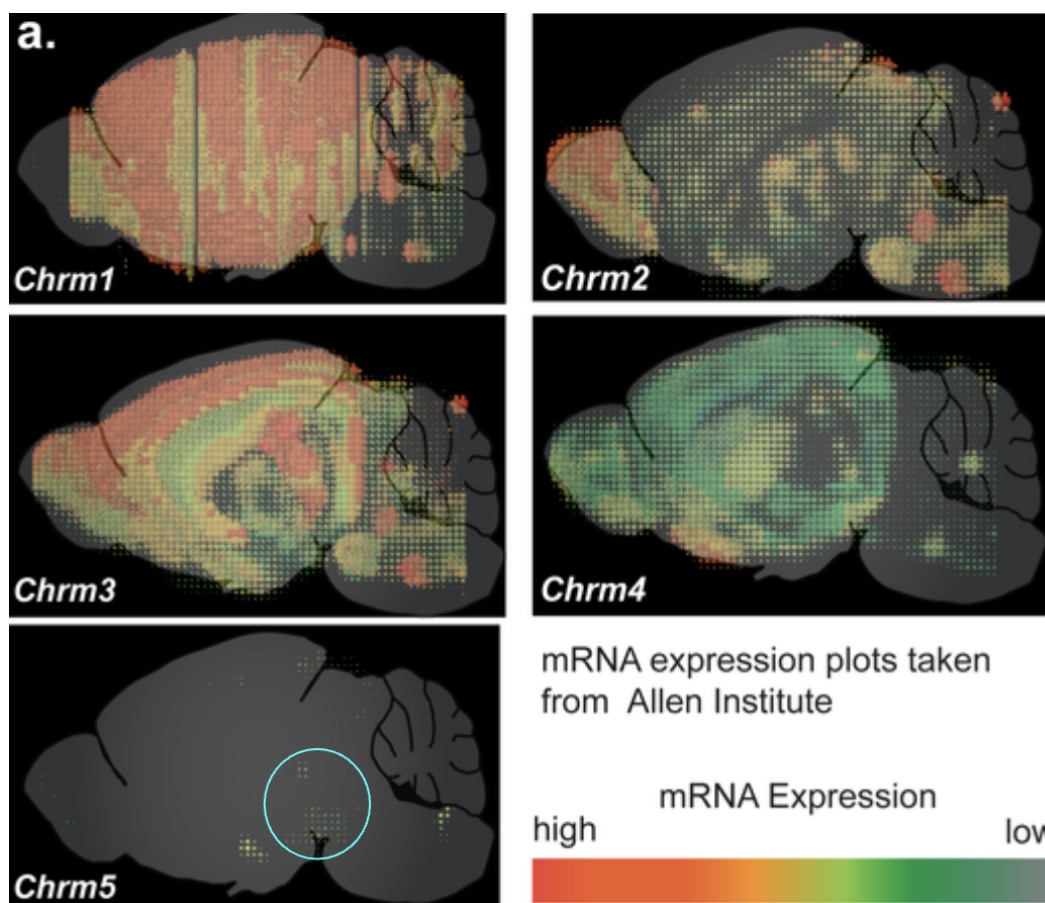


Figure 2. Expression of *Chrm5* in the mouse brain in comparison to other muscarinic acetylcholine receptors (M1-M4). The expression of *Chrm5* is in significantly fewer places than the expression of *Chrm1-4*. The limited expression of *Chrm5* makes M5 receptors an intriguing target. Figure from Razidlo et al., 2022. Referencing Figure 1's, the lateral habenula, the expression seen near to the center of the mouse brain in the *Chrm5* image, sits above the VTA, where M5 is also expressed. These approximate positions are circled in blue.

Research has shown for decades that the M5 receptor, when activated, can influence the activation and excitation of dopamine neurons both at the soma and at the terminals. Activation of M5 receptors via acetylcholine presynaptically leads to excitation of dopamine neurons in the VTA, which releases dopamine (Weiner, Levey & Brann, 1990). Research has also found the

same pattern on M5 receptors on dopamine neurons in the ventral tegmental area (VTA), a key area of the brain in the dopamine system, as VTA neurons with M5 receptors regulate dopamine release in the nucleus accumbens (Nunes et al., 2023). Similarly, this pattern has also been found in the nucleus accumbens, another area of the brain implicated in affecting reward-related behavior. It turns out that M5 receptors are actually needed for prolonged dopamine release within the accumbens (Razidlo et al., 2022). In the absence of M5 receptors, there was no prolonged dopamine release within the accumbens, showing the direct importance of the M5 receptor in dopamine regulation within the accumbens. This provides yet another reason that M5's effect on dopamine neurons in other areas of the brain should be analyzed for potential therapeutic effects.

There are some interesting research studies that show the role of M5 regulation on the ventral tegmental area (VTA), which provides some interesting background information for this study. The VTA is a key part of the dopamine system and has a major role in dopamine regulation (Hou et al., 2024). Additionally, imaging directly in the VTA utilizing high-resolution 7-Tesla MRIs shows that the VTA is directly important to neurological motivation patterns in those that have already been diagnosed with the neuropsychiatric disorders of depression and anxiety (Morris et al., 2022). Therefore, it logically follows that M5 receptors are present in the VTA, as M5 receptors are the only muscarinic acetylcholine receptor that is localized specifically to dopamine neurons (Garzón and Pickel, 2013). It is important to note that M5 receptors, while localized specifically to dopamine neurons, are not found exclusively on dopamine neurons, and are also found on other neurons, such as PACAP neurons. It is likely that the M5 receptor doesn't only play a role in the dopamine system, as it is not found exclusively on dopamine neurons, and the potential therapeutic effects of targeting this receptor outside of the world of dopamine leave

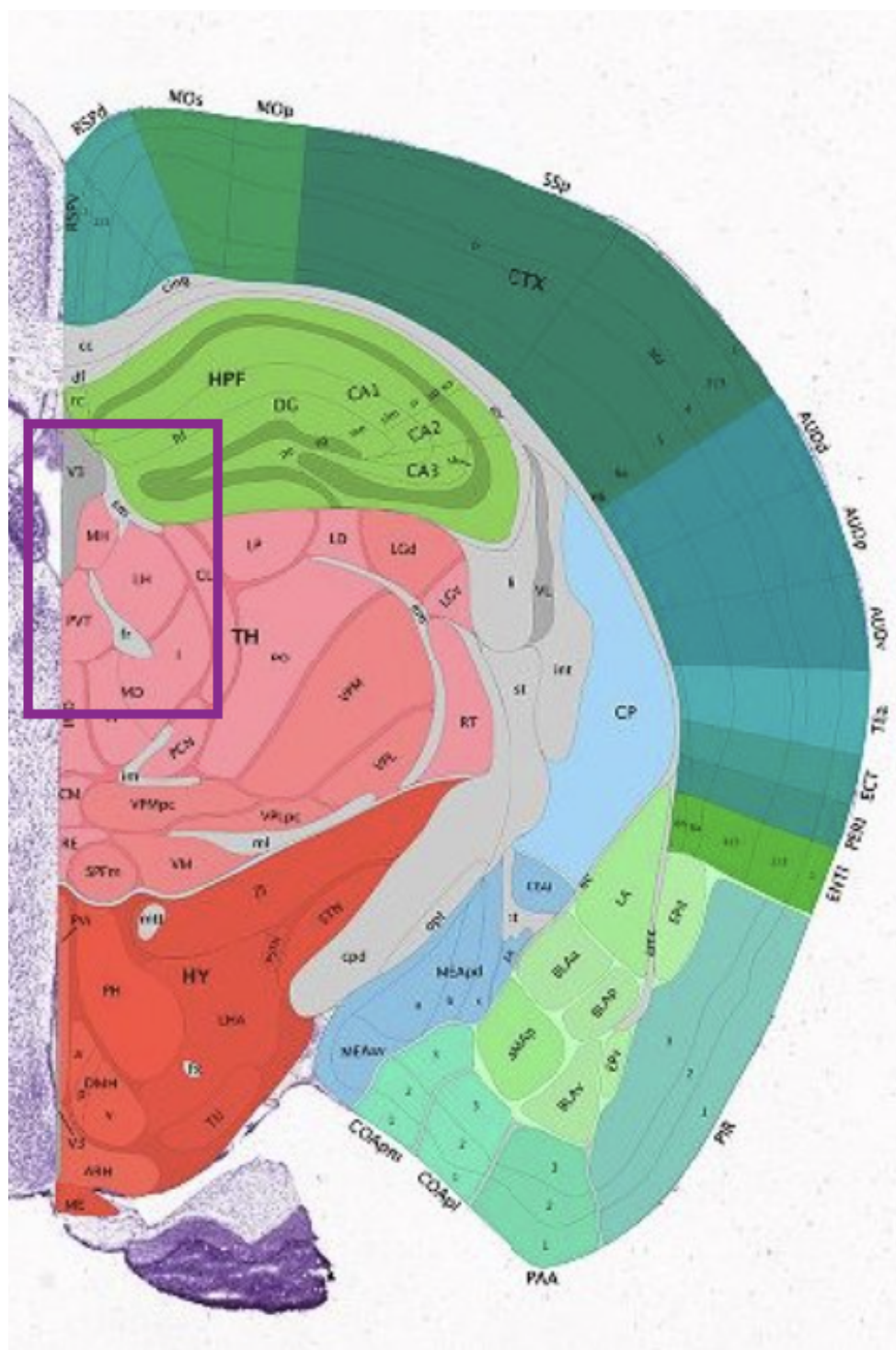
much to be discovered. Circuitry outside of the dopaminergic circuits must be explored in further research on the M5 receptor. Interestingly enough, the lateral habenula may be an interesting place to start for researching the potential therapeutic effects of the M5 receptor.

