

**There's Always Room for Dessert:
MCH Neuron Regulation of Non-Homeostatic Feeding Behavior**

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Neuroscience)
in the University of Michigan
2025

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Dedication

This dissertation is dedicated to all of the fantastic teachers and educators I've had over the years, who fostered a love of learning in me that could only be satisfied by the highest degree I could achieve. Without teachers, mentors, and role models, this would never have been possible. In particular, this dissertation is dedicated to the following educators, who made up the academic village that raised me.

- Mrs. Jean Sheninger, First Grade, Francis A. Desmares Elementary School.
- Mrs. Pratima Patil, Dr. Derek Weber, Mrs. Cathy Fortunato, Dr. Louise Tokarsky-Unda, Mrs. Maureen Lawler, Mrs. Teresa Morelli, Sra. Daysi Lakhilif, Sra Margaret Cross, and Dr. John Cleary of The Academy for Health and Medical Sciences at Somerset County Vocational & Technical High School.
- Dr. Chiye Aoki, Dr. Margarita Kaplow, Dr. Paul Glimcher, Dr. Wei Ji Ma, and Dr. Oliver Vickbladh of New York University Center for Neuroscience.
- Dr. Mariann Weierich, Mrs. Kizzy Vasquez, and Dr. Glenn Schafe of CUNY Hunter College BP-ENDURE fellowship program.

Acknowledgements

I would like to begin by acknowledging that the work presented in this thesis was conducted at the University of Michigan – Ann Arbor, located on the traditional territory of the Anishinaabe people. In 1817, the Ojibwe, Odawa, and Bodewadami Nations made the largest single land transfer to the University of Michigan. This was offered ceremonially as a gift through the Treaty at the Foot of the Rapids so that their children could be educated. Further, I acknowledge that this country would not exist were it not for the free, enslaved labor of Black people. Black Lives Matter.

I am immensely proud of my accomplishments within this thesis, but a doctoral dissertation cannot be complete without the village it takes to complete it. As I write this document, I can't help but pause to reflect on my journey, my community, my growth, and my unbridled joy at having made it to this point. There was a time when I legitimately didn't think I would get through it. If you are a PhD student reading this in the future, know that it takes an army of support to pull you out from that place, but you don't have to look far to find it. The fact that I am here, typing my way across the finish line, is largely due to those who supported me when I could not support myself.

Thank you Christian, for being exactly the type of mentor I needed. I always said I wanted to join a newer lab because that way I could really grow close with my PI. I like to think we've both been there as support systems for each other, and as our working relationship has ebbed and flowed, I'm glad to be ending this PhD journey with a mentor I can also consider a friend. To put it parsimoniously, I am going to miss you dearly. I am going to miss your office, where I currently write these acknowledgements, with the football field sized monitor, mechanical keyboard, a peanut butter kind bar and a bubbly seltzer, even more.

Thank you Alvin, for absolutely everything. Having you by my side both literally and figuratively has made this journey an absolute joy even in times when I hated it. Hospital lunches with you pulled me back to reality when I needed to be grounded. I could not have done this without you. Five big booms for Alvin. You kept my star jar half full.

Tim Cha, Lex Faulkner, Chris Phillips, Jack Evans, Lucia Kim, Lorelei Baron, Courtney Myers, Jordan McCarthy, and Hannah Lyons: you have blurred my work/life balance into oblivion. Y'all each gave me a reason to look forward to coming to work, because I got to hang out with my friends there too. And thank you to everyone in the Burgess Lab for contributing to the environment that made it so easy to enjoy my time in the lab.

Shelly Flagel, you may never know how important you have been to my PhD journey. I could not have picked a better committee co-chair and role model. Your guidance, both personally and scientifically, has had an impact I cannot put into words. Thank you.

The NGP administrative team is second to none in their support and encouragement. Thank you, Valerie Smith, Audrey Seasholtz, Rachel Harbach, Carol Elias, Carol Skala, Vicky Martin, Adam Iliff, and Ed Stuenkel, for crafting the environment of the RPR and the culture of the NGP that keeps all of us on the right track.

I want to give a huge, enormous thank you to the American taxpayers. Knowing that our tax dollars fund the greatest scientific research in the world is an immense privilege, and one that I am so grateful for.

I am blessed to have had an academic mentoring circle that spans over a decade of my life, and over 3,000 miles of the country. Thank you, Susan Sheng, Katie Yoest, Marina Picciotto, Steve Pittenger, Rick Crouse, Rob Froemke, Naomi Lopez Caraballo, Kathleen Martin, Chiye Aoki, Paul Glimcher, Mariann Weierich, Glenn Schafe, Kizzy Vasquez, and Margarita Kaplow, for teaching me everything I know. With all of you in my corner I have a familiar face cheering me on almost anywhere I go.

Thank you to the Graduate Employee Organization local 3550, a mighty group of people with the most material commitment to justice and dignity. I am proud to have played a small role in securing our community's rights and I am forever grateful for your commitment to graduate workers at this institution.

Thank you to Julie Chen-Moonves, Jeff Probst, and Shonda Rhimes, for giving my second monitor something to do while I work. And for cultivating my love for reality TV – a personal expertise of mine which rivals that recounted in the present thesis. Certainly, an expertise which took orders of magnitude longer to cultivate. Love one another.

A cherry on top of this journey has been that I've gotten to make the best friends in the world. Jenn Jaime, Alvin Chiu, Dan Doyle, Emma Thornton-Kolbe, Marie Walicki, Katie

Emery, Audrey Drotos, Kane York, Marcel Elkouri, Kate Giffin, Amanda Gregolynskyj, Amanda Gibson, Emily Peirent, and my entire 2019 cohort; I am incredibly grateful for all of your friendship. Special shoutout to Nicoleen Boyle and Thea Bourdeau for being my two Ann Arbor friends that aren't in school – you've kept me sane by reminding me there's a real full world out there. Thank you to Edison T. Dalton, for being so small and so stinky, and an unconditional source of love.

I'd like to acknowledge the Ann Arbor District Library, the best library in the world. And I specifically want to mention the AADL Summer Game, for being the most reliable source of joy for myself, Emma Thornton-Kolbe, and Katie Emery. I cherish the time we've spent with our friend Summer. If you're reading this, go get a library card.

I am so glad the world brought me my best friends Nikita Reddy, Nishi Shah, Priscilla Kim, Saloni Lad, Maya Schlesinger, and Lauren Lisiewski, BEFORE I embarked on this grad school journey. Completing a PhD is treacherous, rocky, and scary, and this group of incredibly strong women has been there to ground me through all of it. And even before them, my family gave me the fundamentals I would need to make it through this. Thank you to my mom, my dad, my sister Sarah, my Aunt Nanci & Uncle Ben, Uncle Ray, Aunt Mary, and Cousin John.

Warren Yacawych, thank you for being my home. I wish I worked as hard as you do, but I'm so glad I don't. You are the most dedicated individual I've ever met, and I am overjoyed to be one recipient of your dedication. Cheers to one of us finally getting a raise

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Abstract

Regulation of food intake and energy balance is critical to survival. Hunger develops as a response to energy deficit and drives food-seeking and consumption. While the primary evolutionary reason to consume food is to maintain an individual's nutrition and energy balance, there are reasons to eat beyond homeostatic necessity. There is an increasing prevalence of both eating disorders and obesity in the US, which is likely due to hi-jacking and dysregulation of specifically non-homeostatic drives to feed by external stimuli.

Melanin-concentrating hormone (MCH) neurons have been implicated in the regulation of feeding behavior, in addition to a number of other fundamental behaviors including sleep, anxiety, and maternal behavior. Several studies suggest that MCH peptide increases food consumption, while studies of MCH neuron activity show effects only on cued feeding, and others show no effect of MCH neuron manipulation on feeding. MCH neuron cell bodies are located in the lateral hypothalamus (LH) and zona incerta (ZI), and MCH neurons have widespread projections to diverse downstream brain regions including the nucleus accumbens (NAc), of particular relevance to this work. Yet few studies have investigated the function of specific projections or differentiated the behaviors they regulate.

Here we use terminal-specific optogenetics, in combination with high-throughput behavioral analysis, to elucidate the role of MCH projections to the NAc in sleep and feeding behavior. We show that activation of MCH cell bodies in LH leads to increased REM sleep, while activation of MCH neuron terminals in NAc does not induce changes in REM sleep. Neither population induced a significant change in baseline feeding. These results suggest that the role of MCH neurons diverges based on anatomical target, and that not all MCH neurons play a role in feeding or in sleep/wake architecture.

We also investigate the role of NAc projecting MCH neurons in non-homeostatic feeding behavior by developing the optogenetics-reinforced consumption assay (ORCA). ORCA allows mice to voluntarily nose poke in their choice of four ports, which deliver all-pairwise combinations of food availability and MCH optogenetic stimulation. We observe that mice

receiving stimulation of MCH cell bodies in LH prefer ports that deliver food over those that deliver stimulation, while mice receiving MCH terminal stimulation in NAc significantly prefer the port delivering both a food pellet and optogenetic stimulation simultaneously. These results lead us to conclude that MCH → NAc stimulation serves to enhance the rewarding value of a temporally paired food. In a pilot experiment, we also assess the MCH → LC projection using the ORCA paradigm. We observed that mice receiving MCH → LC terminal stimulation showed a preference for ports delivering optogenetic stimulation regardless of food availability. Understanding the MCH → NAc circuit as a neural circuit which attributes reward value to food may help contribute to clinical therapeutics for eating disorders and obesity.

Chapter 1 Introduction

1.1 Project Overview

Food consumption is a behavior that organisms must engage in frequently and effectively. The ability to discern when to eat, and which foods to eat to maintain an appropriate balance of energy and nutrients, is crucial to an organism's health. However, the choice to consume food is not purely due to homeostatic need – there are non-homeostatic contributors to one's decision to eat, regardless of hunger or energy state. Humans commonly crave sugary or fatty foods even when sated (i.e., “there is always room for dessert”). Dysregulation of both homeostatic and non-homeostatic motivators to eat can lead to malnutrition, disordered eating, or obesity, and an understanding of the neural circuitry underlying hunger and motivation is essential to clinically treat these conditions.

Melanin-concentrating hormone (MCH) neurons of the lateral hypothalamus (LH) and zona incerta (ZI) are a relevant neural target for both homeostatic and non-homeostatic motivators to eat. MCH neurons project widely throughout the brain, including to areas involved in feeding and reward, such as the arcuate nucleus, nucleus accumbens, and basolateral amygdala (J. C. Bittencourt et al., 1992; Jackson C. Bittencourt, 2011), areas which also express MCH receptors (Able et al., 2009; Saito et al., 2001). Both MCH neurons and MCH peptide have been implicated in feeding behavior and energy balance (Dilsiz et al., 2019; Hausen et al., 2016; Ito et al., 2009; Mul et al., 2011; Qu et al., 1996; Rossi et al., 1997; Sherwood et al., 2015; Shimada et al., 1998). Early work using MCH knockouts showed that mice lacking the *Pmch* gene exhibit hypophagia, and an anorexic-like phenotype (Shimada et al., 1998). Additionally, chronic intracerebroventricular infusion of MCH peptide has been shown to lead to obesity in mice (Gomori et al., 2003). Recent work has shown that optogenetic activation of MCH neuron cell bodies in the LH promotes feeding, although only when stimulation is specifically paired with consumption (Dilsiz et al., 2019). Previous work largely treats MCH neurons as a homogeneous

population, focusing on collective effects of the entire population of neurons rather than effects of individual projections/subpopulations. There remains ambiguity about the role that MCH neurons play in feeding behavior – ambiguity which is likely confounded by different subpopulations of MCH neurons promoting different effects on feeding. The broad objective of this thesis is to understand the role of specific MCH circuits in both homeostatic and non-homeostatic drives to consume food.

MCH neurons also regulate non-feeding behaviors, including reward, learning, reproduction, and other consummatory behaviors (Blanco-Centurion et al., 2019; Clegg et al., 2003; Duncan et al., 2005; Sherwood et al., 2012; Vetrivelan et al., 2016), although their role in regulating sleep and arousal is the most well-characterized. Optogenetic stimulation of MCH neurons in mice has been shown to increase time spent in rapid eye movement (REM) sleep as well as the number of transitions from Non-REM (NREM) to REM sleep (Blanco-Centurion et al., 2019; Vetrivelan et al., 2016). One study demonstrates activation of terminals in medial septum and dorsal raphe can also promote sleep (Jego et al., 2013). Additionally, ablation of MCH neurons decreases REM sleep during the light cycle, when mice are normally asleep (Adamantidis et al., 2008; Varin et al., 2016). It is not known how MCH neuron activity regulates both sleep and feeding (along with the variety of other behaviors MCH is implicated in), but it is likely through a network of MCH efferents projecting to different downstream brain regions. To better understand the function of MCH neurons, a more thorough investigation of the functional heterogeneity of different subpopulations of MCH neurons is needed, and this is a major objective of the present thesis.

The majority of functional investigations of the MCH system treat it as a homogeneous group of neurons. This approach has been unsuccessful in fully understanding the role of the MCH system. Depending on the variables measured, different studies have concluded that MCH activity regulates sleep (Adamantidis et al., 2008; Blanco-Centurion et al., 2019; Jego et al., 2013; Varin et al., 2016; Vetrivelan et al., 2016), feeding (Ito et al., 2009; Mul et al., 2011; Qu et al., 1996; Rossi et al., 1997; Sherwood et al., 2015; Shimada et al., 1998), drinking (Baird et al., 2006; Clegg et al., 2003; Duncan et al., 2006; Johnson, 2018), or consumption as a whole (Barson et al., 2013; Dilsiz et al., 2019; Duncan et al., 2005; Johnson, 2018; Mul et al., 2011). Furthermore, it is unclear whether MCH-mediated feeding is a homeostatic drive based on energetic need, or a non-homeostatic drive based on perceived reward value. Despite the breadth

of literature treating MCH neurons as a uniform population, studies have demonstrated that there are distinct populations of MCH neurons based on their genetic profile and anatomical projections (V. Cvetkovic et al., 2003; Vesna Cvetkovic et al., 2004; Harthoorn, 2007). The experiments conducted in this thesis intend to identify whether MCH populations can be further subclassified based on their role in specific behavior(s).

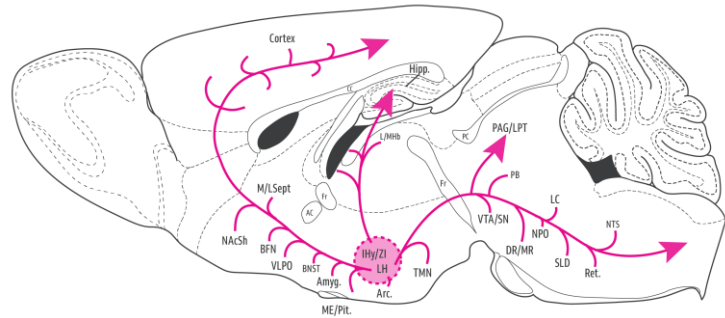


Figure 1.1 Diagram of MCH anatomy in a sagittal section of the mouse brain.

MCH neurons' cell bodies are located in the lateral hypothalamus (LH) and zona incerta (ZI). Their terminals project widely throughout a vast array of brain areas.

1.2 Melanin-Concentrating Hormone

Melanin-concentrating hormone (MCH) is a 19-amino acid cyclic neuropeptide derived from the propeptide pre-pro-melanin-concentrating hormone (ppMCH) and encoded by the *Pmch* gene (Nahon et al., 1989; Vitale et al., 2003). MCH gets its name from the role it plays in the scales of teleost fishes, where it facilitates adaptive changes to scale color by aggregating melanin, causing scales to become more pale (Kawauchi et al., 1983). In mammals, however, MCH expression is primarily localized to the brain, but may also appear in peripheral tissues outside the central nervous system, including lung, thyroid, and gastrointestinal tissues (Breton et al., 1993). MCH-expressing neurons originate in the lateral hypothalamus (LH) and zona incerta (ZI), areas classically involved with energy balance (J. C. Bittencourt et al., 1992; Elias et al., 1998; Sita et al., 2007). These neurons project widely to many areas across the brain (Figure 1.1), including areas involved with learning and reward, such as the hippocampus, lateral septum, amygdala, nucleus accumbens, and ventral tegmental area (J. C. Bittencourt et al., 1992). Subcellularly, MCH peptide is predominantly located within the soma and around the nucleus (Diniz et al., 2019).

The mammalian *Pmch* gene produces at least two other peptides, the 13-amino acid neuropeptide glutamic acid isoleucine (NEI) and the 19-amino acid neuropeptide glycine glutamic acid (NGE) (Borsu et al., 2000; Moldovan et al., 2012; Nahon et al., 1989; Toumaniantz et al., 1996). In rodents and primates, most MCH neurons co-express NEI and NGE (Mickelsen et al., 2019), as well as genes involved with GABA and glutamate synthesis, packaging, and release (Mickelsen et al., 2019, 2017; Nectow et al., 2017), though the specifics

of MCH neuron co-transmission with fast neurotransmitters is unclear. Many MCH neurons are glutamatergic, often co-releasing some combination of MCH, NEI, and NGE (Abrahamson et al., 2001; J. C. Bittencourt et al., 1992; M. J. S. Chee et al., 2015). Some MCH neurons also express cocaine-and-amphetamine-related transcript (CART) and nesfatin, though the expression profiles of these are non-uniform among MCH neurons (Broberger, 1999; Mickelsen et al., 2019; Oh-I et al., 2006).

This non-uniformity among MCH neuron gene expression profiles suggests that MCH neurons may be separable into different subpopulations based on their gene expression profiles. Indeed, there are other characteristics including anatomical projections, chemical markers, and electrophysiological properties by which MCH neurons can be subclassified. Some MCH neurons coexpress both CART and neurokinin-3 (NK3), while others completely lack these markers (Vesna Cvetkovic et al., 2004). CART and NK3 expression may be one neurochemical axis along which MCH neurons are also anatomically subdivided – MCH+/CART+/NK3+ neurons project toward the telencephalon and dorsal brainstem, while MCH+/CART-/NK3- neurons target the caudal brainstem and spinal cord (Vesna Cvetkovic et al., 2004). These cells differ electrophysiologically as well; MCH+/CART+/NK3+ neurons have a smaller maximum change in voltage over time (dV/dt ratio) and shorter action potential half-width time than MCH+/CART-/NK3- cells, suggesting that the latter are more excitable and produce action potentials more quickly than the former (Fujita et al., 2021).

1.3 The MCH Receptor

MCH binds two G-protein coupled receptors (GPCRs), MCHR1 and MCHR2 (Presse et al., 2014). These two MCH receptors have been identified in humans and primates, though MCHR2 is not present in rodents, and therefore is not thoroughly understood (Hill et al., 2001; Sailer et al., 2001; Tan et al., 2002; S. Wang et al., 2001). MCHR1 is a 353-amino acid rhodopsin-family GPCR previously known as the orphan receptor SLC-1 or GPR24 (Bächner et al., 1999; Chambers et al., 1999; Lakaye et al., 1998; Lembo et al., 1999; Saito et al., 2001, 1999; Shimomura et al., 1999). Within the nervous system, MCHR1 mRNA is observed broadly throughout the brain, including in olfactory structures, limbic structures, hippocampus, neocortex, hypothalamus, and spinal cord (Lembo et al., 1999). Peripherally, MCHR1 is found in brown adipose tissue, pancreas, and pituitary gland tissue (Balber et al., 2019; Presse et al.,

2014). Broadly, MCH fibers typically terminate in areas where MCHR1 expression is high, although some MCH fibers reach areas showing little to no MCHR1 expression (Saito et al., 2001). This suggests that MCH neurons may have different effects exerted by either MCH, other neurotransmitters, or co-release of multiple neurotransmitters along with MCH.