The Habenula and Expanding Research Outside Dopaminergic Neurons

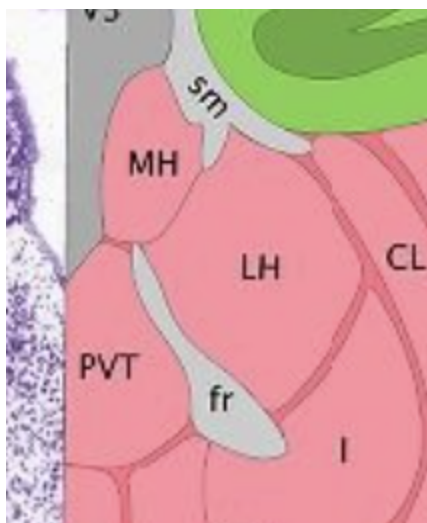
The lateral habenula in the rat brain has M5 receptors present. Using in situ hybridization, mRNA coding for the M5 receptor was found in the lateral habenula (Vilaró et al., 1990). To our knowledge, this is the only study that has shown this. Moreover, little is understood about the connection between the M5 receptor and its regulation of neuronal groups in the habenula. This project aims to close that gap, focusing on M5's possible regulation of circuits in the habenula, specifically in the lateral habenula, and its possible role as a therapeutic treatment for neuropsychiatric disorders by targeting the habenula.

On a more basic level, the habenula has had increasing interest in the last decade or so due to some of its unique properties. The lateral habenula in particular has received significant interest. The lateral habenula, sometimes referred to as the brain's "antireward center", underlies negative emotional state in animal models of drug withdrawal, as well as major depression. The lateral habenula is one of just a few regions in the brain that has control over both the serotonergic and the dopaminergic system (Hu et al., 2020). It also has a role in encoding negative reward responses when omission of reward occurs (Matsumoto and Hikosata, 2007). The lateral habenula also receives projections from the VTA, and when the lateral habenula is suppressed, spontaneous firing of dopamine neurons in the VTA increases (Hu et al., 2020). In addition, activation of cholinergic neurons in the habenula excites dopamine neurons in the VTA (Han et al., 2017). In regards to its own projections, the lateral habenula projects to the dorsal raphe nucleus, which influences anxiety related behavior (Nishitani et al., 2019). Research has

shown that this has its own behavioral effects as well. Glutamatergic neurons in the lateral habenula project to the dorsal raphe nucleus, which ends up promoting aggressive arousal in mice models (Takahashi et al., 2022). The lateral habenula's pattern of projections make it a new valuable area of research because of the projections it receives, as well as the projections that it sends. Because the lateral habenula plays a significant role in so many areas of the nervous system, it is a common area for study and research.



3A



3B

Figure 3. Habenula of the mouse brain. **A**, an outline of the anatomy of half of the mouse brain from the Allen Brain Atlas. **B**, a zoomed-in image of the red box in Figure 3A. LH represents lateral habenula, and MH represents medial habenula. Although they will not be referenced further in this paper, PVT represents the paraventricular nucleus of the thalamus, sm is the stria medullaris, fr is the fasciculus retroflexus, CL is the central lateral nucleus of the thalamus, and l is the lateral part of the mediodorsal nucleus of the thalamus (Allen Institute for Brain Science) (Daigle et al., 2018) (Lein et al., 2007) (Harris et al., 2019) (Oh et al., 2014).

For the purposes of the research in this paper, it is important to narrow down the areas of the lateral habenula to study. Due to the M5's uniqueness as the only muscarinic acetylcholine receptor localized to dopamine neurons, research thus far has focused on M5 receptors on dopamine neurons in the VTA that project to the lateral habenula. This is because researchers have found a circuit between the lateral habenula and VTA that may lead to depression, although the neural circuit does not appear to be affected by chronic mild stress (Moreines et al., 2017).

Therefore, the lateral habenula's role in the dopamine system as it relates to neuropsychiatric disorders such as depression and anxiety has made it a recent hotspot for possible therapeutic treatments. That being said, dopamine is not the only notable neurotransmitter in the lateral habenula that affects behavior. Other non-dopaminergic neurons have not been investigated nearly enough. Therefore, this paper focuses on a largely unstudied portion of the M5 receptor outside of the dopaminergic circuits. Outside of the world of dopamine, there are many other promising proteins and neurotransmitters that could be analyzed for possible therapeutic treatments.

Pituitary Adenylate Cyclase-Activating Peptide (PACAP)

Pituitary adenylate cyclase-activating peptide (PACAP) is a neuropeptide neurotransmitter encoded by the *Adcyap1* gene that appears to play a role in neuropsychiatric disorders and is expressed in several limbic and hypothalamic brain regions (Xu et al., 2016). PACAP neurons in the ventromedial hypothalamus (VMH) play a direct role in glucose homeostasis, where stimulation of PACAP neurons inhibits insulin secretion, leading to reduced glucose tolerance (Khodai et al., 2018). Although this study tested glucose and not necessarily a drug as intense as cocaine, it shows that PACAP has a role in neuropsychiatric behavioral patterns as well. Additionally, PACAP neurons in the lateral habenula can be rewarding or can diminish negative consequences of aversive events (Levinstein et al., 2022). This new research actually challenges the original dogma of the role of the lateral habenula. This opens up the possibility for stimulation of PACAP neurons in the lateral habenula to also be used as a therapeutic target due to their role in establishing tolerance and their ability to be rewarding or diminish negative consequences of behavior. However, a gap of knowledge is present as to the possible overlap of PACAP and M5 in the habenula, and if present, the role those neurons would

play on behavior. Looking at colocalization of the M5 receptor on PACAP neurons in the lateral habenula is worth investigating, as behavioral research surrounding both the M5 receptor and PACAP neurons often present similar results.

Present Behavior Research Surrounding M5

Recent research has shown that M5 receptors may have a role in reward specific behavior. Grasing (2016) has shown that activation of M5 receptors may possibly contribute to reward enhancement using mice models, and when the M5 receptor is activated, the effects of a reward are felt more strongly. The reverse effect is also shown in Grasing (2016), where M5 knockout mice self-administered certain doses of cocaine at a rate that was around half the rate of normal, wild-type animals. The results here show that the M5 receptor's activation or absence played a direct role in the reward behavior shown by mice when presented with cocaine. The modulation behind this is not yet known for certain, although the author states that it may be due to the activation of dopamine neurons, likely in the VTA. Further research on the M5 receptor is needed to better understand these results to help understand and support those struggling with reward-related neuropsychiatric disorders.

Additionally, the M5 receptor has been shown to play an important role in exploratory behavior, and better understanding exploratory behavior helps craft a better understanding of the motivation patterns of those struggling with disorders such as depression or anxiety. It has been shown that M5 receptors played a role in modulating anxiogenic and depressive behaviors in three separate kinds of tests: the sucrose preference test, the elevated plus maze, and the forced swim test in rat models (Nunes et al., 2020). However, it is worth noting that other studies performed the same tests, but did not find the same results, although that could be due to species difference or differences in acute versus chronic M5 disruption (Razidlo et al., 2022) Similarly in

mice models, other studies have shown that when the M5 receptor was knocked out, exploratory behavior was reduced, which is commonly seen in those experiencing depression and anxiety disorders. These mice also did not adapt to changing environments well, and had reduced response to appetitive novel stimuli. These behaviors were also proven not to be due to general stress-induced downregulation of dopamine neurons where M5 is expressed, which infers even more that the M5 receptor specifically, rather than just the general dopamine system, may have a major role in modulating exploratory behavior (Razidlo et al., 2022). With the rates of depression and anxiety increasing across the globe at a rate faster than access to modern treatment is increasing, research surrounding the modulation of exploratory behavior is incredibly important, and emerging research points more and more toward the M5 receptor as a great place to start.

Additionally, the M5 appears to enhance locomotor activity in a way that is linked to schizophrenic behavior patterns. De Luca et al, (2004) found linkage of M5 muscarinic receptor genes and another type of gene ($\alpha 7$ -Nicotinic Receptor Genes) on 15q13 which is known to be related to schizophrenia, and it is very possible that the linkage between the two genes may hint at a possible influence of the M5 receptor and schizophrenic behavior. Regardless, more research should be done to investigate this relationship further. One study from Stiedl and Yeomans (2009) showed that M5 receptor KO mice showed reduced morphine-induced locomotion patterns, suggesting that the M5 receptor plays a role in regulating locomotion activity and increasing it. This relationship is still not fully understood, and something that should be investigated more.

Present Behavioral Research Surrounding PACAP

Prior research has also shown that in mice models where PACAP is knocked out, mice

exhibited hyperactive behaviors in an open field, in addition to increased exploratory behavior (Hashimoto et al., 2001). These results show that PACAP does play a direct role in altering psychomotor behavior in mice models. However, in this study, there was a full constitutive knockout of PACAP from the generation of mice, not in certain areas of the brain. This left researchers unable to determine which region of the brain contained PACAP neurons responsible for the behavioral changes, and led to more research investigating the behavioral effects of PACAP neurons. PACAP neurons also have selective distribution on both excitatory glutamatergic and inhibitory GABAergic neuronal populations, which is incredibly interesting (Zhang et al., 2021). It has a really wide role in supporting both excitatory and inhibitory neurotransmission within the nervous system, which leaves it open for a wide variety of possible therapeutic applications.

PACAP has also been shown to regulate behavioral stress responses surrounding anxiety-like behavior in mice models. It has been shown that the application of multiple small molecule PACAP receptor antagonists (PA-9 and PA-915) displayed anxiolytic effects (Shintani et al., 2022). This opens the door for PACAP related treatments as a possible future treatment for anxiety related behaviors. In this study, the PACAP receptor antagonists were given intraperitoneally, so the researchers were not quite sure where these antagonists were acting to cause a change in behavior. In prior research, it has been seen that PACAP is upregulated following chronic stress in the bed nucleus of the stria terminalis (BNST) (Boucher et al., 2021). It has also been seen that PACAP regulates anxiety-related behavioral responses through corticotropin-releasing factor (CRF) neurons in the paraventricular nucleus (PVN) (Ebner et al., 2024). These two areas of the brain show some promise having an impact on PACAP influenced behavior.

PACAP also plays a role in addiction related behaviors. In addition to PACAP being upregulated in the BNST following chronic stress, it is also seen that PACAP mediates heavy alcohol drinking in mice models. A recent study showed that inhibiting PACAP neural afferents going to the BNST actually lowered heavy consumption of ethanol (Lepeak et al., 2023). In addition, there appears to be a role for PACAP in causing amphetamine induced psychomotor effects in male mice, as PACAP knockout mice showed abnormal jumping behavior and a prepulse inhibition deficit (reduced ability to suppress startle reflex) that were both reversed with amphetamine (Tanaka et al., 2006). Therefore, PACAP neurons have mediated effects of behavior using multiple different substances, showing that PACAP neurons also play a role in behavioral patterns surrounding reward and addiction related behavior.

Linking the M5 Receptor and PACAP Neurons

This research focuses on studying the M5 receptors beyond their established role in dopaminergic signaling by exploring alternative mechanisms which may contribute to promising therapeutic interventions. PACAP neurons are commonly present in the lateral habenula, and are interesting because they have molecularly diverse cells that oppose actions performed by the rest of lateral habenula neurons by either being rewarding or by diminishing the negative consequences of aversive events (Levinstein et al., 2022). Based on all of the prior research and gaps in knowledge presented above, the aim of this project is to gain a better understanding of the relationship between the M5 receptors and PACAP neurons in the lateral habenula, and researchers hypothesize that there is coexpression between PACAP neurons and the M5 receptor that drives regulation of anxiety-like and depression-like behavior. To test this hypothesis, research was conducted testing whether or not anatomically the M5 receptor is expressed on PACAP neurons, and whether or not that occurs in the lateral habenula, occurs somewhere else

in the brain, or does not occur at all. RNAScope on coronally sliced flash-frozen mice brains and quantitative analysis of images taken of those slides was performed to discover possible colocalization. In addition, conditional *Chrm5^{loxP/loxP}* generated by the Lemos laboratory were used and LHb-specific M5 knock-down was achieved using viral injection of Cre-recombinase in the lateral habenula. These mice were compared to empty vector control mice. Locomotor activity in the open field and time and frequency in the center of the open field, a measure of anxiety-like behavior, were assessed.

MATERIALS AND METHODS

RNAScope

Mouse brains were rapidly dissected, and immediately flash frozen in isopentane on dry ice. Frozen brains were kept in a -80°C freezer. Before slicing, brains were equilibrated to the cryostat temperature either overnight in an alternate freezer at -20°C or in the cryostat for at least 2 hours. Brains were sliced coronally at a size of 16µm and then thaw mounted onto Superfrost plus slides using a Leica CM 1900 cryostat maintained at -16°C. Slides were then stored back in the -80°C freezer once slices were cut and mounted. RNAScope was conducted according to the Advanced Cell Diagnostics (ACD) user manual and as previously reported (Lemos, Shin and Alvarez, 2019). To repeat Lemos, Shin and Alvarez, 2019, Superfrost plus slides were fixed in 10% neutral buffered formalin for 20 min at 4°C. Then, slides were washed 2×1 min with 1× PBS, before dehydration with 50% ethanol (1× 5 min), 70% ethanol (1× 5 min), and 100% ethanol (2× 5 min). After being dried for 10 minutes, a hydrophobic barrier was drawn around the sections for analysis using a hydrophobic pen, and then dried for 15 minutes at room

temperature. Sections were then incubated with Protease Pretreat-4 solution for 20 min at RT and washed afterwards with double distilled water (2× 1 min).

For this procedure, the following probes were purchased from ACD: Mm-*Chrm5*-C1, Mm-*adcyap1*-C2, Mm-*Esr1*-C3. Probes were placed on slides at a 50:1:1 ratio (C1:C2:C3), and were incubated at 40°C using the HybEZ oven from ACD for two hours. After probe incubation, slides had a series of amplification steps at 40°C in the HybEZ oven. First, Amp1 for 40°C for 30 minutes, Amp2 for 40°C for 15 minutes, Amp3 for 40°C for 30 minutes. Then, a DAPI-containing (4',6-diamidino-2-phenylindole) solution was added to sections at room temperature for 30 seconds; slides were then covered using ProLong Gold Antifade mounting media and stored at 4°C.