MCHR1 typically mediates an inhibitory effect by activating Gai and Gao subunits, therefore suppressing neuronal activity (Presse et al., 2014), but is capable of multiple actions which seem to contradict each other (Chung, Saito, et al., 2009; Hawes et al., 2000). MCHR1 inhibits adenylyl cyclase, which suppresses cAMP accumulation (Saito et al., 1999), but also mobilizes intracellular Ca²⁺ ions in high concentrations of MCH (Bächner et al., 1999; Saito et al., 1999). This suggests that MCH may couple to both Gai/o and Gao, though it has demonstrably higher affinity to Gai proteins (Eberle et al., 2004; Hawes et al., 2000; Lembo et al., 1999; Saito et al., 1999). Thus, the post-synaptic effect of MCHR1 action is variable across different contexts and target regions, potentially dependent on the availability of different G-protein subunits in those contexts and regions (Gao, 2009).

Given that MCH has well-established roles in both metabolism and sleep, an expected characteristic of the MCH system is that *Pmch* and *Mchr1* expression are modulated by energy states and circadian rhythms. Leptin-deficient *ob/ob* mice display increased levels of *Pmch* expression which is reversed by administration of leptin (Mondal et al., 2002; Qu et al., 1996). One species of chicken displays increased *Pmch* levels with starvation, which decrease as the animals become more and more sated (Simon et al., 2018). *Mchr1* mRNA expression is elevated during the light phase (when rodents typically sleep) in rat frontal cortex (Dias Abdo Agamme et al., 2015), and sleep disruption results in fluctuations of CSF levels of MCH with circadian rhythms (Dias Abdo Agamme et al., 2015).

1.4 Functions of MCH

1.4.1 Role of MCH in Feeding and Metabolism

It has long been accepted that a major role for the MCH system is in promoting feeding. Evidence in favor of this has been around since the 1990s, when it was reported that MCH knockout mice display an anorectic-like phenotype, observed to be hypophagic and lean compared to their WT counterparts (Shimada et al., 1998). Around this time, it was also

observed that MCH mRNA is upregulated in periods of food withdrawal, or in obese ob/ob mice which lack functional leptin (Qu et al., 1996). Furthermore, mice which overexpress MCH at a two-fold increase from WT (MCH-OE mice) eat more food and gain more weight when fed a high fat diet than their WT counterparts over a period of weeks (Ludwig et al., 2001). This early work with genetic manipulation of MCH clearly suggested a role for MCH in feeding.

MCH pharmacological work has deepened the understanding of its role in feeding. Multiple research groups have used intracerebroventricular infusion of MCH peptide and observed increased levels of feeding in both mice (Gomori et al., 2003; Mashiko et al., 2005; Mul et al., 2011) and rats (Della-Zuana et al., 2002; Georgescu et al., 2005). This effect is MCHR1 dependent, as MCHR1 antagonism consistently leads to decreased food intake and body weight (Georgescu et al., 2005; Karlsson, Aziz, et al., 2016; Mashiko et al., 2005), and MCHR1 agonism does the inverse (Guesdon et al., 2009).

Although often considered separately, the experience of feeding is inseparable from the experience of reward. Motivation to feed includes homeostatic signals such as hunger and energy deficit, as well as signals having to do with a food's reward value, palatability, and availability. It was recently observed that requiring animals to perform a simple action in order to receive food was able to significantly reduce their food consumption (Barrett et al., 2024). Similarly, adding a palatable tastant to an otherwise aversive food can increase consumption (Tolliver et al., 1988). Recent work seems to indicate that MCH signaling may be less homeostatic in nature, instead contributing to this variety of signals which determines an organism's motivation to feed.

Despite consistent findings showing a positive relationship between MCH receptor pharmacology, food intake, and body weight, more recent work using neuron-specific techniques complicates this finding. Using both chemogenetics and optogenetics, it has been shown that activation of MCH neurons does not lead to a significant increase in feeding (Dilsiz et al., 2019; Hausen et al., 2016), but can lead to an increase in feeding when paired specifically with food intake (Dilsiz et al., 2019). This suggests a reward/reinforcement effect of MCH rather than a feeding one. Terrill and colleagues used chemogenetic activation of MCH terminals in NAc to show a modest increase in feeding specific to male rats, and not females (Terrill et al., 2020). A number of studies have shown that similar interventions in the MCH system lead to other behavioral changes, including but not limited to reproduction, maternal behavior (Alachkar et al., 2016) anxiety, locomotion, memory, and social behaviors. Notably, however, the bulk of these

studies focus only on one behavioral readout, and fail to observe whether changes in feeding occur simultaneously.

Observed effects of MCH on feeding may be secondary to effects of MCH on general consummatory behavior; a broader category. For example, early work showed that intracerebroventricular MCH infusion stimulates a significant increase in water intake even in a context where food was unavailable (Clegg et al., 2003). This is in contrast to Agouti-Related Peptide (AgRP), which stimulated water intake only in a manner proportional to food intake (Clegg et al., 2003). Intracerebroventricular MCH also dose-dependently increases intake of both alcohol and palatable sucrose solution independent of food availability (Duncan et al., 2005). Changes in liquid/water consumption may be due to changes in licking microstructure, namely increases in lick rate and number of lick bursts early in a meal (Baird et al., 2006). These changes in licking behavior apply to both caloric and non-caloric liquid consumption, and are indicative of animals' "hedonic taste evaluation" rather than their drive to consume (Baird et al., 2006).

Taken together, these findings seem to suggest that the MCH system is not necessarily important for food intake, but instead for a higher-level learning/reward attribution process which may be shared by numerous aspects of consumption. Understanding feeding behavior as having a reward component rather than a simple, reflexive response to hunger can help contextualize the way MCH may be promoting feeding.

1.4.2 Role of MCH in Reward and Learning

Given the relationship between feeding and reward, and the recent evidence that stimulating MCH neurons may not drive increased food consumption, it is possible that MCH promotes feeding simply as a byproduct of enhancing the rewarding experience of consumption. Sherwood and colleagues trained WT and MCHR1-KO mice on a Pavlovian conditioning paradigm, in which a food reward was preceded by a conditioned stimulus tone (CS+). Mice of either genotype were able to learn this task with equal accuracy, but when later tested with both a CS+ and CS-, only WT mice showed an overeating response to the CS+, while MCHR1-KO mice failed to behaviorally discriminate between the two tones (Sherwood et al., 2015). While this demonstrates that MCHR1-KO mice have an impaired overeating response to a learned food cue, it is known that MCH-KO mice show an unimpaired overeating response to starvation, an unconditioned, homeostatic stimulus (Shimada et al., 1998).

Indeed, recent work using optogenetic and chemogenetic stimulation shows no effect of long-term MCH neuron activation on food intake (Dilsiz et al., 2019; Hausen et al., 2016). However, when allowed to engage in operant responding for MCH photostimulation, mice voluntarily nose-poked to receive MCH photostimulation in the absence of a food reward (Dilsiz et al., 2019), suggesting that MCH neuron activity may be rewarding in and of itself. In this same study, despite observing no effect of chronic MCH stimulation on food intake, animals' food intake significantly increased when optogenetic stimulation of MCH neurons was paired discretely with food consumption, rather than continuously delivered (Dilsiz et al., 2019). Importantly, optogenetic or chemogenetic stimulation of MCH neurons may not involve MCH peptide signaling, as these methods serve only to activate the neurons, and it is unclear whether this type of activation evokes MCH peptide release. There is one study showing an electrophysiological postsynaptic effect of optogenetically evoked MCH release which is blunted by an MCHR1 antagonist (J.-J. Liu et al., 2022), but there are notable methodological differences (mice versus rats, changes in optogenetic stimulation parameters) between this work and the aforementioned study. MCH neurons are known to signal through both MCH peptidergic signaling and fast neurotransmission via GABA (M. J. Chee et al., 2019) and glutamate (Schneeberger et al., 2018). Therefore, these findings do not necessarily contradict earlier pharmacological work showing that MCH peptide infusion leads to increased food intake.

Irrespective of its involvement with food intake, there is significant evidence that MCH has a role in reward processing related to consumptive behaviors. Multiple research groups have shown a connection between MCH and drug intake. Administration of MCH peptide has been shown to increase alcohol consumption in rats (Duncan et al., 2006), and MCHR1 antagonism decreases alcohol intake in rats (Cippitelli et al., 2010; Karlsson, Aziz, et al., 2016). Interactions between alcohol and MCH expression are inconclusive, with two groups showing that MCH expression in hypothalamus increases with acute ethanol consumption (Morganstern et al., 2010; Pickering et al., 2007), one of which also showed that chronic ethanol intake decreases MCH mRNA expression in LH (Morganstern et al., 2010). Further, during protracted abstinence following intermittent access to alcohol, decreased expression of MCH mRNA was observed simultaneously with increased expression of MCHR1 mRNA (Karlsson, Aziz, et al., 2016). Another group showed that injection of NMDA into the LH increases animals' ethanol intake,

and that this is accompanied by changes in *Pmch* gene expression in the zona incerta (Chen et al., 2014).

There has also been work linking MCH to drug-related stimuli, specifically pertaining to drug predictive cues. MCH injection into the nucleus accumbens (NAc) inhibits reinstatement of a morphine conditioned place preference (CPP) but not a food CPP (D. Wang et al., 2020), suggesting that MCH has differential and dissociable effects on responding to reward cues but not food cues. A similar experiment showed no difference between MCH-KO and WT mice in development of an amphetamine CPP (Tyhon et al., 2008), and another showed that MCH deletion and pharmacological blockade can decrease an alcohol-induced CPP (Karlsson, Rehman, et al., 2016), so there may be drug-specific differences. This finding complements the aforementioned work showing that MCH-KO mice have an impaired overeating response to a food-related cue (Sherwood et al., 2015), yet an unimpaired overeating response to starvation (Shimada et al., 1998), and suggests that the role of MCH in food consumption may be related to food cues, rather than to food. In fact, there have been multiple findings linking MCH to cue-induced reinstatement. MCHR1 antagonism suppresses cue-induced reinstatement of alcohol-seeking (Cippitelli et al., 2010) and cocaine-seeking (Chung, Hopf, et al., 2009) behaviors, but does not block footshock-induced reinstatement of alcohol-seeking (Cippitelli et al., 2010). The finding that a footshock can still lead to alcohol relapse/reinstatement in the face of MCH loss-of-function points to a role for MCH specifically in rewarding, rather than aversive behaviors. Further supporting this idea is the finding that MCHR1-KO mice fail to discriminate between cocaine-paired and unpaired sides of a chamber, while WT mice do so with no issue (Chung, Hopf, et al., 2009).

There is significant work to suggest that MCH may increase or sensitize the effects of abusive drugs themselves. When measuring orofacial reactions to a palatable sucrose tastant, both MCH injection and opioid injection into the NAc or lateral ventricle increase the positive hedonic “liking” reaction to a palatable taste (Lopez et al., 2011). When MCH is co-injected with an opioid antagonist, the increase in positive hedonic liking reactions is not observed, suggesting that effects of MCH may be opioid-dependent (Lopez et al., 2011). Furthermore, when MCH is co-administered with cocaine, the drug-induced increase in locomotor activity observed is greater than that induced by cocaine alone (Chung, Hopf, et al., 2009). As MCH alone does not increase locomotor activity, this suggests that MCH has the ability to sensitize the locomotor

effect of cocaine (Chung, Hopf, et al., 2009). This effect has been replicated across multiple drugs of abuse; MCHR1 antagonism reduces alcohol self-administration (Cippitelli et al., 2010) and decreases lever pressing for alcohol during protracted abstinence (Karlsson, Aziz, et al., 2016), while MCH injection increases alcohol self-administration, whether or not water is also available (Duncan et al., 2006). Interestingly, one group has also found that both optogenetic and chemogenetic MCH neuron activation during sleep, as well as intra-NAc MCH peptide infusion during a withdrawal period, can decrease propensity toward cocaine relapse after withdrawal (Guo et al., 2022) - a finding which is possibly contradictory to the others mentioned. This contradiction may be explained by differences in timing of the MCH manipulation, as this study was focused on interventions during sleep.

The aforementioned rewarding effects of MCH on drug consumption are independent of effects on food consumption, though they apply to systems involved with food consumption as well. While MCH injection alone increases feeding, MCH co-injected with a kappa opioid receptor antagonist decreases feeding below control levels, and MCH co-injected with a mu opioid receptor antagonist increases feeding above control levels (Lopez et al., 2011). MCH also activates the kappa opioid receptor (Romero-Picó et al., 2018), and injection into kappa opioid receptor-deficient mice fails to induce feeding (Romero-Picó et al., 2018). Geuzaine and colleagues observed that WT mice spend more time in an amphetamine-paired chamber when food restricted than when free-fed, but that MCHR1-KO eliminates the potentiating effect of food restriction on amphetamine place preference, with MCHR1-KO mice spending comparable amounts of time in the amphetamine-paired chamber regardless of their hunger state (Geuzaine et al., 2014).

Much of the work focused on the role of the MCH system in reward consumption is focused on interactions between MCH and mesolimbic dopamine circuits. LH MCH neurons have dense projections to the NAc (J. C. Bittencourt et al., 1992; Hervieu et al., 2000; Saito et al., 2001), which contains medium spiny neurons (MSNs) that receive dopaminergic input from the ventral tegmental area (VTA). MCH neurons also project directly to the VTA (J. C. Bittencourt et al., 1992; Hervieu et al., 2000; Saito et al., 2001). The mesolimbic dopamine pathway is involved in reward behaviors, with classical roles in responses to drugs of abuse (Koob & Bloom, 1988; Wise & Bozarth, 1985) and to food reinforcers (Hoebel et al., 1989; Kelley & Berridge, 2002). A number of research groups have investigated the molecular

mechanism of MCH signaling in NAc. MCHR1 is abundantly expressed in NAc medium spiny neurons (MSNs) (Georgescu et al., 2005). Further, MCHR1 colocalizes with both D1 and D2 dopamine receptors, and MCH peptide interacts with both D1R and D2R to increase spike frequency in NAc shell neurons (Chung, Hopf, et al., 2009). MCH delivery into NAc shell (NAcSh) increases food intake (Georgescu et al., 2005), as does AMPAR antagonist (Cs & Ae, 1995; Stratford et al., 1998) or GABAR agonist delivery (Reynolds & Berridge, 2001; Stratford & Kelley, 1997, 1999) into NAcSh. This suggests that a broad decrease in NAcSh activity may be a primary driver of feeding, and this mechanism may explain how MCH acts on the NAc to promote feeding. Indeed, MCHR1 has been shown to couple with Gi/Go to reduce phosphorylation of GluR1 in the NAc shell (Sears et al., 2010). It has also been observed that MCHR1 is expressed in GABAergic neurons within the NAc, and that deletion of MCHR1 from those GABAergic NAc neurons leads to increased locomotion (M. J. Chee et al., 2019).

MCH administration locally into NAc has reward effects and feeding effects consistent with central administration of MCH, suggesting that the NAc may be the primary driver of such effects. Acute administration of MCH to the NAcSh in adult *Pmch*-KO rats, which otherwise have decreased levels of feeding, can elevate feeding behavior to WT levels (Mul et al., 2011). Intra-NAcSh injection of MCH peptide attenuates the development (but not expression) of methamphetamine-induced sensitization, and intra-NAcSh injection of MCH antagonist increases the development (but not expression) of methamphetamine-induced sensitization (L.-L. Sun et al., 2013). MCH interacts with opioids in the NAc to influence food intake and hedonic reactions to food palatability (Lopez et al., 2011). MCH coadministration with a subthreshold dose of dopamine agonist apomorphine leads to increased firing of NAcSh neurons (Hopf et al., 2013), an effect which is blocked by dopamine receptor antagonists, MCHR1 antagonists, and PKA antagonists alike (Hopf et al., 2013).

There is work to suggest that MCH effects on reinforcement of consumption may be dependent on dopamine itself. Domingos and colleagues showed that, while mice typically prefer caloric, nutritive sucrose over noncaloric sucralose, pairing MCH optogenetic stimulation with sucralose consumption is sufficient to invert their preference to sucralose instead (Domingos et al., 2013). They built upon this work showing that MCH optogenetic stimulation during sucralose consumption increases dopamine release in response to sucralose, which normally does not invoke dopamine release (Domingos et al., 2013). And while dopamine normally is released

during consumption of the nutritive, caloric sucrose, after ablation of MCH neurons with diphtheria toxin, sucrose consumption no longer induces dopamine release (Domingos et al., 2013). They show that this is not an effect of either solution's sweet taste, as sweet-blind *Trpm5*^{-/-} mice still prefer sucrose over sucralose, and this preference is abolished with MCH neuron ablation (Domingos et al., 2013).

Much work has been done to understand overlapping circuitry of MCH and dopamine. Early work showed that CART increases the concentration of DOPAC, a dopamine metabolite, and that MCH administration blocks the CART-induced increase in DOPAC (Yang & Shieh, 2005). MCHR1 deletion attenuates DARPP-32 activation as a response to alcohol consumption, which implies involvement of dopaminergic pathways in this effect (Karlsson, Rehman, et al., 2016). More recently it has been shown that MCHR1 is robustly expressed on multiple VTA cell types, including dopaminergic, GABAergic, and glutamatergic cells, and that MCH directly inhibits dopaminergic cells (Duncan Spencer et al., 2024). The idea that MCH inhibits dopaminergic signaling is somewhat supported by MCH-KO studies. It has been observed that MCH-KO mice are super-sensitive to a D1R agonist, showing significantly more evoked ambulation than WT counterparts (Smith et al., 2005). These same MCH-KO mice are also hyperambulatory compared to WTs without pharmacological intervention (Smith et al., 2005), an effect which is likely mediated by dopamine. MCH-KO mice show increased electrically-evoked DA release (Mul et al., 2011; Pissios et al., 2008), and MCHR1-KO mice similarly show increased mesolimbic dopaminergic tone and locomotor activity (M. J. Chee et al., 2019).

MCH also has effects on the dopamine transporter (DAT). Elevated expression of DAT is observed in the NAc of MCH-KO mice, along with consistent enhancement of DAT mediated reuptake in MCH-KO mice (Pissios et al., 2008). Inhibition of DA reuptake led to significantly more enhancement of locomotor activity in MCH-KO mice than WT mice (Pissios et al., 2008).

Effects of dopamine on MCH cells are also documented, with dopamine inducing an outward current in MCH neurons (Alberto et al., 2011; Conductier et al., 2011). This evoked outward current is not mediated by DA receptors, but is instead mediated through α_2A adrenergic receptors and subsequent GIRK channel opening (Alberto et al., 2011; Conductier et al., 2011). Dopamine should, then, lead to a decrease in MCH neuron excitability (Conductier et al., 2011), likely by acting as a partial agonist of α_2A adrenergic receptors on MCH neurons (Alberto et al., 2011). Another group has shown that NAc MSNs do not directly innervate MCH-

containing neurons, but that they may indirectly signal MCH-containing neurons via glutamatergic neurons in the anterior LH (Sano & Yokoi, 2007).

1.4.3 Role of MCH in Sleep

While the major focus of the present thesis has to do with the role of the MCH system in feeding and consummatory behaviors, the next most well-characterized role for the MCH system is in sleep/wake regulation. MCH is thought of as having a role in promoting and maintaining REM sleep, the phase of sleep associated with near-complete atonia of voluntary muscles. Major features of REM sleep include muscle atonia, dreaming, and hippocampal memory consolidation (Hutchison & Rathore, 2015).

The exact mechanism by which MCH promotes or maintains REM sleep remains unclear. Early work showed that sleep deprivation, whether specific to REM sleep or to all sleep states, resulted in increased levels of Fos expression in MCH neurons of both rats and mice (Modirrousta et al., 2005; Verret et al., 2003). There is a large body of work showing that MCH neurons are maximally active during REM sleep with less activity in NREM or Wake, measured using various techniques including in-vivo electrophysiology (Hassani et al., 2009) and fiber photometry (Blanco-Centurion et al., 2019; Izawa et al., 2019; Y. Sun & Liu, 2020). It is unknown whether the observed sleep effects are due to MCH peptide signaling rather than other neurotransmitter release from MCH neurons, but two groups start to implicate MCH peptide. Naganuma and colleagues saw that chemogenetic stimulation of MCH neurons leads to increased amounts of REM sleep even in transgenic mice which lack glutamatergic signaling (Naganuma et al., 2019). Another group used microdialysis in human subjects to reveal that MCH peptide release is higher during sleep than most waking experiences (Blouin & Siegel, 2013), although this group did not differentiate between specific phases of sleep.