Microscopy

Images of RNAScope slides were obtained via use of a confocal microscope (Stellaris 8), and three tiled 20x images were taken for each mouse (3 males, 3 females). These images were around 5 micrometers thick and the magnification on the confocal was 20x for the images showing *Chrm5*, *Adcyap1* and *Esr1*. Slices were taken evenly from Bregma -1.22 to -1.70 along the rostral-caudal axis, and this was reflected in images collected from the Stellaris 8 microscope as well. The focus was on obtaining images of the lateral habenula for coexpression, but medial habenula was analyzed as well. Coexpression, as well as individual expression, for *Chrm5* and *Adcyap1* was quantified, but not for *Esr1* due to low expression.

Quantitative Analysis of RNAScope

Using Indica HALO software, images were quantitatively analyzed for expression patterns of the three probes mentioned in the microscopy section. Each probe in the ACD catalog also has a unique and different sensitivity, so in regards to the settings on the Indica HALO

software, each probe had its own unique values for puncta size, number of puncta, and minimum intensity to be counted. That being said, across all sections for each probe within a single RNAscope run, the same imaging and analysis settings were always kept constant. Between RNAscope runs, the settings were adjusted as appropriate, and medial and lateral habenula were both outlined following a specific constant procedure using common brain landmarks. Statistical analysis occurred using data exported from Indica HALO, and was performed using a combination of Microsoft Excel imported into Prism (GraphPad) for the running of specific statistical analyses as well as the creation of graphs. Data was reported as mean +/- SEM.

M5 Flox Mice Preparation

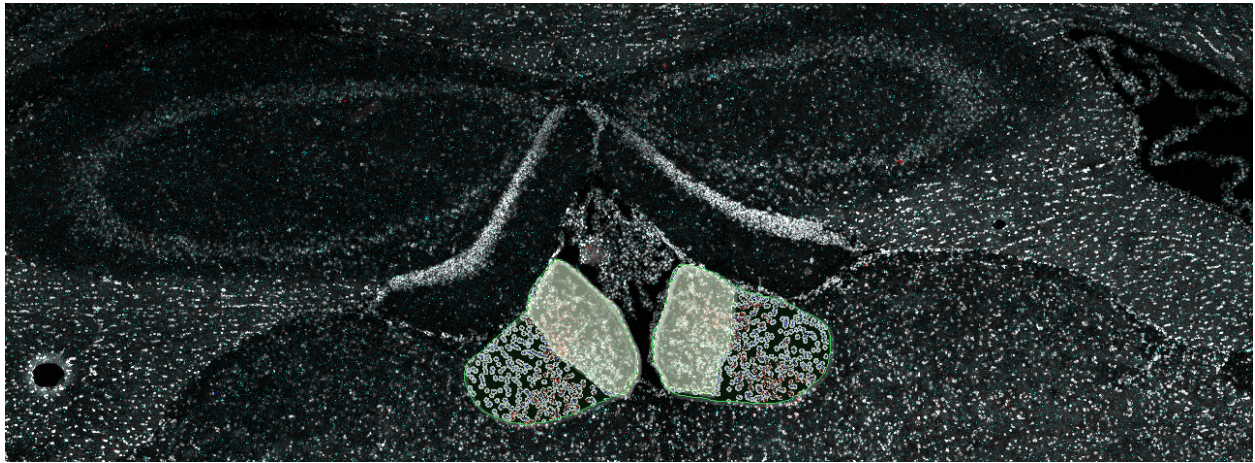
Chrm5^{loxP/loxP} male mice were used for behavioral testing. These mice were generated in the Lemos lab and surgeries were done by Dr. Julia Lemos. Mice received either an injection of Cre virus or a control injection in the habenula through surgical procedures. Behavior tests were performed as a measure to test anxiety-like and depression-like behavioral patterns in both the M5 flox with Cre injections and M5 flox mice with control injections.

Open Field Testing

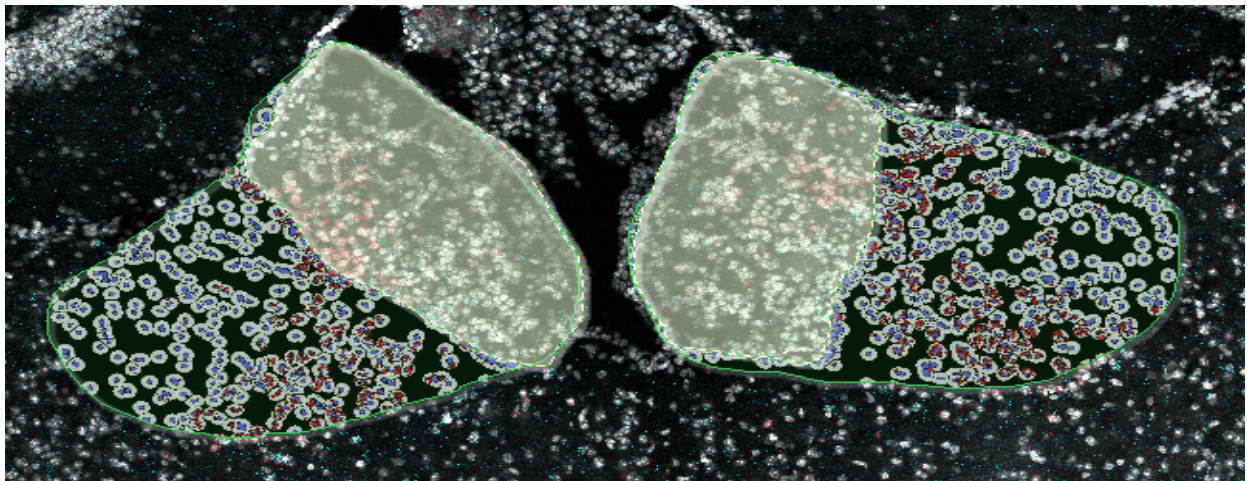
Behavior testing for open fields was performed in two custom-built sound-attenuated chambers. Each chamber had an overhead light with a dimmer switch, as well as a white noise fan. Male mice explored for 30 minutes in a circular arena placed in the center of each chamber. Each circular container had a diameter of 50cm and a height of 40cm. During the open field testing, mice were monitored using a video camera. All data was both acquired and analyzed utilizing Noldus EthoVision Version 14 Software. Data was imported into Prism (GraphPad) for the creation of graphs. Data was reported as mean +/- SEM.

Imaging and Outlines

Medial and lateral habenula were outlined following anatomical recommendations from Allen Brain Atlas for mice, utilizing Indica HALO software for actual quantitative analysis. 17 images were analyzed, with three female mice each having three images, and three male mice each having three images (excluding one mouse, from whom only two quality images could be obtained). Medial habenula and lateral habenula were both quantitatively analyzed using Indica HALO software. DAPI+ was utilized in order to confirm the presence of a cell where M5+ and PACAP+ expression was indicated.



A



B**Figure 4. Imaging and outlining of medial and lateral habenula.**

The yellow / white shaded outlines represent the medial habenula space, and the green outlines represent the lateral habenula space. Outlines were drawn on each image following anatomical recommendations from the Allen Brain Atlas for mice. White dots represent DAPI+ coverage meaning a cell is in that space, blue dots represent M5+ coverage, and red dots represent PACAP+ coverage. **B**, is a zoomed in image of **A**.

Quantitative Analysis

Once each image was analyzed, the percent of each type of cell (M5 + and PACAP +) was represented in comparison to total cells (measured by DAPI+). Each was analyzed separately in the medial and lateral habenula. Presence of each RNA in a cell was measured by the level of fluorescence, indicative of the level of protein. For this analysis, the standard measure of fluorescence was a puncta of 8 or greater in order to indicate that a cell was positive for that type of RNA transcript. Slides were taken from 3 male mice and 3 female mice. 3 sections from each slide were utilized, with the exception of one female mouse, who only had 2 usable sections due to cryostat error.