MCH gain-of-function experiments show that MCH stimulation leads to increased time spent in REM sleep and increases the number of NREM-to-REM sleep transitions, both through optogenetic stimulation of MCH (Blanco-Centurion et al., 2016; Jego et al., 2013; Konadhode et al., 2013; Tsunematsu et al., 2014) and chemogenetic stimulation (Vetrivelan et al., 2016). However, some of the same research groups have shown that MCH neuron stimulation during NREM specifically does not prolong a NREM sleep episode (Jego et al., 2013), and that a more continuous stimulation of MCH during sleep has inconclusive effects on NREM sleep propensity. Some have observed that continuous MCH stimulation leads to a longer duration of

NREM sleep (Blanco-Centurion et al., 2016; Konadhode et al., 2013), while others have shown that continuous MCH stimulation leads to a decrease in NREM sleep in favor of REM sleep (Varin et al., 2016).

MCH loss-of-function studies have had conflicting results. Two studies show no effect on sleep architecture after continuous MCH optogenetic inhibition (Jego et al., 2013; Tsunematsu et al., 2014). Another group used changes in ambient room temperature in order to modulate REM sleep propensity – higher ambient temperature is typically associated with increased amounts of REM sleep (Komagata et al., 2019). This group observed that, when higher ambient temperature was combined with MCH neuron activation, animals showed an increase in REM sleep greater than that induced by increased temperature alone. They also observed that when higher ambient temperature was combined with MCH neuron inactivation, the expected increase in REM sleep was abolished (Komagata et al., 2019). As a result of projection-specific optogenetic inhibition of MCH terminals in the ventro-lateral periaqueductal gray (vlPAG), REM sleep was reduced (Kroeger et al., 2019). When this vlPAG specific MCH optogenetic inhibition was combined with chemogenetic activation of MCH, it blocked the expected increase in REM sleep (Kroeger et al., 2019). Pharmacologic loss of function seems to have a straightforward effect, with subcutaneous injection of an MCHR1 antagonist decreasing total sleep time in both REM and NREM, and increasing latency to enter sleep (Ahnaou et al., 2008). However, when delivered orally, an MCHR1 antagonist did not affect sleep/wake architecture (Able et al., 2009). Transgenic mice lacking MCHR1 paradoxically demonstrated an increase in REM sleep (Adamantidis et al., 2008), although this may be a result of changes to other MCH mediated behaviors rather than a direct effect of MCHR1 deficiency on sleep/wake architecture.

Effects of MCH on sleep may confound effects of MCH on feeding, reward, and other behaviors. Without understanding whether there is overlap between the circuitry involved in MCH regulation of feeding and MCH regulation of sleep, it is difficult to entirely understand either of these behavioral roles for MCH. Further, studies which focus on sleep effects without addressing feeding effects, or those which focus on feeding effects without addressing sleep effects, fail to fully characterize the behavioral effects in question. Very little work has addressed the interplay between MCH-mediated sleep and feeding, ostensibly mutually exclusive behaviors, and therefore both remain incompletely understood.

1.4.4 Other functions of MCH

There are a number of different known functions of MCH in behavior. While the most well-characterized of these functions include promoting food intake, promoting REM sleep, and contributing to reward learning (as characterized above), there are studies to suggest other behavioral roles of MCH. Some of the less-understood roles of MCH are locomotion, anxiety, reproductive function, learning and memory, among others.

Locomotion/Energy Balance

Interactions within the MCH system between feeding, energy balance, and locomotion have been reported. MCH neurons are known to project to areas like the pedunculopontine tegmental nucleus and motor cortex, which are involved in somatomotor integration and response (Elias et al., 2008), making these neurons well-positioned to have a role in locomotion. There is also evidence that physical exercise directly affects MCH expression, as mice which underwent chronic restraint stress produced an increase in *Pmch* gene expression in the LH, and this increase in MCH expression was abolished with access to running wheel exercise (Kim & Han, 2016), though this finding may be more related to the stress and anxiety induced by chronic restraint than to the lack of physical exercise.

Somewhat surprisingly, loss of MCH functioning in either mice with *Mchr1* KO or mice with DTR-mediated MCH neuron ablation leads to mice eating more food than WT, while remaining lean with comparatively less fat mass due to increased hyperactivity and metabolic alterations (Marsh et al., 2002; Whiddon & Palmiter, 2013). *Mchr1* KO mice were hyperactive compared to WT counterparts on two strain backgrounds (C57BL/6J or 129/SvEv) (Lalonde & Qian, 2007). The question of whether this hyperactivity is a standalone effect of MCH neuron activity or if it is a broader metabolic effect related to food intake remains unknown. DTR-mediated MCH neuron ablation leads to different effects on glucose tolerance than does MCH peptide deficiency, with MCH neuron ablation leading to improved glucose tolerance compared to MCH deficient or WT mice (Whiddon & Palmiter, 2013), so effects of MCH on locomotion may be explained, at least in part, by differences in metabolism.

The effects of MCH on locomotion may be mediated by GABA, as evidenced by the finding that deleting *Mchr1* from GABAergic neurons leads to decreased body weight and increased locomotion (M. J. Chee et al., 2019). When this *Mchr1* deletion is specific to GABAergic neurons in the NAc, increased locomotion is observed without changes to body

weight (M. J. Chee et al., 2019). While this finding did not quantify which type of locomotion was observed to have increased, another research group observes MCH effects specifically on exploratory rearing in mice. When measuring MCH calcium activity with fiber photometry during a multitude of different behaviors, MCH activity increased most significantly during exploratory rearing, with no change observed in MCH activity during forward locomotion or turning (Concetti et al., 2024). Following MCHR1 antagonism with SNAP-94847 hydrochloride, mice spent less time rearing, and following optogenetic stimulation of MCH neurons mice spent an increased amount of time rearing, with neither intervention leading to a change in the other behaviors measured (Concetti et al., 2024).

Anxiety/Depressive-like Behaviors

Numerous research groups have investigated the effects of MCH on anxiety and depressive-like phenotypes in rodents, yet results are contradictory. Using a variety of rodent behavioral assays, different research groups have found both anxiolytic and anxiogenic effects of MCH action. Intracerebroventricular infusion of MCH peptide into brain areas involved with mood regulation has largely shown an anxiogenic effect in behavioral assays (Georgescu et al., 2005; Lagos et al., 2011; Urbanavicius et al., 2019, 2016, 2014; Ye et al., 2018), but there are a few studies showing anxiolysis and antidepressive effects (Monzón & De Barioglio, 1999; Oh et al., 2020). Either systemic or intracerebroventricular injections of MCHR1 antagonists are anxiolytic in a wide variety of behavioral assays (Borowsky et al., 2002; Chaki et al., 2005; David et al., 2007; Gehlert et al., 2009; Georgescu et al., 2005; Lee et al., 2011; Millan et al., 2008; Ploj et al., 2016; Smith et al., 2006, 2009; Ye et al., 2018), supporting the idea that MCH itself is anxiogenic. In searching for a mechanism of this relationship between MCH and anxiety/depression, many reports have shown that exogenous MCH peptide administration can stimulate release of stress hormones such as adrenocorticotrophic hormone and corticosterone in mammals (Jezová et al., 1992; Kennedy et al., 2003; Smith et al., 2006, 2009).

Social/Parental Behaviors

Effects of MCH on social and parental behaviors are vast and sex-specific. Sex hormones interact heavily with MCH activity; the orexigenic effect of MCH is lessened in the presence of estrogen, and is most strongly observed in female rodents in low-estrogen stages of the estrus cycle compared to high-estrogen stages (Jessica Santollo & Eckel, 2008). Increased estradiol

leads to decreased MCH expression in LH, despite the finding that MCH neurons do not co-express estrogen receptor 1 (ER1) in either male or female rats (J. Santollo & Eckel, 2013).

A unique population of neurons was discovered in the female mouse which transiently express MCH only during lactation in the medial preoptic area (mPOA), periventricular area (PeA) and periventricular nucleus (PVN) (Knollema et al., 1992), areas known to regulate parental behavior (Diniz & Bittencourt, 2017). This transient MCH expression correlates temporally with the progress of the lactation period in females, and corresponding areas in male mice do not express MCH (Knollema et al., 1992). Indeed, MCH neuron count in the mPOA in postpartum female mice correlates directly with the number of pups in the litter, and can be decreased by removing pups from the litter postpartum (Ferreira et al., 2017). This correlation seems to suggest that transient MCH expression is required for the energetic demands of maternal care, as less of it is required for a smaller litter size. Multiple maternal behaviors (pup retrieval, mouthing, nest building) are impaired by MCH injection directly into the mPOA of early postpartum female rats (Benedetto et al., 2014, 2013; Knollema et al., 1992; Rondini et al., 2010).

Social behaviors in rodents are heavily dependent on olfaction, a sensory input which is critical to social interaction, predator behaviors, and parental behaviors (Lévy et al., 2004; Schultz & Tapp, 1973). The MCH system seems to interact with olfaction, and therefore could play a role in social behavioral aptitude in rodents. MCH KO mice are significantly less likely to locate buried food than WT counterparts (Adams et al., 2011). MCH impairment via either localized conditional MCH KO or full genetic KO leads to impaired parental behavior, including longer pup retrieval times and failure to distinguish a chamber containing pups from one without (Alhassen et al., 2019). However, only a full genetic KO of MCH leads to a failure to recognize novel non-pup-related smells, such as lemon or banana (Alhassen et al., 2019). Impaired olfactory sensation following MCH loss of function likely has numerous downstream effects on sociability, predator awareness, and other social behaviors in addition to effects on parental care.

1.5 Dissertation Preview

It is short-sighted for researchers to treat MCH neurons as a homogeneous population, when multiple subpopulations have been identified based on neurochemical, anatomical, and electrophysiological differences. The work presented in this dissertation aims to justify this, by

showing that individual subpopulations of MCH neurons with different behavioral roles can be distinguished on the basis of their anatomical projection target. We focus on the MCH projection to the nucleus accumbens (NAc), and develop a behavioral paradigm to test the role of this projection in feeding behavior, and to differentiate that role from the role of the broad population of MCH neurons.

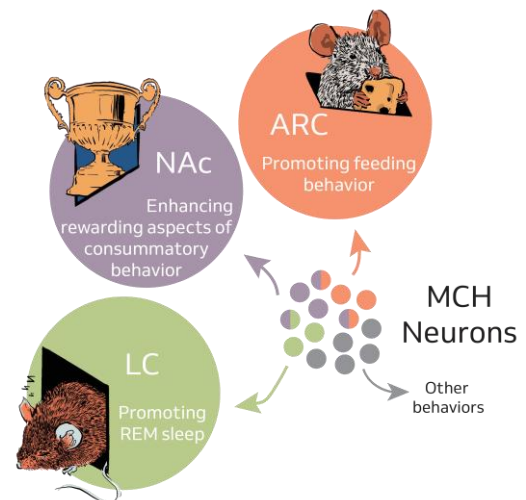


Figure 1.2 MCH projection hypothesis.

1.5.1 Hypothesis

We believe that the complex and contradictory roles of the MCH system in various aspects of mammalian behavior can be explained by heterogeneity of MCH neurons, leading to different subpopulations of MCH neurons which play distinct roles in behavior (See Figure 1.2). Specifically, we hypothesize that MCH projections to the NAc will play a role in non-homeostatic feeding, by affecting the attribution of reward value to food rather than influencing the homeostatic drive to consume, and that MCH projections to the NAc will have no significant role in REM sleep. Diverging roles for different anatomical populations of MCH neurons may explain the vast set of behaviors which implicate MCH in their neural circuitry.

We hypothesize that individual subpopulations of MCH neurons can be defined by their anatomical projection target, and that this may explain why the MCH system influences such a broad array of behaviors.

1.5.2 Objectives

The broad objective of the present thesis is to distinguish the role of the NAc-terminating MCH neuron population from the role of MCH neurons as a whole. We do this by combining high-throughput behavioral assays with optogenetic terminal stimulation, giving us the capability to activate only those neurons which project to a particular downstream area and observe how this affects behavior.

In Chapter 2, we focus on food intake and sleep/wake architecture. We modified the mouse homecage in order to continuously monitor their behavior. This modification includes addition of a Feeding Experimentation Device 3 (FED3) food hopper which automatically tracks

the mouse's food intake, as well as surgical implantation of both an optic fiber at the terminal area of interest, as well as an EEG/EMG headcap enabling us to measure the mouse's sleep/wake state. Within this modified homecage, we optogenetically stimulate either MCH neuron cell bodies in LH, or MCH neuron terminals in NAc, and allow mice to otherwise behave normally. We found that activation of MCH cell bodies in LH, but not of MCH terminals in NAc, resulted in increased REM sleep, and that neither activation affected food intake levels. This led us to conclude that the role of MCH neurons can differ based on the downstream projection target, as well as that the MCH → NAc projection does not play a role in regulation of sleep/wake architecture.

In Chapter 3, we developed a new behavioral paradigm with the goal of understanding more nuanced aspects of feeding, and interrogating the involvement of MCH → NAc neurons in the rewarding aspects of food intake. In our Optogenetics-Reinforced Consumption Assay (ORCA), the mouse homecage is modified to include two FED3 devices and a total of four nose poke ports. The four ports are programmed to trigger different outcomes; either food pellet delivery, optogenetic stimulation, both, or neither. Between each of these four ports, mice may choose among all-pairwise combinations of food and optogenetic stimulation. From the ORCA paradigm, we observed that mice receiving MCH terminal stimulation in NAc significantly preferred the port delivering both food and optogenetic stimulation, while mice receiving MCH cell body stimulation in LH preferred the two ports delivering a food pellet, with no difference in preference based on optogenetic stimulation. This led us to conclude that the MCH → NAc projection is involved in attributing reward value to paired food, rather than promoting increased food consumption.

In Chapter 4, we aimed to further modify the ORCA paradigm into a more acute version, in order to increase throughput and allow a wider variety of experimental interventions. We outline the process of doing so, including preliminary results which informed our decision-making process as we made more modifications to the paradigm. After successfully developing the acute ORCA paradigm, we used it in a pilot experiment with the goal of both reproducing our original finding in Chapter 3, as well as investigating an additional MCH projection to the locus coeruleus (LC). We observed that, when receiving MCH terminal stimulation in the LC, mice seem to find this stimulation reinforcing, as they showed a preference for the two ports

delivering optogenetic stimulation rather than for those delivering a food pellet. This was unexpected, and must be replicated with a bigger sample size.

The present thesis characterizes the various behavioral roles of the MCH system as a function of its anatomy, by focusing on the individual projection targets of MCH neurons which may be responsible for different behaviors. In characterizing the system this way, we aim to contribute to the broader field by helping separate the mechanisms by which MCH activity promotes sleep from those by which it promotes feeding. This will ultimately lead to better therapeutics for eating disorders, obesity, and sleep disorders.

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Chapter 2 Melanin Concentrating Hormone Projections to the Nucleus Accumbens Do Not Induce Feeding or REM Sleep

Portions of this chapter have been published:

Furman et al 2025 Journal of Neuroscience 5 March 2025, 45 (10)

DOI: 10.1523/JNEUROSCI.1725-24.2024

2.1 Abstract ¹

Regulation of food intake and energy balance is critical to survival. Hunger develops as a response to energy deficit and drives food-seeking and consumption. However, motivations to eat are varied in nature, and promoted by factors other than energy deficit. When dysregulated, non-homeostatic drives to consume can contribute to disorders of food intake, adding to the increasing prevalence of restrictive eating disorders and obesity. Melanin-concentrating hormone (MCH) neurons have been implicated in the regulation of feeding behavior, in addition to a number of other fundamental behaviors including sleep, anxiety, and maternal behavior. Several studies suggest that MCH peptide increases food consumption, while studies of MCH neurons show effects only on cued feeding, and others show no effect of MCH neuron manipulation on feeding. MCH neurons have widespread projections to diverse downstream brain regions, yet few studies have investigated the function of specific projections or differentiated the behaviors they regulate. Here we use optogenetics, in combination with high-throughput behavioral analysis, to elucidate the role of MCH projections to the nucleus accumbens (NAc) in sleep and feeding behavior. We show that activation of MCH cell bodies in LH leads to increased REM sleep, while activation of MCH neurons projecting to the NAc does not induce changes in REM sleep. Neither population induced a significant change in baseline feeding. These results suggest

¹ Electrophysiological experiments shown in Figure 2.1 were carried out by Dr. Limei Zhu in the laboratory of Dr. Joanna Mattis. Rabies tracing experiments shown in Figure 2.2 were carried out by Jayeeta Manna and Lorelei Baron in the Burgess Lab. Behavioral experiments shown in subsequent figures were designed and executed by Katherine Furman and Christian Burgess, with assistance from Jack Evans and Hannah Lyons.

that the role of MCH neurons diverges based on anatomical target, and that not all MCH neurons play a role in feeding or in sleep/wake architecture.

2.2 Background

Consuming different types of food in appropriate amounts and at appropriate times is crucial to an organism's survival. Several different neuronal populations are responsible for integrating sensory signals, interoceptive cues, and other information about an animal's environment to govern the choices and behaviors necessary to maintain adequate energy balance and nutrition. One such population is Melanin concentrating hormone (MCH) neurons, which have a known role in feeding and energy balance. Knockout of *Pmch* from neurons in the lateral hypothalamus (LH) and zona incerta (ZI) results in mice that are hypophagic and lean, while overexpression or injection of MCH peptide into the brain induces feeding and can induce weight gain (Georgescu et al., 2005; Guesdon et al., 2009; Ludwig et al., 2001; Rossi et al., 1997; Shimada et al., 1998). There is work to indicate that MCH neuron activity reflects nutritive value of different types of food (Domingos et al., 2013), as well as work to indicate that MCH neuron activity is rewarding, and may not simply promote homeostatically regulated food intake (Dilsiz et al., 2019; Sherwood et al., 2015).

MCH neurons project widely throughout the brain, terminating in brain areas with roles in a wide range of behaviors (V. Cvetkovic et al., 2003; Vesna Cvetkovic et al., 2004; Diniz & Bittencourt, 2017; Potter & Burgess, 2022), including to the nucleus accumbens (NAc). The NAc is classically involved with reward processing, and with attributing reward value to learned or conditioned stimuli. This well-characterized role for the NAc, and the density of MCH projections to this region, suggests that MCH projections to NAc could play a role in consummatory behavior. Indeed, one research group has shown that chemogenetic activation of MCH neurons in the NAc increases feeding behavior in a sexually-dimorphic manner (Terrill et al., 2020).

In addition to their role in feeding, MCH neurons have established roles in reward processing, learning & memory, reproduction, maternal behavior, and consummatory behaviors more broadly (B. G. Beekly et al., 2023; Blanco-Centurion et al., 2019; Chaki et al., 2005; Clegg et al., 2003; Concetti et al., 2024; Duncan et al., 2005; Potter & Burgess, 2022; Sherwood et al., 2012; Vetrivelan et al., 2016). Of these non-feeding behaviors, however, the role of the MCH

system in REM sleep is the most well-characterized. Mice spend more time in rapid eye movement (REM) sleep and have more transitions into REM sleep when receiving optogenetic stimulation of MCH neurons (Blanco-Centurion et al., 2019; Vetrivelan et al., 2016). Activation of MCH terminals in specific downstream areas including medial septum and dorsal raphe can also promote sleep (Jego et al., 2013). When MCH neurons are ablated, mice show a decrease in REM sleep time during the light cycle, the phase when they are typically asleep (Adamantidis et al., 2008; Varin et al., 2016).

As most investigations into the MCH system have focused only on one behavioral readout at a time and treated the population as homogeneous, it is unclear exactly how the MCH system regulates feeding and whether this is independent of effects on other behaviors, like sleep. In this chapter, we use optogenetic activation of both MCH neuron cell bodies and their terminals in the NAc to demonstrate that MCH stimulation does not enhance food intake either via cell bodies in the LH or via MCH terminals in the NAc. Furthermore, stimulation of MCH terminals in the NAc does not elicit REM sleep, while stimulation of MCH neurons in the LH does. These data provide insights into the role of the MCH system in consummatory behavior and demonstrate the utility of investigating specific subpopulations and/or efferent projections of MCH neurons to more comprehensively elucidate their function across disparate behaviors.

2.3 Methods

2.3.1 Mice

All mice were housed in a University of Michigan vivarium in a temperature-controlled environment (12 h light and 12 h dark cycle; lights on at 2 AM) with *ad libitum* access to food and water. All animal protocols were approved by the University of Michigan's Institutional Animal Care and Use Committee and are in accordance with NIH guidelines for the use and care of laboratory mice. For behavioral experiments, Pmch-ChR2-eYFP mice (n = 60) were made by crossing Ai32 mice (Strain #: 024109) to Pmch-Cre (RRID: IMSR_JAX:014099). Control mice were Cre-positive, ChR2-negative littermates. For rabies tracing experiments, Pmch-iCre positive mice (n = 5) were used (RRID: IMSR_JAX:014099, (Bethany G. Beekly et al., 2020)). Both male and female mice were used in all experiments. Overall health of experimental mice

was monitored daily and any mice that displayed obvious signs of distress or weakness were removed from the study.

2.3.2 Surgery

Mice were deeply anesthetized by inhalation of 2% isoflurane and placed on a Kopf stereotaxic apparatus (Tujunga, CA). Following standard disinfection procedure, the skin was retracted to expose the skull, and a small hole was drilled into the skull unilaterally at defined positions to target NAc or LH. An optic fiber (400- μ m diameter core; NA 0.5; RWD) was implanted unilaterally over NAc (A/P: 1.0 mm, M/L: -0.8 mm, D/V: -4.2 mm) or LH (A/P: -1.4 mm, M/L: -1.0 mm, D/V: -4.7 mm). Fibers were fixed to the skull using dental acrylic. A subset of mice was also implanted with two stainless-steel screws (Frontal: A/P: 1.5 mm, M/L: -1.5 mm; Temporal: A/P: -3.5 mm, M/L: -2.8 mm) and a pair of multi strand stainless steel wires inserted into the neck extensor muscles. EEG and EMG leads were wired to a small electrical connector attached to the skull with dental cement.

Following surgery, mice were kept on a warming pad until fully ambulatory and fed a regular chow diet throughout the experimental period unless otherwise noted. All mice were given analgesics (5 mg/kg Meloxicam) prior to the end of surgery and 24 hours after surgery. Mice were given a minimum of 2 weeks for recovery before being used in any experiments. After the completion of the experiments, mice were euthanized, and the locations of optic fiber tips were verified histologically.

For rabies tracing experiments, Pmch-iCre positive mice underwent an initial round of injections following anesthesia induction with 2-4% isoflurane. Mice were placed in a stereotaxic apparatus (KOPF Model 963). After exposing the skull via a small incision, a hole was drilled for injection. A pulled glass pipette (~20 μ m tip diameter, Friedrich & Dimmock, Inc.) was inserted into the brain, and AAV-hSyn-DIO-TVA(66T)-tdTomato was injected using a picospritzer. The pipette was kept in place for 5 minutes before withdrawal. Injections targeted the LH (A/P: -1.6 mm, M/L: -1.0 mm, D/V: -5.4 mm and -5.0 mm) and ZI (A/P: -1.6 mm, M/L: -1.0 mm, D/V: -4.6 mm and -4.3 mm). Postoperative care included intraperitoneal meloxicam (5 mg/kg) prior to the end of surgery and 24 h after surgery, as well as close monitoring for ten days to ensure recovery. Four to eight weeks later, 50-60 nL of rabies virus deltaG-N2c-Rabies-GFP

(1×10^8 vg/mL) was injected into the ipsilateral nucleus accumbens shell (NAcSh; A/P: +1 mm, M/L: -0.52 mm, D/V: -4.6 mm). Mice were sacrificed five to eight days post-rabies injection.

2.3.3 Experimental Design and Statistical Analysis

Feeding and sleep experiments

Behavioral experiments began no less than 10 days following surgery. Mice were outfitted with an EEG/EMG tether in their home cage. To give mice time to habituate to the attached EEG/EMG tether, no optogenetic stimulation was delivered during a habituation phase which lasted at least 4 days. Once mice were adequately habituated to the tether (ambulatory, eating and drinking normally) optogenetic stimulation was delivered (20Hz, 10ms pulse width, 1s-ON 4s-OFF) for 3 hours during either the light cycle or the dark cycle, with alternating days of stimulation or baseline recording. Polysomnographic signals were digitized at 1000 Hz, with a 0.3-100 Hz bandpass filter applied to the EEG and a 30-100 Hz bandpass filter applied to the EMG (ProAmp-8, CWE Inc.), with a National Instruments data acquisition card and collected using a custom MATLAB script. EEG/EMG signals were notch filtered at 60 Hz to account for electrical interference from the recording tether. Polysomnographic signals were analyzed using AccuSleep, an open-source sleep scoring algorithm in MATLAB (Barger et al., 2019), and verified by an experienced sleep-scorer blinded to the group/condition. Behavioral states were scored in 5s epochs as either Wake, non-rapid eye movement (NREM) sleep, or rapid eye movement (REM) sleep.

Animals' home cages were modified to include an automatic feeder (FED3; (Matikainen-Ankney et al., 2021)). The FED3 was programmed to release a new food pellet (BioServ dustless precision pellets, Rodent Purified Diet, 20mg) each time the previous pellet was taken by the mouse, such that food was always available. Feeding was quantified as the number of pellets consumed. Comparisons were made between days with and without optogenetic stimulation, as well as between control and ChR2 experimental groups. Sleep behavior was quantified by time spent in each arousal state, number of bouts of each arousal state, and lengths of bouts of each arousal state with a focus on REM sleep.

Feeding Architecture

In addition to raw numbers of pokes and pellets, animals' feeding architecture was quantified. This involved measuring inter-pellet intervals (IPIs) to understand the distribution of consumption over time. A histogram of IPI lengths was created to understand the distribution of IPI lengths for a given animal. This histogram was used to determine the appropriate interbout interval, in order to denote separate feeding "bouts." The majority of IPIs were less than 2 minutes, thus 2 minutes was determined as the interbout interval. Using these criteria, the number of feeding bouts and the number of food pellets per bout were calculated. The skewness of the IPI distribution was also calculated.

2.3.4 Acute slice preparation

Mice were deeply anesthetized using inhaled isoflurane. Brains were removed and then immediately placed in an ice-cold sucrose solution containing (in mM): 87 NaCl, 75 sucrose, 2.5 KCl, 1.0 CaCl₂, 2.0 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose, maintained with 95% O₂ and 5% CO₂. Using a Leica VT-1200S vibratome (Leica Microsystems Inc., Buffalo Grove, IL, USA), coronal slices of 300 μ m thickness were prepared. These slices were then transferred to a holding chamber filled with oxygenated artificial cerebrospinal fluid (ACSF) comprised of (in mM): 125 NaCl, 2.5 KCl, 2.0 CaCl₂, 1.0 MgSO₄, 26 NaHCO₃, 1.25 NaH₂PO₄, and 10 glucose. The slices were allowed to recover at 32°C for 30 minutes, followed by an additional 30 minutes at room temperature before proceeding to recordings.

2.3.5 Slice electrophysiology

Whole-cell patch-clamp recordings were conducted using a SliceScope Pro 6000 electrophysiology system (Scientifica). Slices were transferred to a recording chamber and continuously perfused with oxygenated ACSF at a flow rate of roughly 3 mL/min. All slice physiology experiments were carried out at 31°C. MCH neurons were visualized using a SliceScope microscope (Scientifica) and identified by their YFP expression. Borosilicate glass electrodes, created using a P-97 puller (Sutter Instruments) to achieve a tip resistance of 3-4 M Ω , were used for whole-cell voltage- and current-clamp recordings. Electrodes were filled with a K-Gluconate internal solution, composed of (in mM): 130 K-gluconate, 6.3 KCl, 0.5 EGTA, 1.0 MgCl₂, 10 HEPES, 4.0 Mg-ATP, and 0.3 Na-GTP. The pH was adjusted to 7.30 with KOH and

the osmolarity was calibrated to 285 mOsm with 30% sucrose. Electrodes were maneuvered using a PatchStar manipulator (Scientifica). Signals were sampled at 20 kHz, amplified using a MultiClamp 700B amplifier (Molecular Devices), filtered at 10 kHz, digitized with a DigiData 1550, and captured using pClamp10 software. Data were included in the final analysis only if the cell had a resting potential below -50 mV. Series resistance compensation (bridge balance) was applied throughout the current clamp experiments, with readjustments made as necessary. No correction for liquid junction potential was performed. Activation of ChR2 was achieved through widefield blue (470 nm) LED illumination (CoolLED pE-800; Scientifica).

2.3.6 Immunohistochemistry

Following the conclusion of all experiments, mice were anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by formalin. Once removed, brains were post-fixed in 10% formalin overnight then transferred to 20% PBS-sucrose for ~48 hours. Brains were then frozen and 30µm coronal sections were cut using a freezing microtome. Location of optic fibers was confirmed by histology, and mice in which the fiber did not hit the intended target area were excluded.

For MCH immunohistochemistry, coronal sections were washed with 1x PBS and then incubated in 10% NDS (Normal Donkey Serum) blocking solution for 1 hour at room temperature. Once blocked, sections were incubated in MCH primary antibody solution (Rabbit anti-MCH Polyclonal Antibody (Catalog #H-070-47, Phoenix Pharmaceuticals) (1:8000)) overnight at RT. Following primary antibody staining, sections were washed with PBS-Tween then incubated in secondary antibody solution (Donkey anti-Rabbit Alexa Fluor 647 Conjugated Antibody (Catalog #A-31573, Invitrogen) (1:1250)) for 1 hour at RT. Slices went through a final PBS wash and then mounted onto glass slides using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Images were then captured by a fluorescence microscope (Olympus IX83/Keyence BZ-X800) at 10x magnification.

To increase the brightness of endogenous eYFP fluorescence, we used GFP amplification. Sections were washed with PBS then incubated in 5% NDS blocking solution for 2 hours at RT. After blocking, sections were incubated in primary antibody solution (GFP Polyclonal Antibody, Alexa Fluor™ 488 (Catalog #A-21311, ThermoFisher) (1:1000)) for 48

hours at RT. After primary antibody incubation, sections were washed with PBS then mounted onto slides.

For rabies tracing experiments, brain slices in a 1-in-3 series were mounted on slides and cover-slipped. Images were taken using a BZ-X800 fluorescence microscope. Coronal fluorescent images were registered to the Allen Brain Atlas V3p1 using the Fiji plugin ABBA for accurate mapping. Once registered, images were transferred to Adobe Illustrator to align with the appropriate Allen Brain Atlas template, allowing simultaneous display of the entire brain slice and cell distribution. The fluorescent ABBA brain sections were used to mark all MCH neurons containing mCherry, GFP, or mCherry and GFP with a filled circle using the ellipse tool. The relative positions of these circles were mapped onto the Allen Brain Atlas template. Anything above the NS tract was considered ZI, anything at or below was considered LH (Watson et al., 2014). Neurons were marked only if the soma was clearly visible.

2.3.7 Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad), IBM SPSS Statistics, or Matlab software. Details of statistical tests employed can be found in the relevant figure legends. In all statistical tests, normal distribution and equal variance was established. The data presented met the assumptions of the statistical test employed. Exclusion criteria for experimental mice were (i) sickness or death during the testing period (ii) if histological validation of the injection site demonstrated an absence of reporter gene expression (iii) or if histological validation demonstrated a mistargeted fiber placement. These criteria were established before data collection. N numbers represent final numbers of healthy/validated mice.

2.4 Results

We sought to elucidate the function of MCH neurons and their projections to the NAc by generating an MCH-ChR2 transgenic mouse (Figure 2.1 A). To validate that MCH neurons were sensitive to our optogenetic stimulation parameters, ChR2-expressing MCH neurons were held in voltage clamp (-70 mV) and demonstrated photocurrent when exposed to 1 s pulses of blue light (470 nm, 14.5 mW/mm²; Figure 2.1 B). Current clamp experiments demonstrated sustained trains of action potentials evoked via blue light photostimulation (470 nm, 14.5 mW/mm², 20

Hz, 5 ms pulse width; Figure 2.1 C,D). Not all recorded neurons were able to follow continuous 20 Hz stimulation (Figure 2.1 D). These ex vivo slice electrophysiology recordings from MCH-ChR2 mice confirmed that MCH neurons were sensitive to light stimulation.

MCH neurons send dense projections to the NAc, in particular the medial NAc shell (Georgescu et al., 2005). We used rabies-based retrograde tracing to demonstrate the proportion and location of MCH neurons that send projections to the NAc (Figure 2.2 A). Retrogradely labeled NAc-projecting neurons were prevalent in the MCH field (Figure 2.2 B; n = 5 mice). Cell counts demonstrated that 21.4 $\pm$ 2.2% of all MCH neurons send projections to the NAc (Figure 2.2 B, inset). The majority of MCH neurons projecting to the NAc were located in the LH rather than the ZI (LH: 95.4 $\pm$ 1.1% of NAc-projecting neurons originated in the LH; Figure 2.2 C), with a higher proportion of LH neurons also projecting to NAc (LH: 24.2 $\pm$ 2.9% of LH neurons project to the NAc; ZI: 10.2 $\pm$ 2.5% of ZI neurons project to the NAc).

2.4.1 MCH projections to the NAc do not modulate REM sleep or baseline feeding

We next aimed to understand the role of MCH neurons and their projections to the NAc on homeostatic behaviors; feeding and sleeping. We recorded polysomnographic signals with EEG/EMG tethers and feeding behavior using a FED3 food hopper (Matikainen-Ankney et al., 2021) as mice freely behaved in their homecage (Figure 2.3 A). Mice had an optic fiber implanted in either the LH or the NAc, and received three hours of optogenetic stimulation (20Hz, 1s on, 4s off) during the light and dark cycle on alternating days. While mice expectedly consumed more food pellets during the dark cycle than the light cycle, the amount of food pellets consumed in each phase was similar regardless of whether they received optogenetic stimulation (Figure 2.3 B,C). We did observe an effect of MCH optogenetic stimulation on REM sleep in LH, but not in NAc (Figure 2.3 E,F). Representative hypnograms of an animal with the optic fiber in the LH are shown in Figure 2.3 D. Animals with optic fibers placed in the LH exhibited a significant increase in REM sleep bout length during the light cycle on days with optogenetic stimulation compared to days without (paired t-test, $t(16) = 2.589$; $p = 0.0198$; Figure 2.3E, top). These mice also showed a trend toward an increase in the number of REM bouts during the dark cycle (paired t-test, $t(16) = 1.567$; $p = 0.1366$; Figure 2.3 E, bottom). No effect of MCH stimulation on REM sleep was observed for mice with optic fibers placed in the NAc (Figure 2.3 F).

Optogenetic stimulation of MCH neurons, regardless of fiber location, did not affect meal architecture (Figure 2.4). When analyzing the number of feeding bouts (Figure 2.4, Left) and the number of pellets per feeding bout (Figure 2.4, Right), no difference was observed between sessions with or without optogenetic stimulation. There was also no effect of optogenetic stimulation on arousal states other than REM (NREM or Wake; Figure 2.5). Mice with optic fibers placed in the LH (Figure 2.5A) or in the NAc (Figure 2.5B) showed similar levels of NREM sleep and Wake whether or not they received MCH optogenetic stimulation, measured both as bout length (Figure 2.5, top) and number of bouts (Figure 2.5, bottom) of each state. Lastly, continuous MCH optogenetic stimulation did not produce any sex-specific effects on REM sleep or pellet consumption (Figure 2.6).

Control mice which did have an optic fiber surgically implanted but did not express ChR2 in MCH neurons showed no effect of optogenetic stimulation on feeding behavior or REM sleep (Figure 2.7). Regardless of fiber location there was no significant effect of optogenetic stimulation on the number of food pellets consumed (Figure 2.7B,C,D, top), the length of REM bouts (Figure 2.7B,C,D, middle), or the number of REM bouts (Figure 2.7B,C,D, bottom) in control mice.

2.5 Discussion

The earliest investigations into the role of MCH demonstrated its ability to increase feeding. Knockout of the *Pmch* gene leads to reduced food consumption and an anorexia-like phenotype while overexpression of MCH results in increased feeding and body weight gain (Ludwig et al., 2001; Shimada et al., 1998). Pharmacological evidence also points to a role for MCH peptide in food consumption; injection of MCH into the cerebral ventricles, and a number of other specific brain regions, results in increased food consumption (Georgescu et al., 2005; Guesdon et al., 2009; Rossi et al., 1997). However, more recent work using optogenetic and pharmacogenetic approaches do not have clear findings linking the MCH system to increased food consumption. Dilsiz and colleagues showed that stimulation of MCH neurons with either chemogenetic or optogenetic approaches does not induce increased feeding; indeed, optogenetic activation could slightly but significantly reduce feeding (Dilsiz et al., 2019). We similarly do not observe increased consumption when optogenetically stimulating MCH neurons in LH. While there has been interest in developing MCH receptor antagonists for treatment of obesity, it

is worth noting that none have reached the clinic and they seem to have modest effect sizes at best (Potter & Burgess, 2022). A key difference between the majority of the early work in this area and the more recent application of specific circuit mapping tools is that recent experiments involve activation of the MCH ‘system’ (i.e., the neurons that express MCH) vs MCH peptide release or MCH receptor activation per se. Some functions of the MCH system are a result of glutamatergic, and potentially GABAergic, release from these neurons and it is unclear the extent to which MCH peptide itself is released in response to in vivo pharmacogenetic or optogenetic activation (B. G. Beekly et al., 2023; J.-J. Liu et al., 2022). Furthermore, it is possible that glutamate, GABA, and/or other peptides released from MCH neurons may have differing or opposing effects to MCH peptide.

MCH peptide injections into the NAc increase food intake (Georgescu et al., 2005; Guesdon et al., 2009), while chemogenetic activation of the MCH neurons that project to the NAc can drive modest increases in feeding in male mice and no change in females (Terrill et al., 2020). Here, we do not see increased food intake with optogenetic stimulation of MCH terminals in the NAc. There are several potential explanations for this apparent discrepancy. Previous work has been performed predominantly in rats, and there is evidence of differences in rat vs mouse MCH systems, at least at the anatomical level (Croizier et al., 2010). Alternatively, optogenetic vs pharmacogenetic manipulation of MCH neurons may result in differential release and/or release patterns of glutamate vs MCH peptide, ultimately resulting in different postsynaptic effects. Those MCH neurons that project to the NAc likely also exhibit axon collaterals in several other brain regions given the widespread nature of MCH projections, whereas optogenetic stimulation of terminals is more likely to limit this activation to the NAc region (though there is evidence that in certain conditions optogenetic activation of terminals may result in antidromic stimulation of cell bodies (Jennings et al., 2013), and we cannot rule this out in the current study). It is also possible that our stimulation was not sufficiently strong to see the subtle feeding effects previously characterized. Our characterization of these mice showed clear light-evoked activation of MCH neurons in ex vivo slice experiments (Figure 2.1), but not all neurons were able to follow the 20Hz light train, suggesting that transmitter release in the NAc may not have been maximal. We do demonstrate notable behavioral effects, but it is possible that more sustained or higher frequency activation in vivo could have also driven a feeding effect.

We also demonstrate that stimulation of MCH terminals in the NAc does not increase food pellet consumption or increase REM sleep. This is despite MCH neuron activation in the LH increasing REM sleep as we show here and as others have previously demonstrated (Jego et al., 2013; Kroeger et al., 2019; Varin et al., 2018). Importantly, our finding that MCH terminals in NAc do not have a role in sleep/wake state is complementary to a long-standing body of literature implicating the NAc in arousal state. As early as 1992 it was observed that NAc neuron firing rates change with arousal state, with the highest firing rates during Wake and REM, and lowest during NREM (Callaway & Henriksen, 1992), a finding that has been replicated recently with more cutting-edge calcium imaging techniques (Luo et al., 2018). These changes in NAc neuron firing activity across sleep states are accompanied by changes in extracellular levels of relevant neurotransmitters across sleep/wake states (Léna et al., 2005). There may even be interactions between NAc involvement in arousal state and motivational state; one research group shows that motivational stimuli (including a toy, a conspecific of the opposite sex, and chocolate) are sufficient to both reduce the amount of time mice spend in NREM sleep and decrease cFos activity in NAc neurons (Oishi et al., 2017).