RESULTS

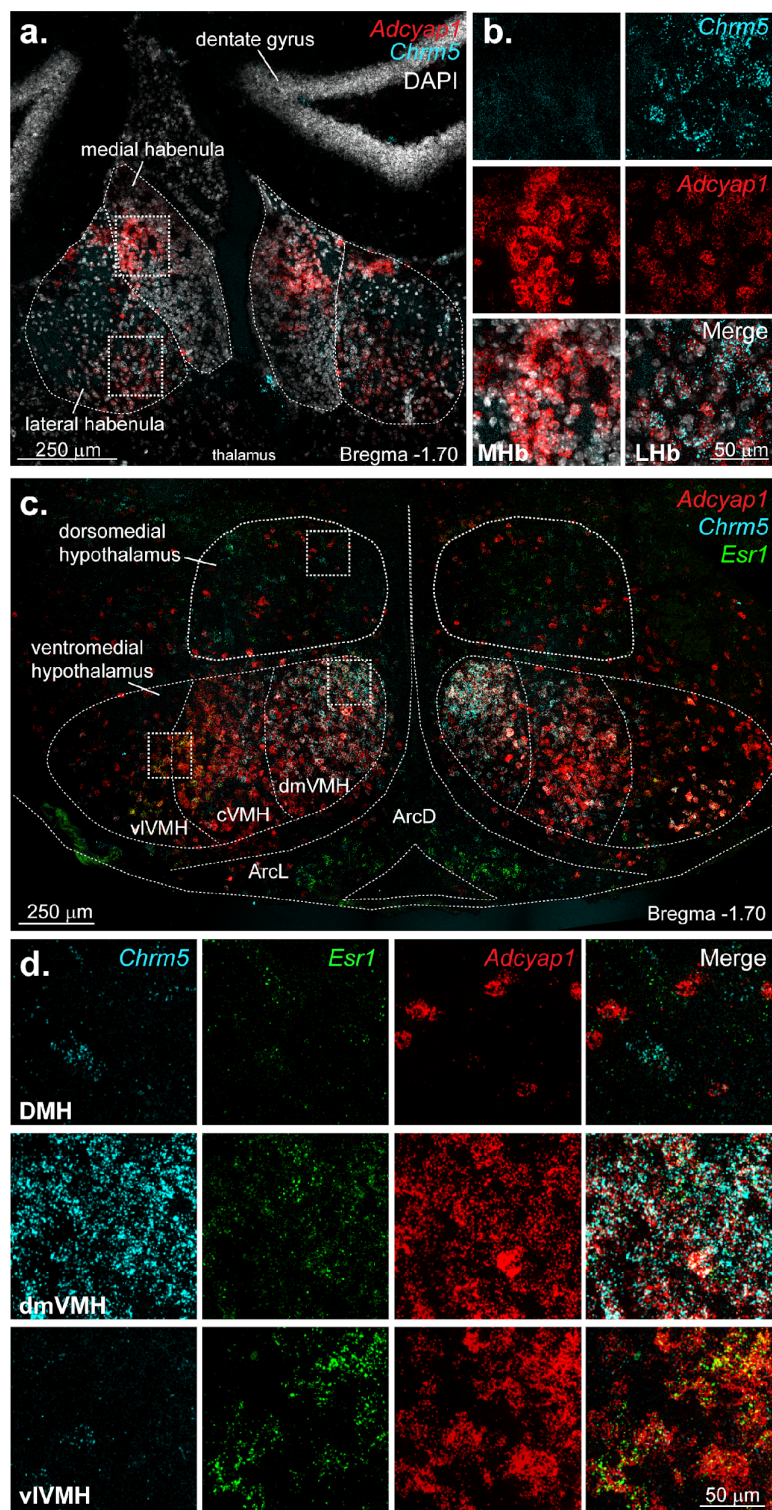


Figure 5. Muscarinic M5 receptors and PACAP neurons are colocalized in the habenula.

The above figure displays imaging from the Stellaris 8 Microscope. **A. B.** Expression of M5, PACAP and DAPI are shown in both the medial and lateral habenula. *Chrm5* (blue) is representative of the M5 receptor, as *Chrm5* is the gene encoding for M5. *Adcyap1* (red) is representative of PACAP expression. *Esr1* (green) is representative of the estrogen receptor 1 (ESR1), but was not analyzed in this particular paper. DAPI is a marker for visualizing cell nuclei and can be seen in white. In B, the left column represents the medial habenula (MHb) and the right column represents the lateral habenula (LHb). **C. D.** Additional fluorescent images of *Chrm5*, *Adcyap1*, and *Esr1*. ArcD represents the dorsal arcuate hypothalamic nucleus and ArcL represents the lateral arcuate hypothalamic nucleus. DMH represents the dorsomedial hypothalamus in the upper row of D, dmVMH represents the dorsal medial region of the ventromedial hypothalamus in the middle row, and vlVMH represents the ventral lateral region of the ventromedial hypothalamus in the bottom row.

Imaging of the habenula, both medial and lateral, provided preliminary evidence visually that there was overlap between the M5 receptor, as shown in blue and produced by the *Chrm5* gene, and PACAP neurons, shown in red and produced by the *Adcyap1* gene. This is a novel finding of incredible interest, even though the overlap does not appear to be complete colocalization. Analysis was broken down further into specific portions for more detailed quantitative results.

In the medial habenula, there was, on average, a significant amount of expression of M5, PACAP, and colocalization present. This effect appeared to be stronger in the lateral habenula, where 7.73% of total cells expressed colocalization of M5 and PACAP.