Relatively few studies of the MCH system have investigated more than one MCH-related behavior, or the role of efferent projections, despite there being evidence of subpopulations of MCH neurons, both at the level of anatomy and gene expression (B. G. Beekly et al., 2023; V. Cvetkovic et al., 2003; Vesna Cvetkovic et al., 2004). At least two subtypes of MCH neurons have been characterized based predominantly on co-expression of MCH and Cocaine and amphetamine regulated transcript (CART), with differing projection and electrophysiological profiles (Vesna Cvetkovic et al., 2004; Fujita et al., 2021), but it is unclear how they differentially impact physiology and behavior. Our data also point to distinct subpopulations of MCH neurons based on anatomical projection profile. Our demonstration that MCH → NAc terminal stimulation does not result in the same REM sleep effect as LH MCH cell body stimulation is suggestive of at least two populations of MCH neurons, one of which drives REM sleep while the other enhances the reward value of consumption. While our results help to understand how the MCH system can regulate many disparate behaviors, including food consumption, more work is needed to fully elucidate the structure-function relationship of the MCH system.

2.6 Figures

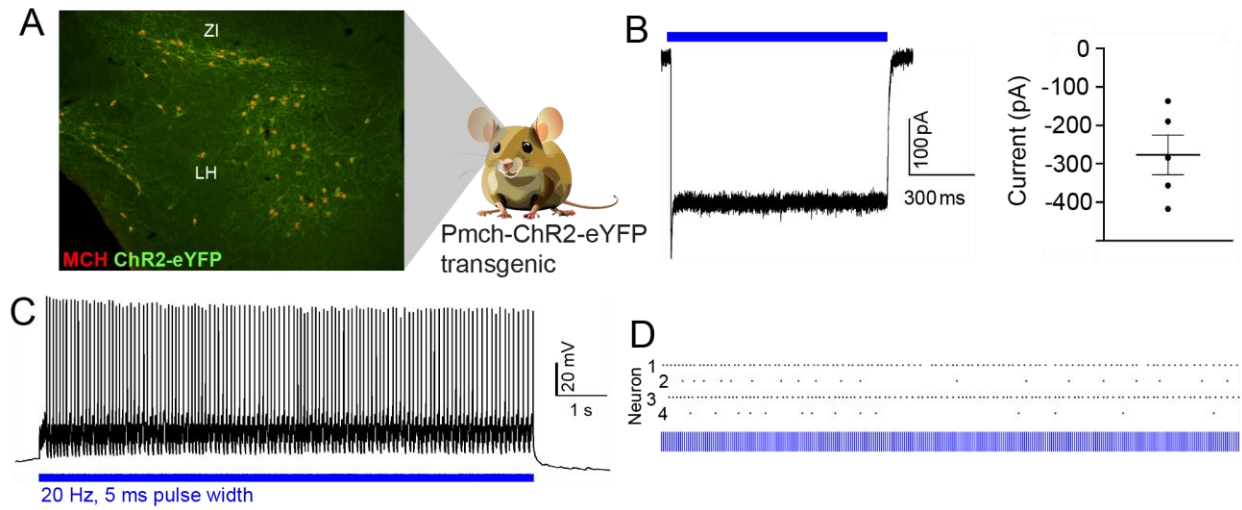


Figure 2.1 Optogenetic activation of MCH neurons in MCH-ChR2 mice.

- A.** MCH-ChR2 mice express eYFP-labeled ChR2 in MCH neurons.
- B.** ChR2-expressing MCH neurons were held in voltage clamp (-70mV) and exposed to 1s pulses of blue light (470nm, 14.5 mW/mm²). Left: a representative photocurrent in an MCH neuron in response to light (blue horizontal bar). Right: steady state photocurrents (-227 ± 52 pA; n=5 cells).
- C.** Representative current clamp trace from a ChR2-expressing MCH neuron in which a sustained train of action potentials was evoked via photostimulation (470nm, 14.5 mW/mm², 20hz, 5 ms pulse width)
- D.** Action potentials (black dots) elicited by photostimulation in four MCH neurons.

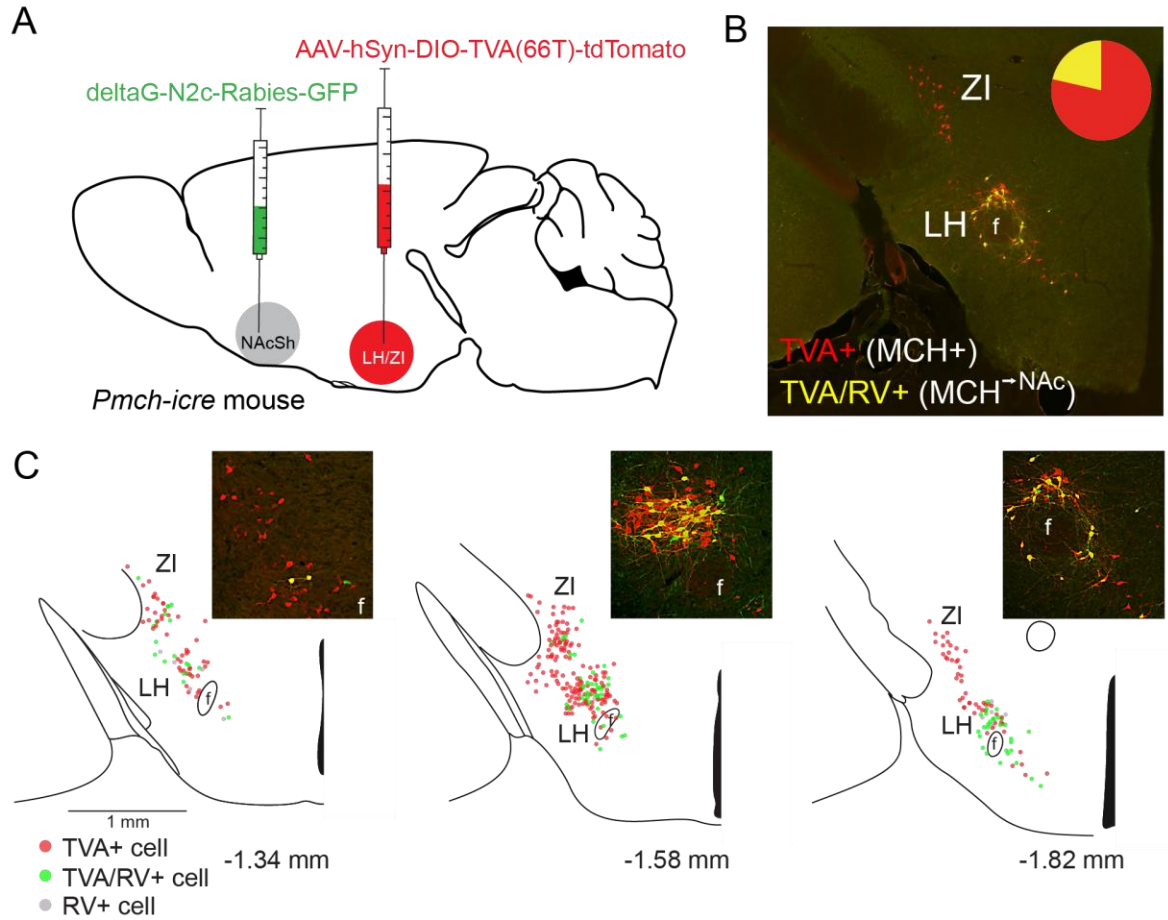


Figure 2.2 MCH neurons send dense projections to the NAc.

A. Schematic of rabies-based retrograde tracing experiments.

B. Example image demonstrating TVA-expressing MCH neurons (red) and retrogradely labeled rabies-expressing MCH neurons (yellow) that project to the NAc. 21.4 $\pm$ 2.2% of MCH neurons project to the NAc (n = 5 mice; inset).

C. Composite images showing the distribution of TVA-labeled neurons and rabies virus-labeled neurons in the lateral hypothalamus (LH) and zona incerta (ZI). 24.2 $\pm$ 2.9% of LH MCH neurons project to the NAc while 10.2 $\pm$ 2.5% of ZI MCH neurons project to the NAc. Insets show individual example images from the LH. f: fornix.

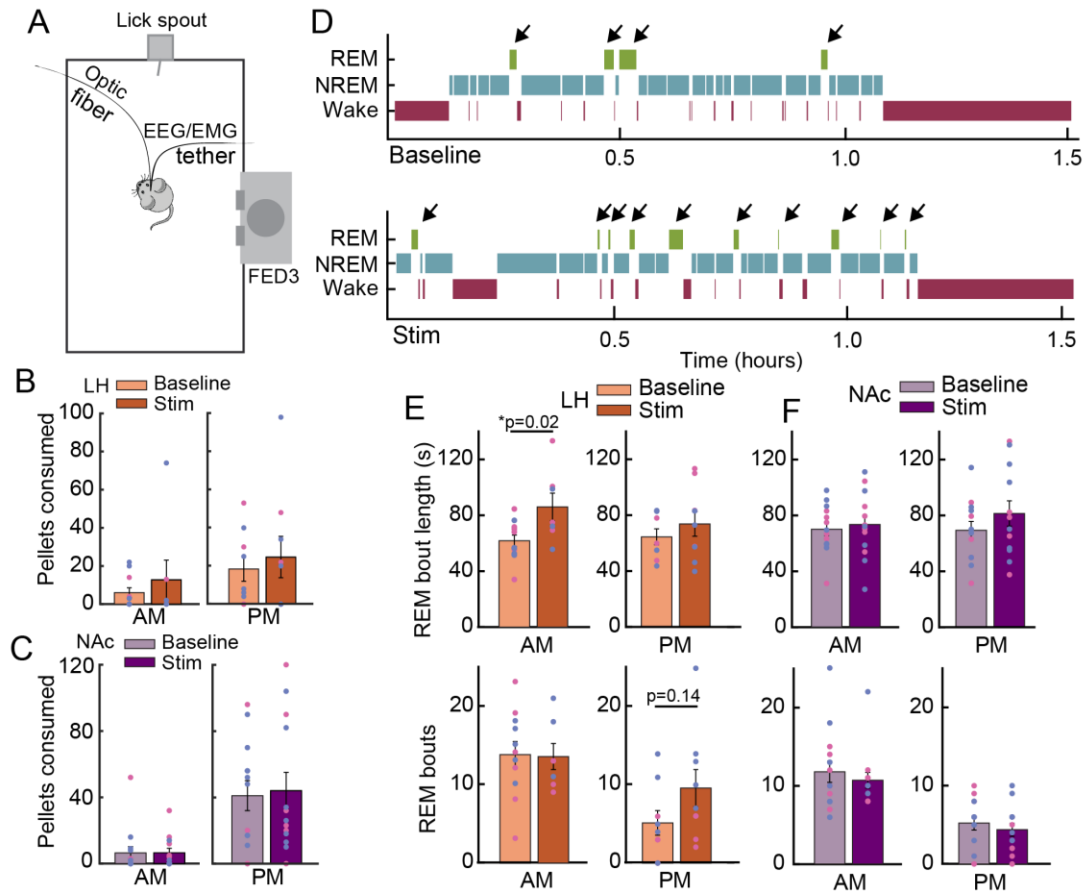


Figure 2.3 Continuous optogenetic stimulation of the MCH system does not increase food intake but can modulate REM sleep.

A. Homecages were modified to include a Feeding Experimentation Device 3 (FED3) from which mice could retrieve food pellets *ad libitum*. Mice were outfitted with both an optic fiber to deliver optogenetic stimulation and an EEG/EMG recording tether to monitor sleep-wake architecture. Fiber implant locations are shown in Figure 2.7.

B. Optogenetic stimulation of MCH neurons in the LH did not increase food pellet intake during either the light or dark phase (paired t-test, $p > 0.05$). Control mice with fiber in LH also showed no change in feeding behavior (Figure 2.7 B). There was no significant change in feeding architecture as a result of LH MCH optogenetic stimulation (Figure 2.4 A,B), and feeding behavior was similar for both males and females (Figure 2.6 A, right). Pink and blue dots denote female and male mice, respectively.

C. Optogenetic stimulation of MCH terminals in the NAc did not increase food pellet intake during either the light or dark phase (paired t-test, $p > 0.05$). Control mice with fiber in NAc also show no change in feeding behavior (Figure 2.7 C). There was no significant change in feeding architecture as a result of MCH optogenetic stimulation (Figure 2.4 C,D), and feeding behavior was similar for both males and females (Figure 2.6 B, right). Pink and blue dots denote female and male mice, respectively.

D. Example hypnograms taken during the dark cycle from a MCH-ChR2 mouse with optic fiber implanted into the LH with optogenetic stimulation off (top) or on (bottom). Arrows denote REM sleep bouts.

E. Optogenetic stimulation of MCH neurons in the LH led to a significant increase in REM bout length during the light cycle (top, paired t-test, $t(16) = 2.589$; $p = 0.0198$), and a non-significant change in the number of REM sleep bouts during the dark cycle (bottom, paired t-test, $t(16) = 1.567$; $p = 0.1366$). Both male and female mice had similar changes in REM sleep architecture as a result of MCH optogenetic stimulation (Figure 2.6 A). Pink and blue dots denote female and male mice, respectively.

F. There was no significant difference in REM sleep architecture with optogenetic stimulation of MCH terminals in NAc (paired t-test, $p > 0.05$). Both male and female mice had similar changes in REM sleep architecture as a result of MCH optogenetic stimulation (Figure 2.6 B). Pink and blue dots denote female and male mice, respectively.

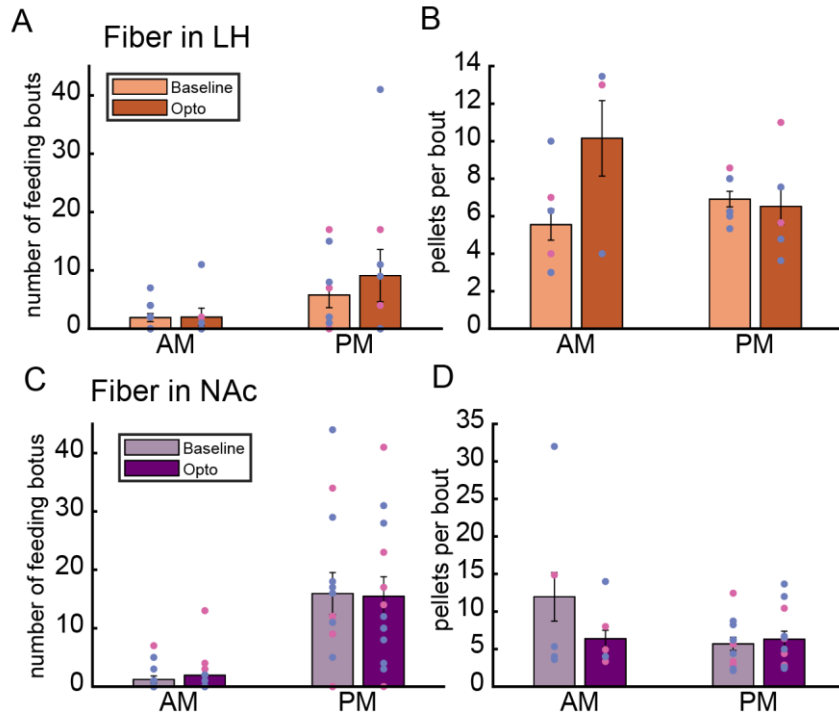


Figure 2.4 Feeding architecture was not altered as a result of MCH optogenetic stimulation

A. Feeding architecture measurements for mice receiving MCH optogenetic stimulation. “Feeding bouts” were calculated by specifying an interbout-interval of 2 minutes. Pellet consumption events that were more than 2 minutes apart were separated into separate “bouts” of feeding, while those closer than 2 mins together were considered part of the same “bout.” Number of feeding bouts did not significantly change with optogenetic stimulation in LH (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

B. Number of pellets per feeding bout did not significantly change with optogenetic stimulation in LH (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

C. Number of feeding bouts did not significantly change with optogenetic stimulation in NAc (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

D. Number of pellets per feeding bout did not significantly change with optogenetic stimulation in NAc (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

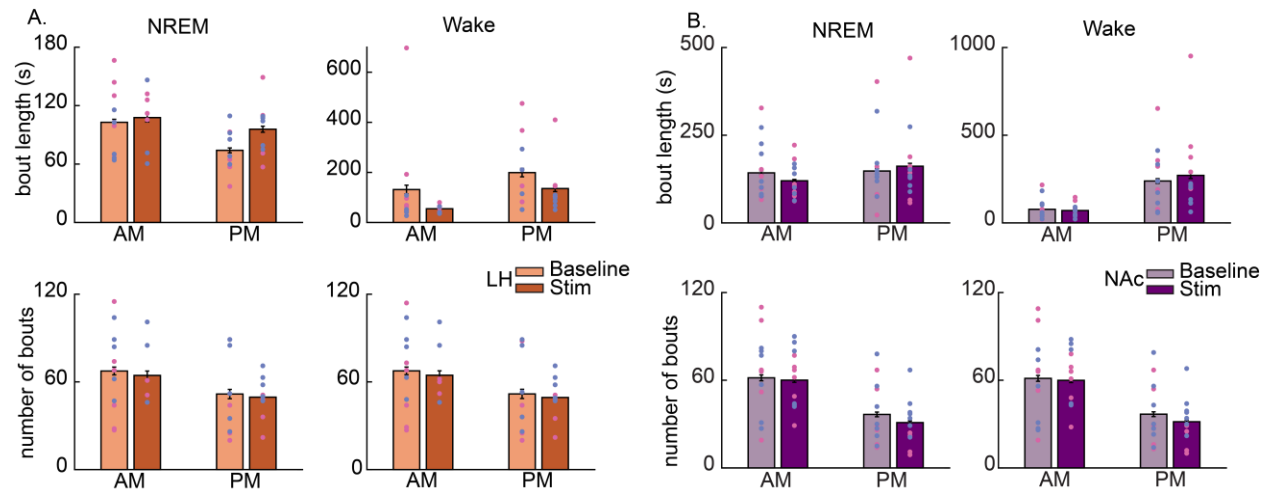


Figure 2.5 Optogenetic stimulation of MCH neurons did not produce changes in NREM or Wake.

A. There were no differences in NREM (left) or Wake (right) bout length (top) or number of bouts (bottom) with continuous optogenetic stimulation of MCH cell bodies in LH.

B. There were no differences in NREM (left) or Wake (right) bout length (top) or number of bouts (bottom) with continuous optogenetic stimulation of MCH terminals in NAc.

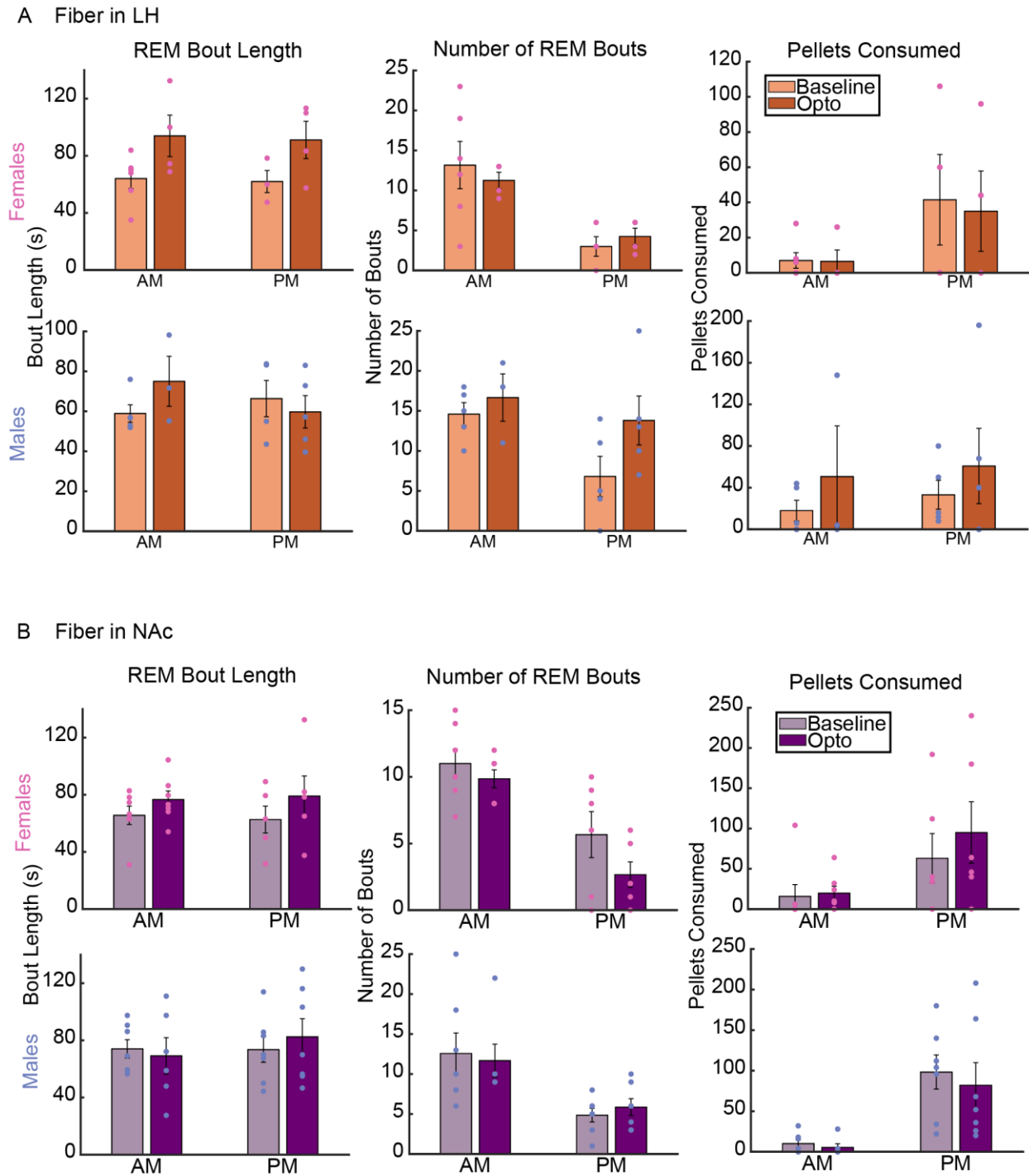


Figure 2.6 Optogenetic stimulation of MCH neurons did not produce sex-specific differences in REM sleep or pellet consumption.

A. There were no sex-specific differences in REM bout length (left), number of REM bouts (middle) or pellets consumed (right) with MCH optogenetic stimulation in LH (paired t-test, $p > 0.05$).

B. There were no sex-specific differences in REM bout length (left), number of REM bouts (middle) or pellets consumed (right) with MCH optogenetic stimulation in NAc (paired t-test, $p > 0.05$).

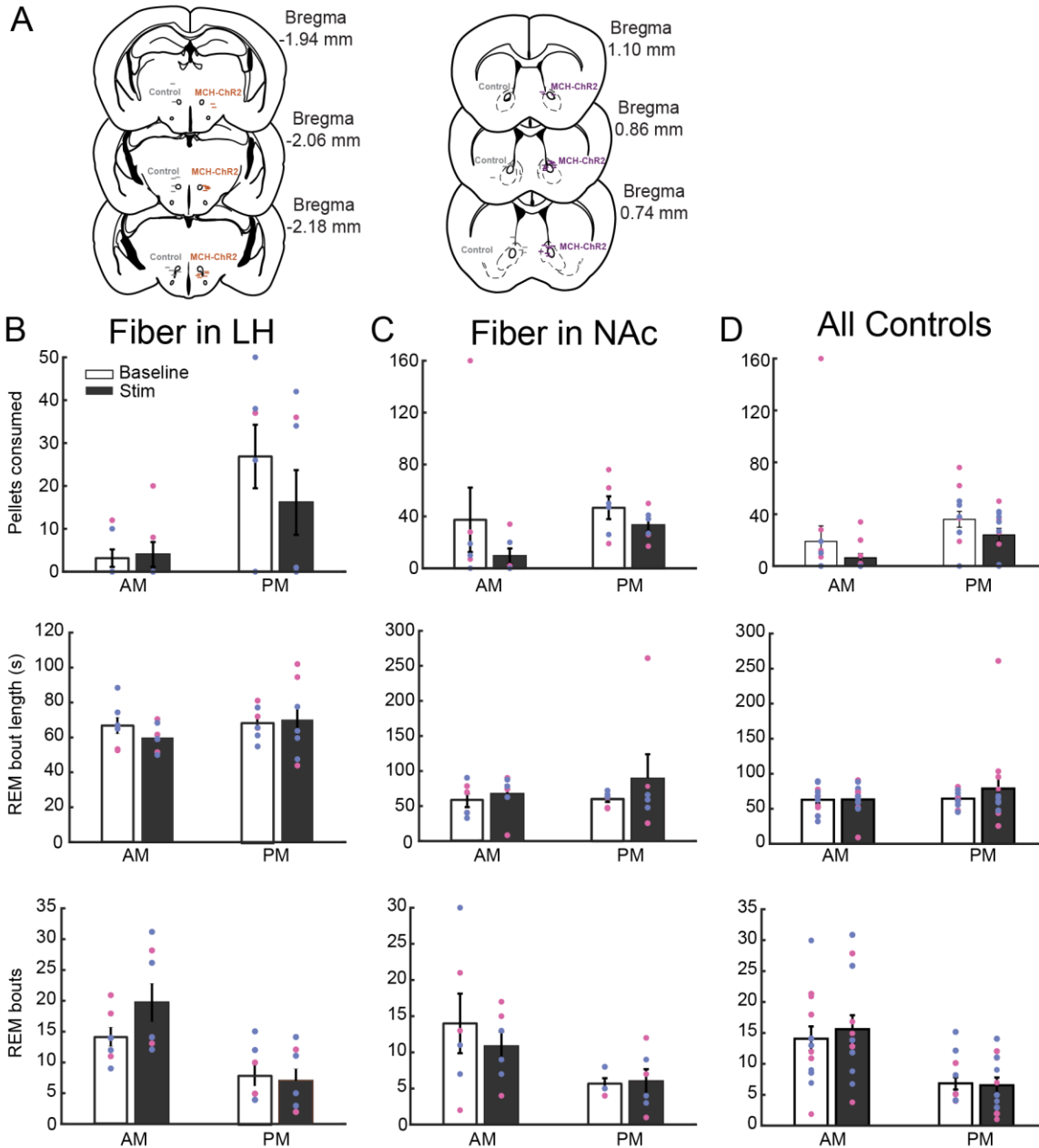


Figure 2.7 Optogenetic stimulation of the MCH system did not change feeding or REM sleep behavior in control mice.

A. Schematics showing fiber placements in the LH and NAc

B. Control mice with fiber in LH show no significant change in pellet consumption (top) REM bout length (middle) or REM bout number (bottom) with light stimulation regardless of fiber location (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

C. Control mice with fiber in NAc show no significant change in pellet consumption (top) REM bout length (middle) or REM bout number (bottom) with light stimulation regardless of fiber location (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

D. Control mice with fiber locations combined show no significant change in pellet consumption (top) REM bout length (middle) or REM bout number (bottom) with light stimulation regardless of fiber location (paired t-test, $p > 0.05$). Pink and blue dots denote female and male mice, respectively.

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Chapter 3 Melanin Concentrating Hormone Projections to the Nucleus Accumbens Enhance the Reward Value of Consummatory Behavior

Portions of this chapter have been published:

Furman et al 2025 Journal of Neuroscience 5 March 2025, 45 (10)

DOI: 10.1523/JNEUROSCI.1725-24.2024

3.1 Abstract²

While the primary evolutionary reason to consume food is to maintain an individual's nutrition and energy balance, there are reasons to eat beyond homeostatic necessity. Non-homeostatic motivators to feed are dysregulated and exacerbated in the case of eating disorders and obesity, leading to patients eating too much or too little food without regard for their homeostatic need. In this chapter, we investigate the role of nucleus accumbens (NAc) projecting MCH neurons in non-homeostatic feeding behavior by developing the optogenetics-reinforced consumption assay (ORCA). ORCA allows mice to voluntarily nose poke in their choice of four ports, which deliver all-pairwise combinations of food availability and MCH optogenetic stimulation. We observe that mice receiving stimulation of MCH cell bodies in LH prefer ports that deliver food over those that deliver stimulation, while mice receiving MCH terminal stimulation in NAc significantly prefer the port delivering both a food pellet and optogenetic stimulation simultaneously. These results lead us to conclude that MCH → NAc stimulation serves to enhance the rewarding value of a temporally paired food. Understanding the neural circuits which attribute reward value to food may help contribute to clinical therapeutics for eating disorders and obesity.

² Mouse surgeries for the behavioral experiments shown in this chapter were performed by Lorelei Baron and Christian Burgess. Behavioral experiments were executed by Katherine Furman, with assistance from Hannah Lyons, Jack Evans, Lucia Kim, and Lorelei Baron.

3.2 Background

Animals must make informed decisions about which foods to eat, and how much, to maintain energy balance. Yet homeostatic need is not the sole factor in the decision to consume food. Humans commonly experience non-homeostatic motivators to eat, such as craving of sugary or fatty foods even when sated (i.e., “there is always room for dessert”). Over the last several decades there has been a steady increase in the prevalence of eating disorders and obesity in the United States, and these conditions are exacerbated by non-homeostatic feeding (Cawley et al., 2021; Hoek, 2016; Imes & Burke, 2014; B.-N. Liu et al., 2021; Sturm & Hattori, 2013; Ward et al., 2019). The specific mechanisms and circuitry through which the brain regulates non-homeostatic food intake are not fully understood.

The melanin-concentrating hormone (MCH) system has been implicated in homeostatic and non-homeostatic motivators to eat, in addition to a number of other fundamental behaviors. Knockout of *Pmch* from neurons in the lateral hypothalamus (LH) and zona incerta (ZI) results in mice that are hypophagic and lean, while overexpression or injection of MCH peptide into the brain induces feeding and can induce weight gain (Georgescu et al., 2005; Guesdon et al., 2009; Ludwig et al., 2001; Rossi et al., 1997; Shimada et al., 1998). MCH KO mice have shown normal overeating behavior in response to food restriction, a homeostatic motivator, but do not overeat in response to non-homeostatic conditioned food cues (Sherwood et al., 2015). More recent applications of optogenetics or pharmacogenetics have had mixed effects; stimulation or inhibition of MCH neurons shows no increase in feeding behavior (Dilsiz et al., 2019; Hausen et al., 2016) or small effects under specific conditions (Terrill et al., 2020). Optogenetic activation of MCH neurons is reinforcing and, when explicitly paired with food availability, can increase consumption (Dilsiz et al., 2019). These data suggest a complex relationship between the MCH system and feeding, where specific projections and products of MCH neurons may drive homeostatic or non-homeostatic consumption.

In Chapter 2 we showed that MCH neuron projections to NAc do not play a role in homeostatically-motivated behaviors, such as baseline food consumption or sleep/wake architecture. Therefore, in Chapter 3 we aim to understand the role for NAc-projecting MCH neurons in a more complex feeding paradigm. We developed the Optogenetics-Reinforced Consumption Assay (ORCA) in order to assess the reward value of MCH stimulation in different brain areas, and in different temporal relationships to food availability. By allowing mice a

choice between food, optogenetic stimulation of MCH neurons, and food paired with optogenetic stimulation of MCH neurons, we assess the degree to which MCH stimulation at various anatomical structures may affect the rewarding value of food consumption. Our data show that MCH projections to NAc serve to enhance the rewarding value of a paired food, while MCH cell bodies in LH do not share this effect.

3.3 Methods

3.3.1 Mice

All mice were housed in a University of Michigan vivarium in a temperature-controlled environment (12 h light and 12 h dark cycle; lights on at 2 AM) with *ad libitum* access to food and water. All animal protocols were approved by the University of Michigan's Institutional Animal Care and Use Committee and are in accordance with NIH guidelines for the use and care of laboratory mice. For behavioral experiments, Pmch-ChR2-eYFP mice (n = 60) were made by crossing Ai32 mice (Strain #: 024109) to Pmch-Cre (RRID: IMSR_JAX:014099). Control mice were Cre-positive, ChR2-negative littermates. For rabies tracing experiments, Pmch-iCre positive mice (n = 5) were used (RRID: IMSR_JAX:014099, (Bethany G. Beekly et al., 2020)). Both male and female mice were used in all experiments. Overall health of experimental mice was monitored daily and any mice that displayed obvious signs of distress or weakness were removed from the study.

3.3.2 Surgery

Mice were deeply anesthetized by inhalation of 2% isoflurane and placed on a Kopf stereotaxic apparatus (Tujunga, CA). Following standard disinfection procedure, the skin was retracted to expose the skull and a small hole was drilled into the skull unilaterally at defined positions to target NAc or LH. An optic fiber (400- μ m diameter core; NA 0.5; RWD) was implanted unilaterally over NAc (A/P: 1.0 mm, M/L: -0.8 mm, D/V: -4.2 mm) or LH (A/P: -1.4 mm, M/L: -1.0 mm, D/V: -4.7 mm). Fibers were fixed to the skull using dental acrylic. A subset of mice were also implanted with two stainless-steel screws (Frontal: A/P: 1.5 mm, M/L: -1.5 mm; Temporal: A/P: -3.5 mm, M/L: -2.8 mm) and a pair of multi strand stainless steel wires inserted into the neck extensor muscles. EEG and EMG leads were wired to a small electrical connector that is attached to the skull with dental cement.

Following surgery, mice were kept on a warming pad until fully ambulatory and fed a regular chow diet throughout the experimental period unless otherwise noted. All mice were given analgesics (5 mg/kg Meloxicam) prior to the end of surgery and 24 hours after surgery. Mice were given a minimum of 2 weeks for recovery before being used in any experiments.

After the completion of the experiments, mice were euthanized and the locations of optic fiber tips were verified histologically.

3.3.3 Experimental Design and Statistical Analysis

Optogenetics-Reinforced Consumption Assay

Animals' home cages were outfitted with two FED3 feeding devices (Matikainen-Ankney et al., 2021). FED3s were programmed to release a food pellet (BioServ Dustless Precision Pellets, Rodent Purified Diet, 20mg) following appropriate nose pokes. In order to pair optogenetic stimulation and pellet consumption, FED3s also acted as a closed-loop optogenetics trigger, activating optogenetic stimulation (20Hz, 10ms pulse width for 15s) upon appropriate pokes in the nose ports. While in the modified home cage, mice could move freely while attached to an optic fiber patch cable.

This paradigm has three phases: Habituation, StimON, and Reversal. The four-day Habituation phase allows mice to habituate to the optic fiber (day 1) and demonstrate any baseline preference among food ports (days 2-4). During Habituation, a poke in any of the four ports results in the same outcome: food pellet delivery. During the StimON phase, the FED3s were programmed to allow all-pairwise combinations of food and optogenetic stimulation at the four different ports. Ports were identified as (1) a Pellet + Stim port, which triggers both pellet delivery and optogenetic stimulation (20Hz for 15s), (2) a Stimulation Only port, which triggers only optogenetic stimulation (20Hz for 15s), (3) a Pellet Only port, which triggers only food pellet delivery, and (4) a No Outcome port, which does not result in either stimulation or pellet delivery. The StimON phase of the experiment lasted 5 days, a length we experimentally validated as it is long enough for the mouse to develop and demonstrate consistent preference. The last phase of the experiment is the Reversal phase, which is one day immediately following the end of the StimON phase. During Reversal, all four ports delivered a food pellet in response to a poke, and none triggered stimulation. In the results and figures, Sham stimulation refers to those opportunities where a mouse could nose poke to a port that either will later (Habituation phase) or had previously (Reversal phase) been paired with optogenetic stimulation, during these phases the LED was powered off.

The FED3 data collection includes data regarding both quantity and timing of pokes at each port. Using this data, the number of pokes in each of the four ports was counted on each

day, and port preference was calculated as a percentage of total pokes that day. We also collected data of the cumulative pokes over time, to measure changes in port preference within-days by comparing the rate of increase across shorter durations.

Preference Index

Mouse poking behavior at rewarded ports was used to calculate a preference index, indicating their preference for the Pellet+Stim port over other available ports. The calculation was as follows:

$$\text{Preference Index} = \frac{\text{Pokes at Pellet+Stim Port} - (\text{Pokes at Pellet Port} + \text{Pokes at Stim Port})}{\text{Pokes at Pellet+Stim Port} + \text{Pokes at Pellet Port} + \text{Pokes at Stim Port}}$$

Preference index was calculated per-animal per-day, and mean preference index was compared across days and groups as a readout of their Pellet+Stim preference.

Feeding Architecture

In addition to raw numbers of pokes and pellets, animals' feeding architecture was quantified. This involved measuring inter-pellet intervals (IPIs) to understand the distribution of consumption over time. A histogram of IPI lengths was created to understand the distribution of IPI lengths for a given animal. This histogram was used to determine the appropriate interbout interval, in order to denote separate feeding "bouts." A majority of IPIs were less than 2 minutes, thus 2 minutes was determined as the interbout interval. Using these criteria, the number of feeding bouts and the number of food pellets per bout were calculated. The skewness of the IPI distribution was also calculated.

3.3.4 Immunohistochemistry

Following the conclusion of all experiments, mice were anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by formalin. Once removed, brains were post-fixed in 10% formalin overnight then transferred to 20% PBS-sucrose for ~48 hours. Brains were then frozen and 30µm coronal sections were cut using a freezing microtome. Location of optic fibers was confirmed by histology, and mice in which the fiber did not hit the intended target area were excluded.

For MCH immunohistochemistry, coronal sections were washed with 1x PBS and then incubated in 10% NDS (Normal Donkey Serum) blocking solution for 1 hour at room temperature. Once blocked, sections were incubated in MCH primary antibody solution (Rabbit anti-MCH Polyclonal Antibody (Catalog #H-070-47, Phoenix Pharmaceuticals) (1:8000)) overnight at RT. Following primary antibody staining, sections were washed with PBS-Tween then incubated in secondary antibody solution (Donkey anti-Rabbit Alexa Fluor 647 Conjugated Antibody (Catalog #A-31573, Invitrogen) (1:1250)) for 1 hour at RT. Slices went through a final PBS wash and then mounted onto glass slides using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Images were then captured by a fluorescence microscope (Olympus IX83/Keyence BZ-X800) at 10x magnification.

To increase the brightness of endogenous eYFP fluorescence, we used GFP amplification. Sections were washed with PBS then incubated in 5% NDS blocking solution for 2 hours at RT. After blocking, sections were incubated in primary antibody solution (GFP Polyclonal Antibody, Alexa Fluor™ 488 (Catalog #A-21311, ThermoFisher) (1:1000)) for 48 hours at RT. After primary antibody incubation, sections were washed with PBS then mounted onto slides.

3.3.5 Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad), IBM SPSS Statistics, or Matlab software. Details of statistical tests employed can be found in the relevant figure legends. In all statistical tests, normal distribution and equal variance was established. The data presented met the assumptions of the statistical test employed. Exclusion criteria for experimental mice were (i) sickness or death during the testing period (ii) if histological validation of the injection site demonstrated an absence of reporter gene expression (iii) or if histological validation demonstrated a mistargeted fiber placement. These criteria were established before data collection. N numbers represent final numbers of healthy/validated mice.