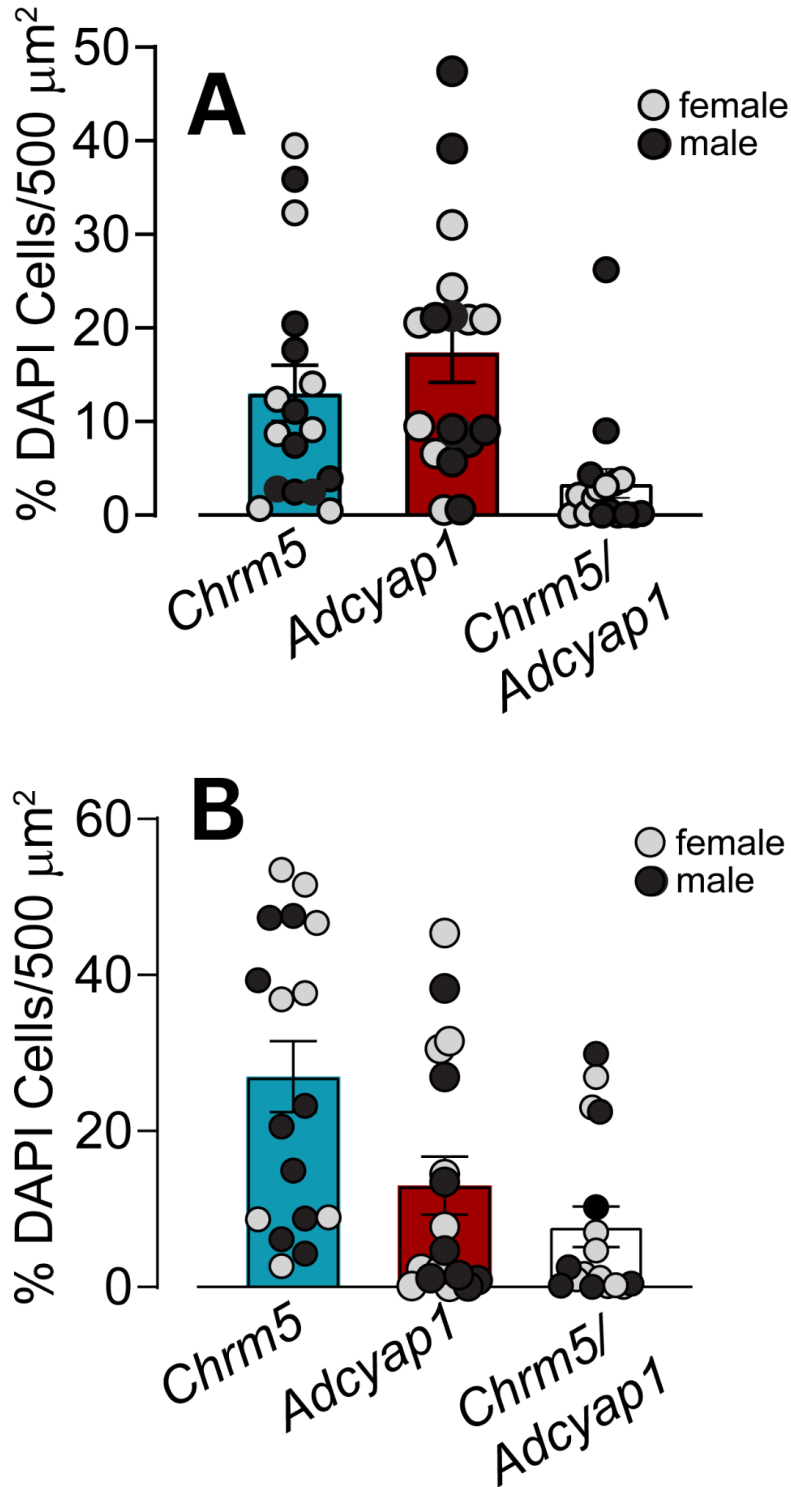


Figure 6. Distribution of *Chrm5* and *Adcyap1* across all cells in medial (A) and lateral (B) habenula.

A. In the medial habenula, there was, on average, 13.0% (SEM $\pm$ 3.006%) of total cells that expressed M5, 17.4% (SEM $\pm$ 3.197%) of total cells that expressed PACAP, and 3.39% (SEM $\pm$ 1.539%) of total cells that expressed colocalization of M5 and PACAP. **B.** In the lateral habenula, there was, on average, 27.0% (SEM $\pm$ 4.550%) of total cells that expressed M5, 13.0% (SEM $\pm$ 3.731%) of total cells that expressed PACAP, and 7.73% (SEM $\pm$ 2.589%) of total cells that expressed colocalization of M5 and PACAP. N = 17 images from six mice.

Next, as this project is all about seeing if coexpression between M5 and PACAP exists on neurons in either part of the habenula, as this coexpression has not been before investigated, analysis was performed in both the medial and lateral habenula to see if there was coexpression at the same standard of fluorescence (8+ puncta).

In the medial habenula, on average, 17.5% of M5 cells expressed PACAP and 13.6% of PACAP cells expressed M5. In the lateral habenula, this effect was even stronger, as 26.0-% of M5 cells expressed PACAP, and 47.2%, nearly half of PACAP cells expressed M5. It appears that more coexpression, overall, was found in the lateral habenula in comparison to the medial habenula. This effect appears to be even more strong in the M5 neurons in the lateral habenula, with a p value of an unpaired t-test being $<.001$.

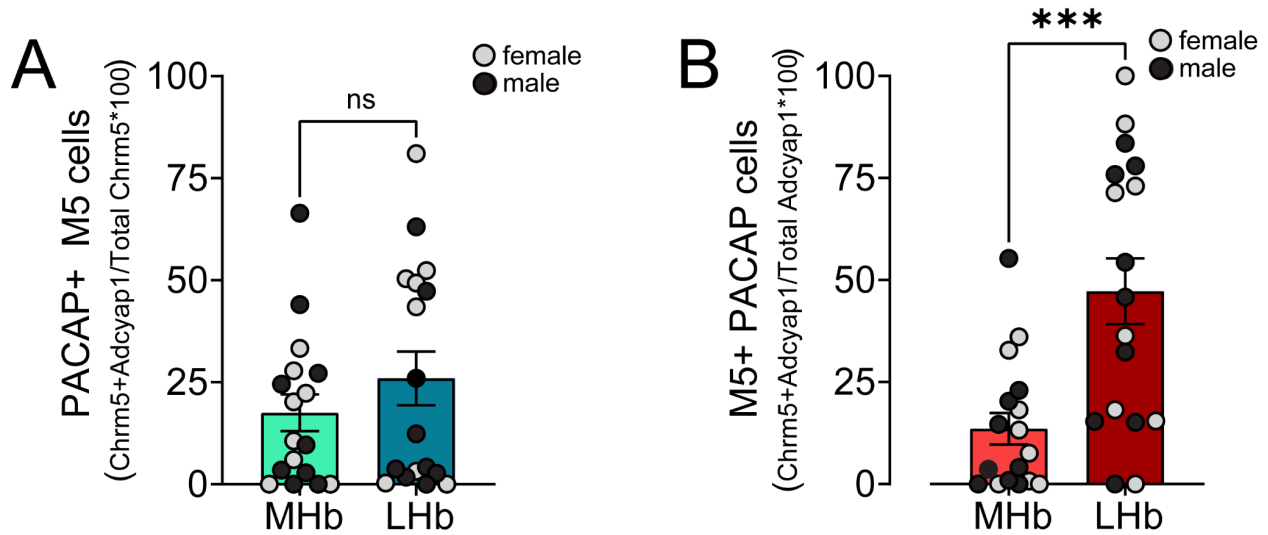


Figure 7. Comparison of co-expression of M5 and PACAP in medial habenula (MHb) and lateral habenula (LHb).

A. Shown is the percentage of M5 positive cells that also express PACAP. In the medial habenula, on average, 17.5% (SEM +/- 4.500%) of M5+ cells expressed PACAP. In the lateral habenula, on average, 26.0% (SEM +/- 6.590%) of M5+ cells expressed PACAP. This was not significant. **B.** Shown is the percentage of PACAP positive cells that also express M5. In the medial habenula only 13.6% (SEM +/- 3.864%) of PACAP+ cells expressed M5, but 47.2% (SEM +/- 8.033%) of PACAP+ cells expressed M5. *** $p < 0.001$, unpaired t-test. N = 17 images, from six mice.

Three male mice and three female mice were used in this set of images. Across all analyses, sex differences were absent, and generally speaking, male and female mice did not have any statistically different results.