3.4 Results

3.4.1 *MCH projections to the NAc enhance the value of consummatory behavior*

As there was no effect of MCH → NAc stimulation on either acute feeding or arousal in Chapter 2, we developed the optogenetics-reinforced consumption assay (ORCA) to assess how consumption behavior changes when mice have voluntary access to self-stimulation of MCH in LH, or MCH terminals in NAc. Following surgical implantation of an optic fiber in either LH or NAc, MCH-ChR2 mice were given the choice between all-pairwise combinations of food and optogenetic stimulation between two automated food hoppers (FED3, (Matikainen-Ankney et al., 2021)) and four total ports. During the Habituation phase of the ORCA paradigm, when all ports resulted in delivery of a pellet and none resulted in optogenetic stimulation (Sham stimulation refers to a port that will later be paired with optogenetic stimulation), mice from all groups showed no significant innate preference for any individual port (two-way ANOVA/Bonferroni multiple-comparison test; LH group: stim, $F(1,16) = 2.144$, $p = 0.165$; pellet, $F(1,16) = 0.5438$; $p = 0.4715$; interaction, $F(1,16) = 0.1320$; $p = 0.7212$; NAc group: stim, $F(1,16) = 0.01224$; $p = 0.9133$; pellet, $F(1,16) = 0.04548$; $p = 0.8320$; interaction, $F(1,16) = 0.1097$; $p = 0.7447$) (Figure 3.1 B,C, left). Following Habituation, the ports delivering stimulation were assigned to the food hopper which the mouse had least preferred during habituation. This accounts for the slight preference Control mice (the MCH-Cre littermates of the MCH-ChR2 experimental mice) had for the two “No Stimulation” ports during their Habituation phase (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,17) = 20.51$; *** $p = 0.0003$; pellet, $F(1,17) = 0.09945$; $p = 0.7563$; interaction, $F(1,17) = 0.018939$; $p = 0.8939$) (Figure 3.1 D, left). After observing no innate preference for any of the four ports during Habituation, the paradigm was changed to allow mice a choice between food, stimulation, or both.

During the StimON phase of the ORCA paradigm, mice had access to all-pairwise combinations of food pellet delivery and optogenetic stimulation. Both MCH-ChR2 mice with the fiber in the LH and controls showed a significant and expected shift towards a preference for the two ports delivering a food pellet, and away from the ports that no longer deliver food pellets (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,14) = 0.5409$; $p = 0.4742$; pellet, $F(1,14) = 16.85$; ** $p = 0.0023$; interaction, $F(1,14) = 8.226e-006$; $p = 0.9978$) (Figure 3.1 B, D right; Figure 3.3). Specifically, animals’ preference for the Pellet+Stim port was significantly higher than for the No Pellet+No Stim port (Bonferroni multiple-comparison test,

$p=0.0390$). However, MCH-ChR2 mice with the fiber placed in the NAc showed a significant main effect of both food and stimulation availability on port preference (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,15) = 5.498$; $*p = 0.0332$; pellet, $F(1,15) = 64.17$; $****p < 0.0001$; interaction, $F(1,15) = 1.338$; $p = 0.2656$) (Figure 3.1 C, right). Mice receiving MCH optogenetic stimulation in the NAc significantly preferred the Pellet+Stim port over the Stim Only port (Bonferroni multiple-comparisons test, $p = 0.0018$), the Pellet Only port (Bonferroni multiple-comparisons test, $p = 0.0217$), and the No Pellet+No Stim port (Bonferroni multiple-comparisons test, $p < 0.001$). The preference for the port delivering both a food pellet and stimulation in the MCH-ChR2 group with MCH $\rightarrow$ NAc stimulation was not due to a change in the total number of pellets consumed, or a change in overall meal architecture (number of feeding bouts, or number of pellets per feeding bout; Figure 3.2) as, similar to homecage optogenetic stimulation, there was no increase in feeding or change in meal structure in the ORCA paradigm.

In addition to our investigation of port preference after each session, we also investigated the time course of nose poking and port preference emergence within ORCA sessions. A representative raster plot from a single mouse shows differences in poking patterns at each port (Figure 3.4 A). For mice receiving MCH stimulation in the LH, both MCH-ChR2 and Control mice develop a persistent preference for ports with a food pellet, regardless of whether the food is paired with stimulation (Figure 3.4 B, left) or not (Figure 3.4 B, right). However, mice receiving MCH $\rightarrow$ NAc stimulation quickly develop a preference for the Pellet + Stim port, emerging during the first StimON dark period and lasting through the end of the StimON phase (Figure 3.4C, left). Control mice receiving sham stimulation in the NAc take longer to develop a preference, and only prefer pellet ports, regardless of whether the food pellet is paired with stimulation (Figure 3.4 C).

The StimON phase of the ORCA experiment is followed by a day-long Reversal phase, during which the four ports all release a food pellet, and stimulation can no longer be triggered by poking (Sham stimulation refers to a port that had previously been paired with optogenetic stimulation). Following reversal of the paradigm, there remained a significant effect of prior food pellet availability in MCH-ChR2 mice receiving MCH optogenetic stimulation in LH (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,16) = 1.844$; $p = 0.1933$; pellet, $F(1,16) = 7.457$; $*p = 0.0148$; interaction, $F(1,16) = 0.06838$; $p = 0.7970$; Figure 3.5 A,B), and a

significant effect of both prior stimulation and prior food pellet availability in MCH-ChR2 mice receiving MCH optogenetic stimulation in NAc. (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,13) = 7.178$; $*p = 0.0189$; pellet, $F(1,13) = 7.458$; $*p = 0.0172$; interaction, $F(1,13) = 0.005975$; $p = 0.9396$; Figure 3.5 A,B). When the poking data of the Reversal phase are viewed cumulatively, it is clear that, regardless of fiber location, mice retain a preference for their previously-preferred port(s) during the first three hours of the Reversal phase (Figure 3.5 C,D, left). However, this preference is entirely abolished by the end of the Reversal phase, as the poking behavior at each port diminishes, with mice failing to prefer any specific port during this time (Figure 3.5 C,D, right).

3.4.2 Sex-specific effects of MCH stimulation

Some previous research has demonstrated sex-specific roles for the MCH system in feeding (Ferreira et al., 2017; Terrill et al., 2020). We sought to investigate whether there was a sex-specific effect of MCH optogenetic stimulation in our ORCA paradigm. During the StimON phase, effects of MCH optogenetic stimulation were more pronounced in males than females, (Figure 3.6), and this effect was stronger in the NAc. Females receiving optogenetic stimulation in LH (Figure 3.6 A, top) showed no significant effect of either stimulation or food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,5) = 0.3427$; $p = 0.5837$; pellet, $F(1,5) = 1.226$; $p = 0.3185$; interaction, $F(1,5) = 1.380$; $p = 0.2930$). Males receiving optogenetic stimulation in LH (Figure 3.6 A, bottom) showed a significant effect of food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,8) = 0.1892$; $p = 0.6750$; pellet, $F(1,8) = 29.87$; $***p = 0.0006$; interaction, $F(1,8) = 1.355$; $p = 0.2779$). The pellet preference emerged over the course of the 12h of stimulation (Figure 3.6 B). We calculated a preference index to quantify the degree to which mice preferred the Pellet+Stim port relative to the other available ports. In this measure, male and female mice receiving LH MCH optogenetic stimulation did not have a significantly different preference for the Pellet+stim port (Figure 3.6C). Females receiving optogenetic stimulation in NAc (Figure 3.6 D, top) showed a significant effect of pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,10) = 2.021$; $p = 0.1856$; pellet, $F(1,10) = 35.00$; $***p = 0.0001$; interaction, $F(1,10) = 0.0002062$; $p = 0.9888$). Males receiving optogenetic stimulation in NAc (Figure 3.6 D, bottom) showed a significant effect of pellet availability (two-way

ANOVA/Bonferroni multiple-comparison test; stim, $F(1,4) = 4.211$; $p = 0.1094$; pellet, $F(1,4) = 58.44$; $**p = 0.016$; interaction, $F(1,4) = 4.045$; $p = 0.1146$). This preference emerged over the course of the 12h of stimulation (Figure 3.6 E). In our preference index measure, male mice receiving MCH optogenetic stimulation in NAc showed a higher, though not significant, preference index than their female counterparts (Figure 3.6F). Our data demonstrates that MCH has similar overall effects in males and females, but supports previous reports that the MCH system may more potently impact behavior in males.

3.5 Discussion

We refer to the effects of MCH on ‘non-homeostatic’ feeding here because we did not see changes in total food consumption with MCH stimulation delivered either continuously in Chapter 2 (Figure 2.3 B,C) or in a food-paired manner here (Figure 3.2A), but we did see reinforcing effects on consumption of food paired with MCH→NAc stimulation (Figure 3.1). It is indeed possible that the MCH system could drive both homeostatic and non-homeostatic consumption based on prior literature.

A previous study demonstrated that MCH-KO mice do overeat in response to food restriction, a homeostatic motivator, but do not overeat in response to conditioned food cues (Sherwood et al., 2015). Our findings seem to complement this work, as we demonstrate an increase in preference for a specific food paired with MCH → NAc terminal stimulation, but not an increase in overall food consumption, suggesting that MCH terminal stimulation in the NAc may serve to reinforce consumption of paired food. The NAc has a well-established role in reward and reinforcement learning, particularly as it relates to mesolimbic dopaminergic inputs (Castro & Bruchas, 2019; Sackett et al., 2017; Salgado & Kaplitt, 2015). The MCH system has been implicated in regulation of dopamine activity, suggesting that this interaction may underlie reinforcing effects of MCH in the NAc (M. J. Chee et al., 2019; Domingos et al., 2013; Duncan Spencer et al., 2024; Sears et al., 2010). A recent study demonstrated that MCH in the ventral tegmental area modulates both midbrain dopaminergic and glutamatergic neurons (Duncan Spencer et al., 2024), but there is also evidence of more local interactions between MCH neurons and dopamine release in the NAc. MCH peptide has been shown to enhance the postsynaptic effects of the dopamine receptor agonist apomorphine in the NAc, and to increase NAc neuron firing when both D1R and D2R are activated (Chung, Saito, et al., 2009; Hopf et al., 2013);

though MCH peptide itself, as opposed to neural activation, likely decreases dopamine release in the NAc and the activity of NAc neurons (M. J. Chee et al., 2019; Sears et al., 2010).

As noted above, MCH neurons release multiple neurotransmitters and peptides in addition to MCH (B. G. Beekly et al., 2023). MCH neurons express genes involved with the classical neurotransmitters glutamate and GABA (Blanco-Centurion et al., 2018; Mickelsen et al., 2019, 2017; Nectow et al., 2017), suggesting that the role of MCH peptide may not be congruent with the role of MCH activity overall. Previous work suggests that MCH neurons mediate the post-ingestive value of sucrose consumption; this work also demonstrates that activation of MCH neurons can increase dopamine release in the NAc (Domingos et al., 2013). Follow up work shows that sucrose preference is mediated by glutamatergic release from MCH neurons, not MCH peptide release (Schneeberger et al., 2018). These studies suggest that the MCH system may act to enhance dopaminergic signaling or postsynaptic neural activity in the NAc, facilitating the reinforcement behavior observed here. However, they also indicate that this effect might not be mediated by MCH peptide release.

It is well-documented that hypothalamic neurons can release both GABA and glutamate, and that they can co-release these with other hypothalamic neuropeptides (Dicken et al., 2015, 2012; Horvath et al., 2001; Mickelsen et al., 2019; Romanov et al., 2017; Venner et al., 2019). MCH and glutamate immunoreactivity colocalize in the rat LH (Abrahamson et al., 2001), and approximately 30% of MCH neurons express GAD67 (B. G. Beekly et al., 2023; Elias et al., 2008; Noble et al., 2018). One study of AgRP neurons, another hypothalamic neuron population involved with feeding and energy homeostasis, showed that GABA and neuropeptide Y (NPY) release from AgRP neurons led to rapid changes in feeding behavior, while release of AgRP peptide itself led to more delayed changes in feeding behavior (Krashes et al., 2013). A similar mechanism might be at play in MCH neurons, potentially dictated by the parameters of neuronal firing, as neuropeptide release is typically observed in response to sustained high-frequency firing (Arrigoni & Saper, 2014). Therefore, we may have seen different results here if we had used different optogenetic stimulation parameters. This type of experiment, along with studies of either GABA receptor, glutamate receptor, or MCH receptor loss of function, would help further elucidate the exact neuronal mechanisms responsible for the effect of MCH → NAc neurons on the reward value of consumption.

3.6 Figures

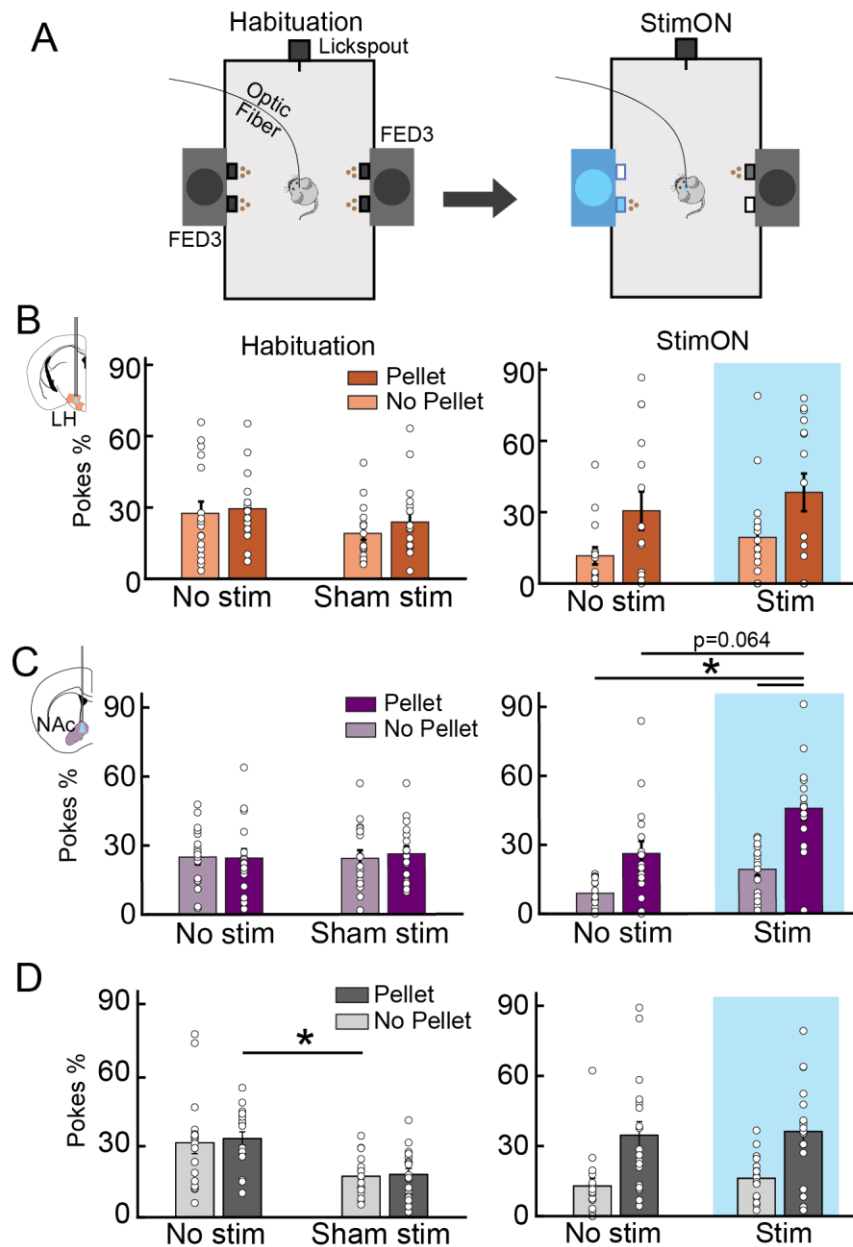


Figure 3.1 Optogenetic stimulation of MCH terminals in the NAc increases preference for a paired food.

A. Optogenetics Reinforced Consumption Assay (ORCA) behavioral schematic. Mouse homecages were modified to include two FED3 food hoppers. During habituation, a poke at either nose port on each FED3 triggers pellet delivery from the pellet well. During StimON, pokes at one nose port on each FED3 trigger food pellet delivery, while pokes at both nose ports on one FED3 trigger optogenetic stimulation.

B. Poke preference data from mice with a fiber implanted into the LH on the final Habituation day (left) and the final StimON day (right). There was no significant difference observed in port preference on the Habituation day (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,16) = 2.144$; $p = 0.165$; pellet, $F(1,16) = 0.5438$; $p = 0.4715$; interaction, $F(1,16) = 0.1320$; $p = 0.7212$). On the final StimON day, there was a significant effect of

Pellet availability on port preference (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,14) = 0.5409$; $p = 0.4742$; pellet, $F(1,14) = 16.85$; $**p = 0.0023$; interaction, $F(1,14) = 8.226e-006$; $p = 0.9978$). Specifically, animals' preference for the Pellet+Stim port was significantly higher than for the No Pellet+No Stim port (Bonferroni multiple-comparison test, $p=0.0390$). There were no significant changes to feeding architecture (Figure 2.9, orange bars). Pink and blue dots denote female and male mice, respectively.

C. Poke preference data from mice with a fiber implanted into the NAc on the final Habituation day (left) and the final StimON day (right). There were no significant differences observed on the Habituation day (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,16) = 0.01224$; $p = 0.9133$; pellet, $F(1,16) = 0.04548$; $p = 0.8320$; interaction, $F(1,16) = 0.1097$; $p = 0.7447$). On the StimON day (left), there was a significant effect of both stimulation and pellet availability on poke preference (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,15) = 5.498$; $*p = 0.0332$; pellet, $F(1,15) = 64.17$; $****p < 0.0001$; interaction, $F(1,15) = 1.338$; $p = 0.2656$). Mice receiving MCH optogenetic stimulation in the NAc significantly preferred the Pellet+Stim port over the Stim Only port (Bonferroni multiple-comparisons test, $p=0.0018$), the Pellet Only port (Bonferroni multiple-comparisons test, $p=0.0217$), and the No Pellet+No Stim port (Bonferroni multiple-comparisons test, $p<0.001$). There were no significant changes to feeding architecture (Figure 2.9, purple bars). Pink and blue dots denote female and male mice, respectively.

D. Poke preference data from control mice on the final Habituation day (left) and the final StimON day (right). Control mice showed a significant preference on the Habituation day (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,17) = 20.51$; $***p = 0.0003$; pellet, $F(1,17) = 0.09945$; $p = 0.7563$; interaction, $F(1,17) = 0.018939$; $p = 0.8939$), and no significant preference on the final StimON day (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,17) = 0.1681$; $p = 0.6869$; pellet, $F(1,17) = 27.35$; $****p < 0.0001$; interaction, $F(1,17) = 0.03477$; $p = 0.8543$). There were no significant differences based on fiber location in Control animals (Figure 2.10). Pink and blue dots denote female and male mice, respectively.

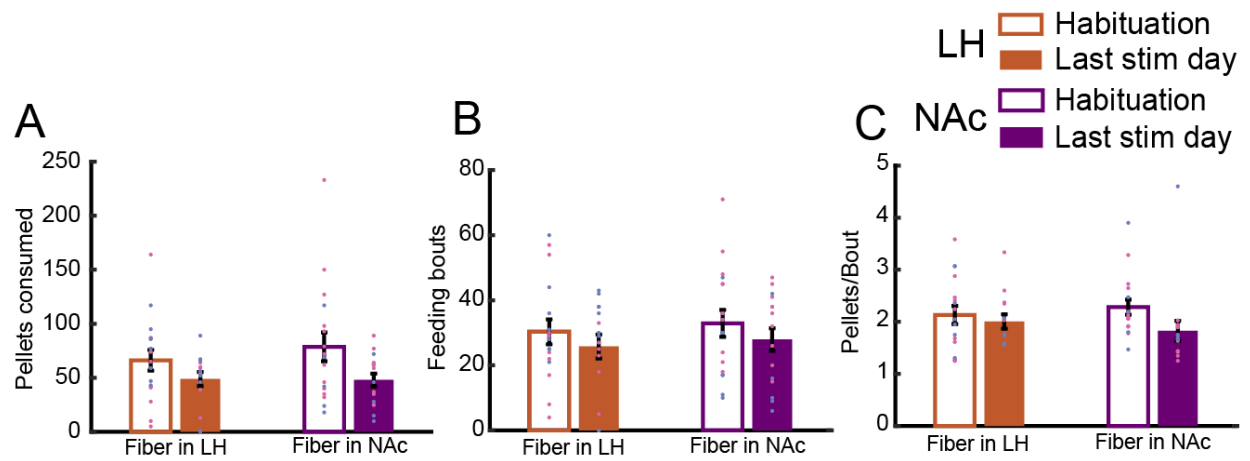


Figure 3.2 Optogenetic stimulation did not significantly impact feeding behavior

A. Optogenetic stimulation of MCH neurons in the LH or MCH terminals in the NAc did not increase the number of pellets consumed during the optogenetics reinforced consumption assay. Pink and blue dots denote female and male mice, respectively.