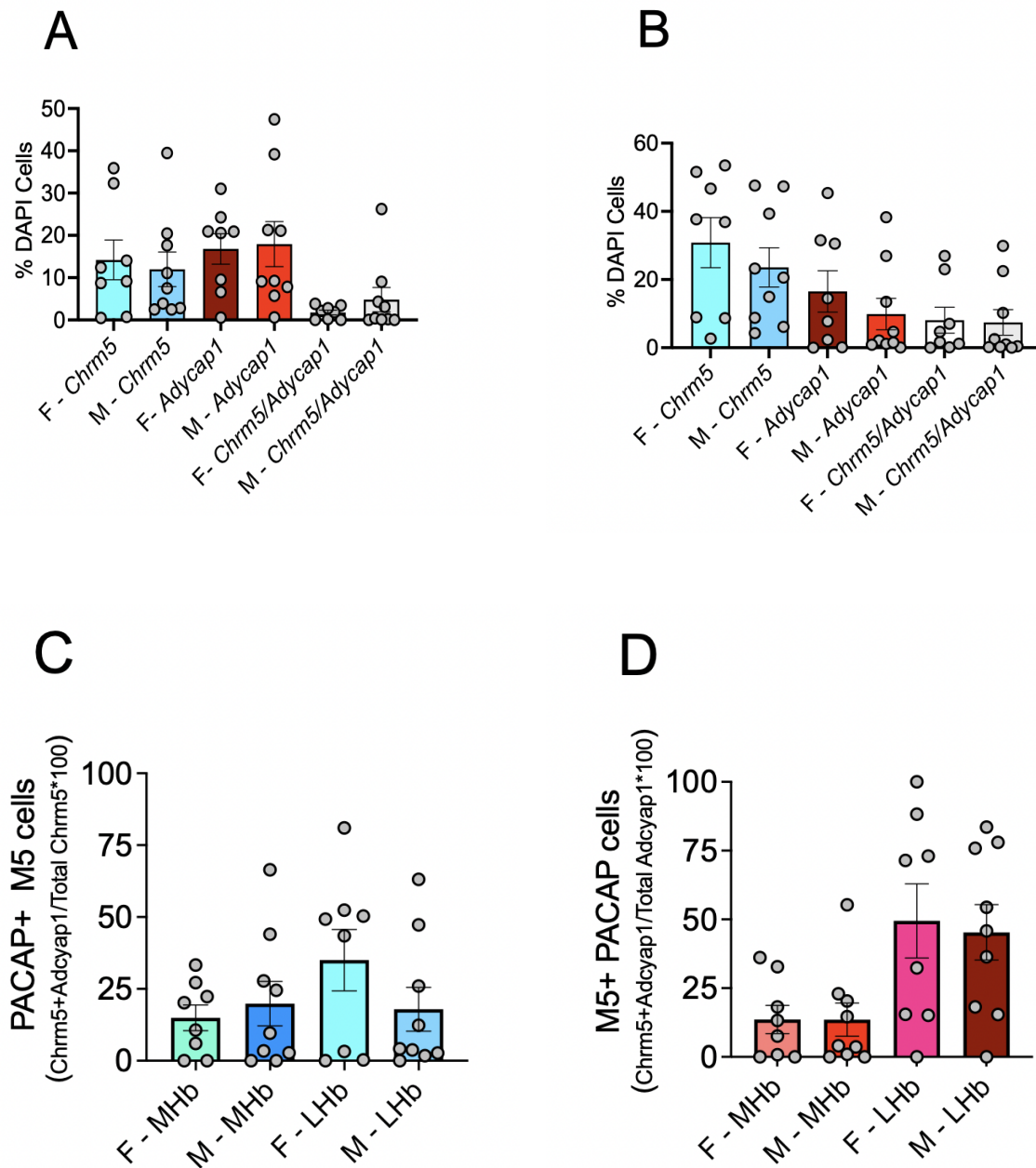


Figure 8. Sex differences in distribution of *Chrm5* and *Adcyap1* across all cells in medial (A) and lateral (B) habenula and comparison of co-expression of M5 and PACAP in medial habenula (MHb) and lateral habenula (LHb).

In all graphs, for females, N=8, and for males, N=9. **A.** There were no significant sex differences in expression of *Chrm5*, *Adcyap1*, or colocalization of *Chrm5* and *Adcyap1* in the medial habenula. Mean and SEM from left to right column: 14.21 +/- 4.679, 11.98 +/- 4.100, 16.80 +/- 3.603, 17.94 +/- 5.333, 1.784 +/- 0.5463, 4.811 +/- 2.859. **B.** There were no significant sex differences in expression of *Chrm5*, *Adcyap1*, or colocalization of *Chrm5* and *Adcyap1* in the lateral habenula. Mean and SEM from left to right column: 30.82 +/- 7.368, 23.57 +/- 5.735, 16.50 +/- 6.091, 9.339 +/- 4.600, 8.072 +/- 3.799, 7.430 +/- 3.751. **C.** As a percentage of M5+ cells that also expressed PACAP, there were no significant sex differences in either the medial or lateral habenula. Mean and SEM from left to right column: 14.97 +/- 4.461, 19.85 +/- 7.722, 35.01 +/- 10.67, 17.90 +/- 7.640. **D.** As a percentage of PACAP+ cells that also expressed M5, there were no significant sex differences in either the medial or lateral habenula across sexes. Both males and females had a higher percentage rate of PACAP+ cells that also expressed M5 in the lateral habenula than in the medial habenula. Mean and SEM from left to right column: 13.59 +/- 5.119, 13.54 +/- 6.002, 49.46 +/- 13.48, 45.29 +/- 10.07.

Open field testing, run solely on male mice, was utilized in order to test whether or not M5 played a role in anxiety-related behavior. The open field testing also provided interesting results, with male mice that had region specific knockdowns of M5 in the lateral habenula displaying less distance traveled ($p = .08$), a slower velocity traveled ($p = .06$) and more time spent in the center ($p = .06$). There was no difference surrounding entries into the center between the control and knockout groups. P values were not below an alpha value of .05, but there were trends, with a relatively small sample size of 5 mice in each group. This indicates promising, yet underpowered results that need to be followed up on with a second replicate cohort.

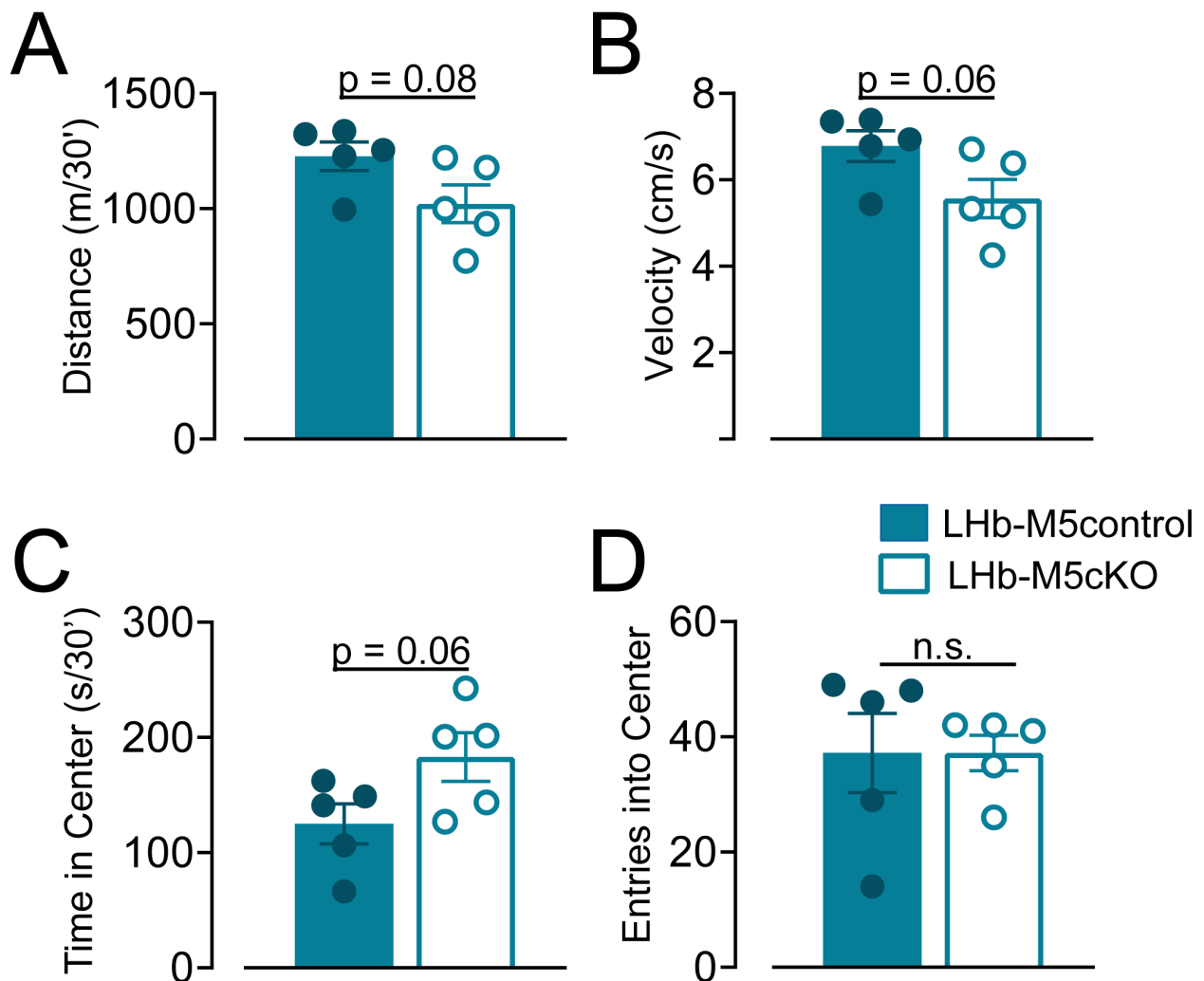


Figure 9. Lhb-M5 knockdown may reduce male mouse distance and velocity and increase time in center.

A. Time in the center of an open field in a 30 min open field session. Lhb-M5 KOs appear to cover less distance. Differences were not significant, but may be with a larger sample size. **B.** Velocity of male mice in a 30 min open field session. Lhb-M5 KOs appeared to travel slower than control mice, and may be statistically significant with larger samples. **C.** Time in center spent by male mice in a 30 min open field session. Lhb-M5 KOs appear to spend more time in

center, which may be statistically significant with larger samples. **D.** Entries into center of an open field during a 30 min session for male mice. No statistical significance present. N = five mice.

DISCUSSION

Up to this point, M5 research has really been focused on the VTA and dopamine transmission, due to the localization of M5 receptors on dopamine neurons (Garzón and Pickel, 2013). In addition, global M5 knockouts or systemic pharmacological manipulations have not been able to determine how M5 regulates particular cells and circuits. However, as shown in this study, M5 receptors are not solely colocalized with dopamine neurons, and are likely involved in the mediation of other circuitry as well. This provides a new, significant field for further research into the possible therapeutic effects of the M5 receptor, possibly focusing on targeting the M5 receptors in PACAP neurons.

Key Imaging Findings

The most major key finding discovered through the research in this paper was the significant (outside of 2+ SEM) presence of M5 and PACAP colocalization in cells in the brain. This was present in both the medial habenula ($p = .0428$) as well as in the lateral habenula ($p = .0087$). Prior to the completion of this study, there had not been colocalization discovered between M5 and PACAP in the medial or lateral habenula. This is particularly interesting because PACAP has been shown to diminish negative consequences of aversive events (Levinstein et al., 2022). In addition, it has already been established that M5 receptors have a role in specific reward behavior, exploratory behavior and locomotor behavior (Grasing, 2016; Steidl & Yeomans, 2009; Razidlo et al., 2022). The connection between M5 and PACAP could

lead to diminishing negative consequences of reward consumption while altering reward related behaviors in the habenula.

Interestingly, the colocalization seen in the medial habenula was not nearly as strong in the lateral habenula. The lateral habenula had much more significant colocalization: as percent of M5+ cells, as percent of PACAP + cells, and as percent of total cells. This would indicate a higher portion of the neurons in the lateral habenula, in comparison to the medial habenula, display colocalization. Currently, our research team is not sure as to what this finding represents, nor the reasoning behind this difference. This is an important finding to note, and it should be researched and investigated further in the future.

Key Behavior Findings

Open field findings have indicated that male mice with M5 knocked down in the lateral habenula move a lesser distance and do so at a slower velocity than regular mice in a 30 minute open field session. Male mice with M5 knocked down in the lateral habenula also spent more time in the center of the circular open field, although there was no significant difference in the amount of entries into the center of the open field across the two groups. In open field testing, the results of less distance traveled and slower velocity traveled are indicative of anxiety-like behaviors (Prut and Belzung, 2003). More time in the center was not expected, as that was a behavior indicative of anxiolytic, not anxiogenic results. However, this could be due to possible confounding effects present between lower velocity, less distance moved, and more time spent in the center. Lateral habenula M5 knockdowns showing a reduction in anxiety-like behaviors is surprising, as it indicates that these M5-containing neurons may play a role in generating anxiety in male mice. However, M5 and PACAP colocalized neurons specifically were not knocked out, simply M5 neurons in the lateral habenula, as the approach was region-specific but not

cell-specific. We also have a viral strategy using the CRISPR-Cas9 system in which we can viral transduce guide RNAs directed toward *Chrm5* in a Cre-inducible manner. We can use *Acpyp1-Cre* driver lines to target M5 knock-down solely to PACAP neurons in the habenula. We have used this strategy successfully in dopamine neurons. We will use this strategy in our future directions.

Study Limitations

A major caveat to this work is that in this research, a viral strategy was used for the M5 knockout mice that was region specific in comparison to a cell-type-specific transgenic strategy. However, we have a future plan to address this caveat, as discussed above. Additionally, another limitation is that the medial and lateral habenula outlines were drawn manually by one researcher following the Allen Brain Atlas. Although this method should be consistent, there is no way to ensure complete consistency regarding outlines of both parts of the habenula on each coronal slice. In the future, we can use an automated registration strategy in collaboration with the University Imaging Core to better score the boundaries of the medial and lateral habenula. Likewise, we did not assess differences along the rostrocaudal axis. Gradients along the rostrocaudal axis of the habenula may account for considerable variance in both *Chrm5* and *Adcyap1* expression.

In addition, the open field testing in this study was only run with male mice. This strategy was utilized to ensure consistency and eliminate the variable of sex possibly playing a role in the behavioral results found. We know that systemic or constitutive manipulations of M5 receptors show profound sex differences (Nunes et al., 2023; Razidlo et al., 2022). Yet, the colocalization results hint that there are likely not sex-related differences at play here surrounding colocalization of M5 and PACAP in the habenula. That being said, open field testing, as well as

possible future behavioral testing, should also be completed with female M5 knockout mice to ensure the absence of sex differences. Having only male mice was a limitation of this study, but one that future research should be able to answer and overcome. Another limitation of the open field behavior was the knockdown of M5 in the lateral habenula.

Finally, the sample size for this study was limited to three female mice and three male mice, with equal amounts of each sex in order to limit effects directly based on sex. Although the %M5+ of PACAP cells results were clearly statistically significant, further research could benefit from having a larger sample size of more mice. The experiments in this study, especially the open field testing, needs to be completed with a larger sample size to confirm the findings and conclusions from this work.

Future Directions

There are many future directions this study could be taken. To begin, as this research presents novel findings, replication of this research must be completed in order to ensure the reliability of the results surrounding colocalization of M5 and PACAP found in this study. The colocalization of M5 and PACAP had not yet been discovered in any part of the brain prior to this study.

M5 is found in many other parts of the brain due to its key role in the dopamine system, and *Adcyap1*, the PACAP encoding gene, is also found in other areas of the brain. There are many future directions to see if PACAP is also colocalized with M5 neurons in other parts of the brain. Further research needs to be completed to see if the colocalization between M5 and PACAP is present in the other parts of the brain, or if this is unique to the habenula. An example of this could be looking at the M5 receptor in the ventral tegmental area and seeing if there is any expression of *Adcyap1* in the ventral tegmental area, and if so, if that coexpression is significant.

Additionally, another future direction for this study here could surround the fact that there was a much more significant effect present in the lateral habenula in comparison to in the medial habenula. The reasoning behind this is currently unknown, and is an important future direction that should be taken out of this study. Similar behavior experiments to the ones run in this study (novel object, novel food, open field, 3 chamber social interaction, and sucrose preference behavior testing) could be completed with more specific knockouts of M5 in either just the medial habenula or just the lateral habenula to see if there are behavioral differences with removal of just one part of the habenula.

Other behavioral experiments may also reveal a further role of M5 knockouts in the habenula in mice models. In this study, only open field testing was completed as a behavioral measurement. Future experiments such as elevated zero maze (EZM) testing and light-dark box testing may also reveal further behavioral differences in patterns of reward-related behavior in these mice. EZM testing and light-dark box testing are commonly used to test anxiety-like behavior in mice (Razidlo et al., 2022), whereas tests such as open field testing and 3 chamber social interaction test social behavior. In addition, sucrose preference testing could also test anhedonia. Behavioral experiments testing a new type of behavior, anxiety-like behavior, in these same knockout mice would be fantastic extensions of the behavior already discovered.

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