B-C. There was no difference in feeding architecture measurements for mice receiving optogenetic stimulation. Pink and blue dots denote female and male mice, respectively.

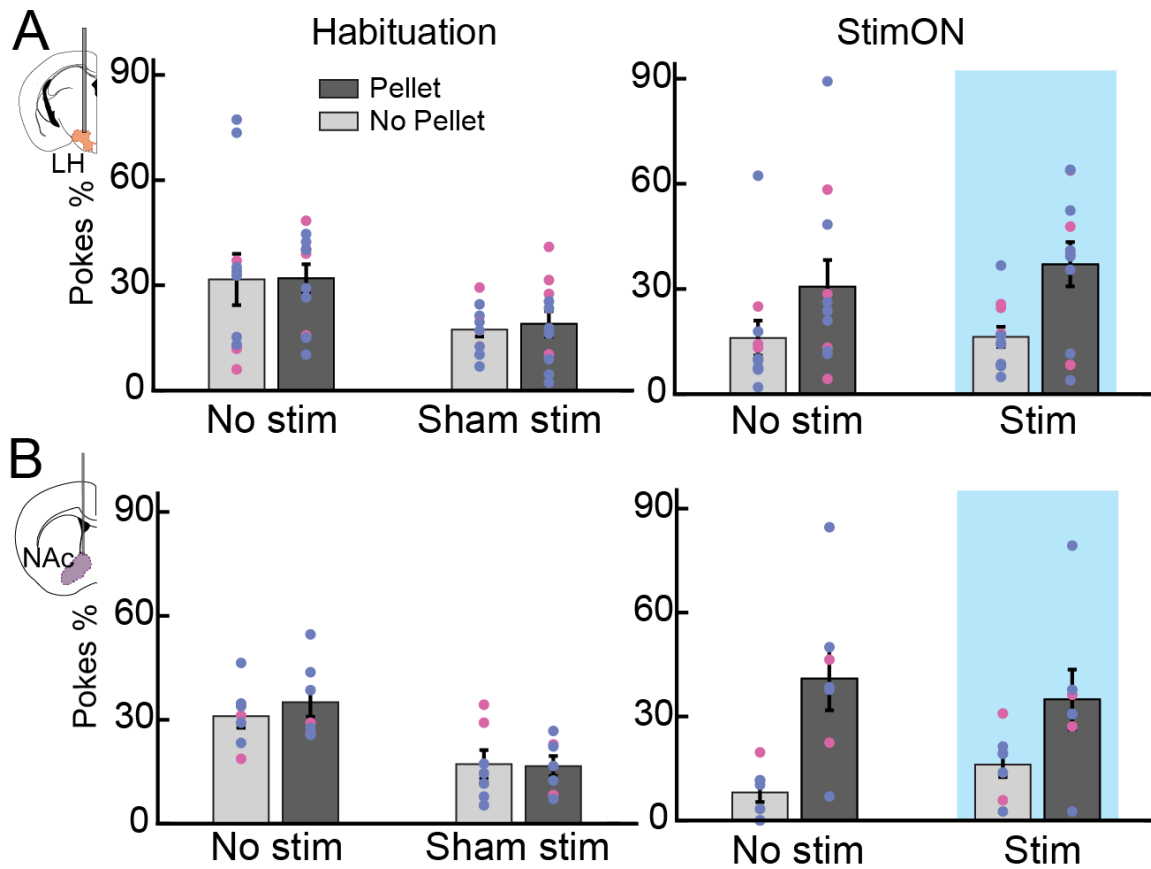


Figure 3.3 Optogenetic stimulation did not cause a preference for stimulation-paired ports in control mice.

A. Control mice with optic fibers implanted in the LH showed a non-significant preference for pellet-paired ports in the StimON phase. Pink and blue dots denote female and male mice, respectively.

B. Control mice with optic fibers implanted in the NAc showed a non-significant preference for pellet-paired ports in the StimON phase. Pink and blue dots denote female and male mice, respectively.

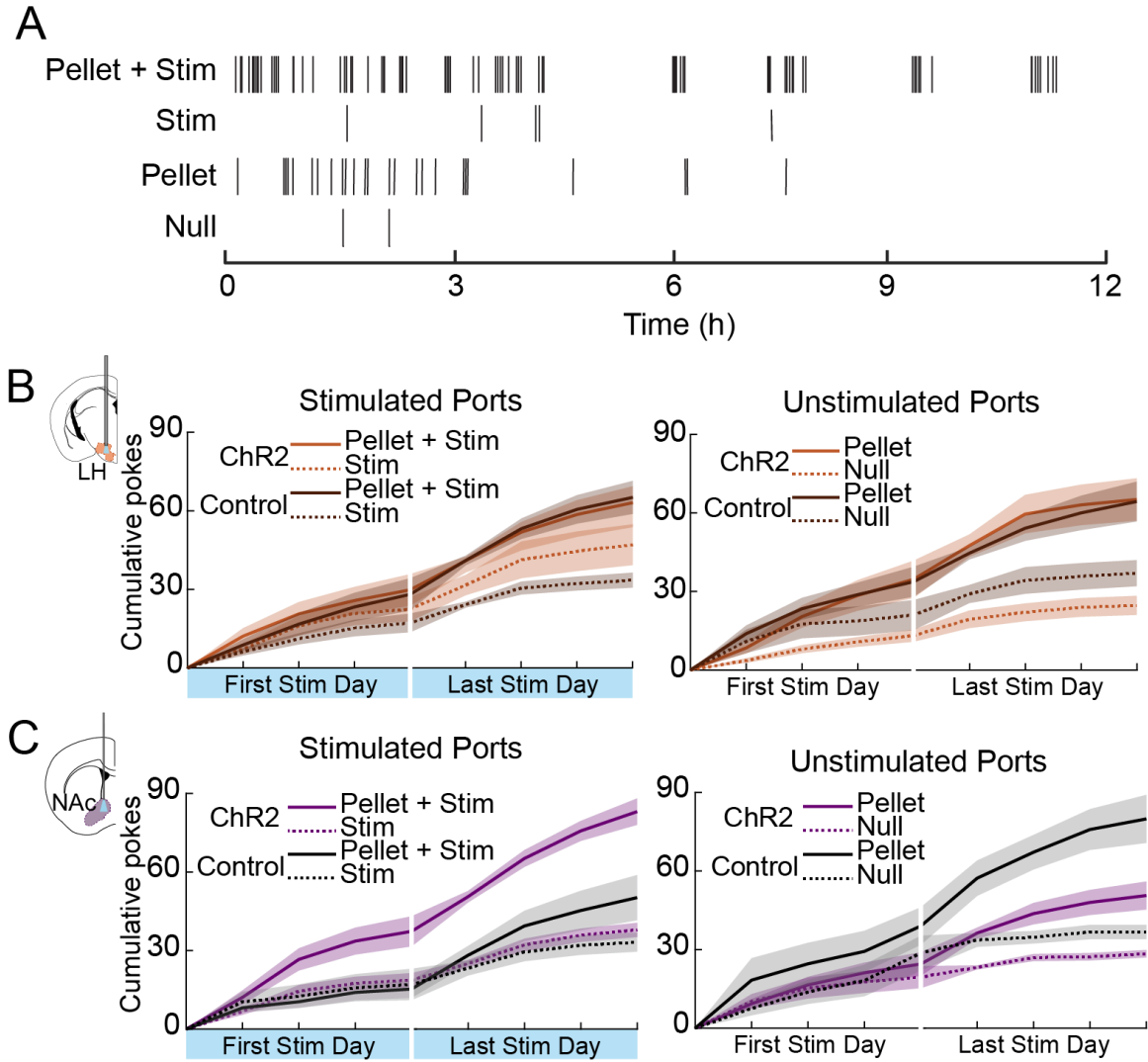


Figure 3.4 Preference for stimulation-paired food emerges more rapidly with MCH → NAc optogenetic stimulation.

A. Raster plot of a representative mouse's poking behavior over the 12 hour dark cycle. Vertical lines represent pokes at the specified port.

B. MCH-ChR2 mice optogenetic stimulation in LH show similar behavior to Control mice. The preference develops by the end of the final day of stimulation.

C. MCH-ChR2 mice receiving MCH optogenetic stimulation in the NAc quickly develop a preference for the Pellet+Stim port, by the middle of the first day of stimulation, which lasts throughout the entire StimON period. Control mice receiving sham stimulation do not develop this preference, preferring the port(s) delivering a food pellet regardless of stimulation availability.

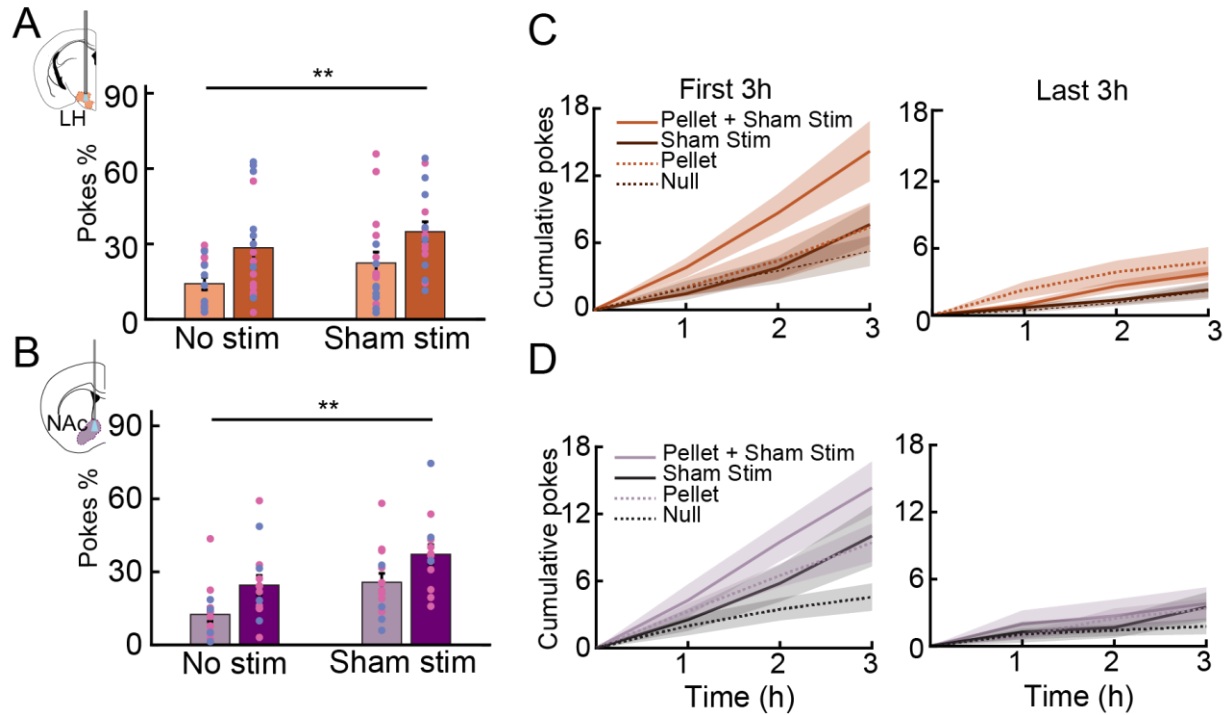


Figure 3.5 Preference persists for a short period after removal of stimulation.

A. Percentage of pokes at each port during the Reversal day of the ORCA paradigm for mice receiving MCH optogenetic stimulation in LH. Following reversal of the paradigm, there remained a significant effect of food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,16) = 1.844$; $p = 0.1933$; pellet, $F(1,16) = 7.457$; $*p = 0.0148$; interaction, $F(1,16) = 0.06838$; $p = 0.7970$). Pink and blue dots denote female and male mice, respectively.

B. Percentage of pokes at each port during the Reversal day of the ORCA paradigm for mice receiving MCH optogenetic stimulation in NAc. Following reversal of the paradigm, there remained a significant effect of both stimulation and food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,13) = 7.178$; $*p = 0.0189$; pellet, $F(1,13) = 7.458$; $*p = 0.0172$; interaction, $F(1,13) = 0.005975$; $p = 0.9396$). Pink and blue dots denote female and male mice, respectively.

C. Cumulative poking behavior over the first three hours (left) and last three hours (right) of the Reversal day of the ORCA paradigm for mice receiving MCH optogenetic stimulation in LH. A preference for pellet ports persisted during the first three hours of the Reversal day but was eliminated by the last three hours.

D. Cumulative poking behavior over the first three hours (left) and last three hours (right) of the Reversal day of the ORCA paradigm for mice receiving MCH optogenetic stimulation in NAc. A preference for the Pellet+Stim port persisted during the first three hours of the Reversal day but was eliminated by the last three hours.

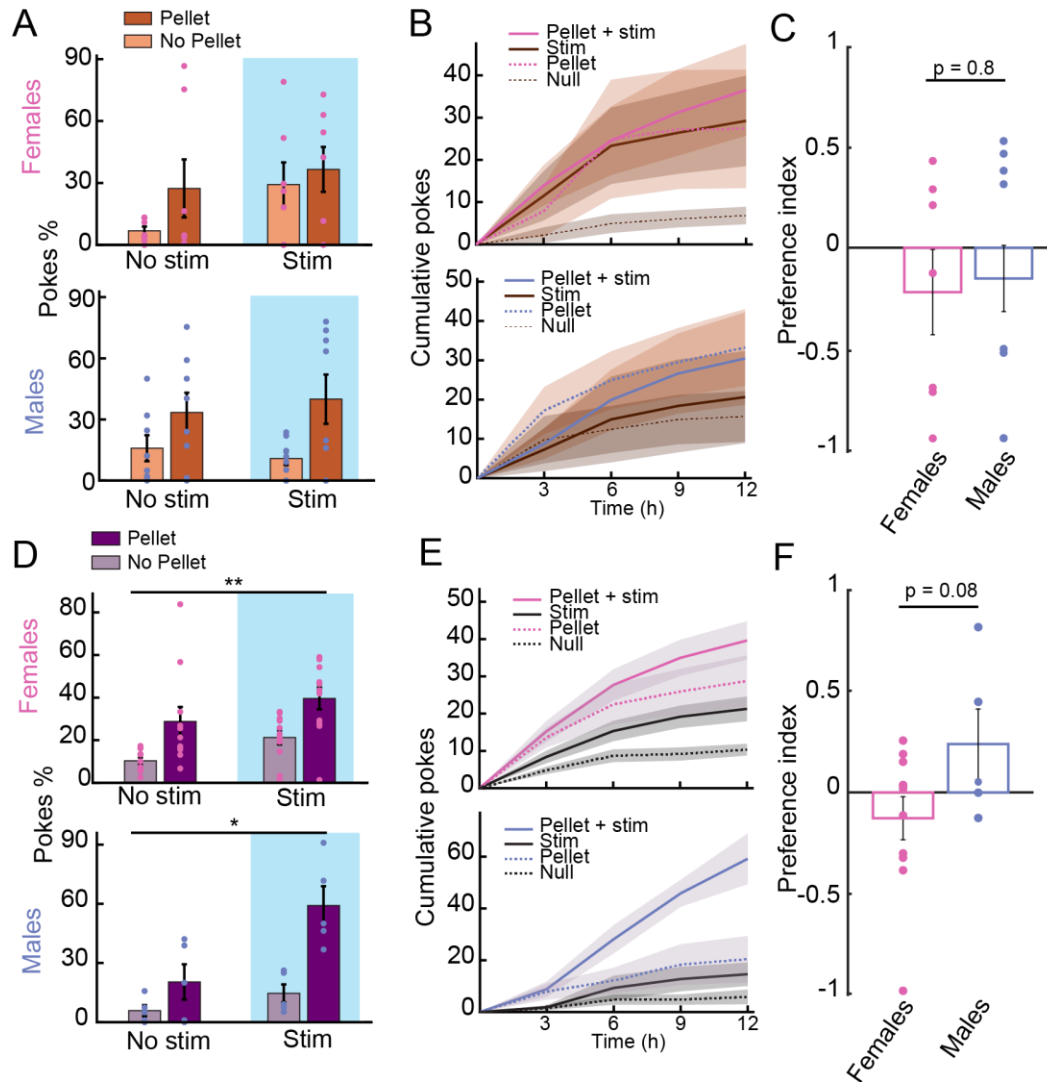


Figure 3.6 Sex-specific effects of MCH stimulation and associated preference.

A. Poke percentages on the final day of the StimON phase of the ORCA paradigm for females (top) and males (bottom) receiving MCH optogenetic stimulation in LH. Females receiving optogenetic stimulation in LH (top) showed no significant effect of either stimulation or food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,5) = 0.3427$; $p = 0.5837$; pellet, $F(1,5) = 1.226$; $p = 0.3185$; interaction, $F(1,5) = 1.380$; $p = 0.2930$). Males receiving optogenetic stimulation in LH (bottom) showed a significant effect of food pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,8) = 0.1892$; $p = 0.6750$; pellet, $F(1,8) = 29.87$; *** $p = 0.0006$; interaction, $F(1,8) = 1.355$; $p = 0.2779$).

B. Cumulative poking behavior over the entire 12hr dark cycle on the final day of the StimON phase of the ORCA paradigm for females (top) and males (bottom) receiving MCH optogenetic stimulation in LH. No significant differences between ports were observed. Females receiving optogenetic stimulation in NAc (top) showed a significant effect of pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,10) = 2.021$; $p = 0.1856$; pellet, $F(1,10) = 35.00$; *** $p = 0.0001$; interaction, $F(1,10) = 0.0002062$; $p = 0.9888$). Males receiving optogenetic stimulation in NAc (top) showed a significant effect of pellet availability (two-way ANOVA/Bonferroni multiple-comparison test; stim, $F(1,4) = 4.211$; $p = 0.1094$; pellet, $F(1,4) = 58.44$; ** $p = 0.016$; interaction, $F(1,4) = 4.045$; $p = 0.1146$).

C. Preference index calculation for females' and males' preference for the Pellet+Stim port over other rewarded

ports for mice receiving MCH optogenetic stimulation in LH. No significant difference was observed (two-sample t-test, $p=0.8313$).

D. Poke percentages on the final day of the StimON phase of the ORCA paradigm for females (top) and males (bottom) receiving MCH optogenetic stimulation in NAc. No significant differences between ports were observed.

E. Cumulative poking behavior over the entire 12hr dark cycle on the final day of the StimON phase of the ORCA paradigm for females (top) and males (bottom) receiving MCH optogenetic stimulation in NAc. No significant differences between ports were observed.

F. Preference index calculation for females' and males' preference for the Pellet+Stim port over other rewarded ports for mice receiving MCH optogenetic stimulation in NAc. No significant difference was observed (two-sample t-test, $p=0.0824$).

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Chapter 4 Investigating the Role of Distinct MCH Efferents in Feeding and Reward

4.1 Abstract³

MCH neurons project to a variety of downstream targets, but the potential functional heterogeneity of these efferents remains understudied. Despite having previously observed that NAc-projecting MCH neurons can be behaviorally distinguished from the complete population of LH MCH neurons (see Chapter 2 and Chapter 3), there remain more MCH neuron projections to characterize. In the pursuit of testing additional anatomical targets of MCH neurons, we sought to modify our existing ORCA paradigm to increase throughput. This involved a number of modifications to the behavioral timeline and experimental design. After troubleshooting, we arrived at an acute version of the ORCA paradigm which sufficiently reproduced our Chapter 3 result, and used it to focus on an additional efferent site: MCH neurons which project to the locus coeruleus (LC). In our pilot acute ORCA experiment, we observed that mice receiving optogenetic activation of MCH terminals in LC seem to prefer food ports triggering optogenetic stimulation, regardless of food availability. This led us to conclude that optogenetic activation of MCH terminals in LC may be reinforcing, regardless of food availability. However, the sample size on this experiment must be increased before finalizing this conclusion.

4.2 Background

In Chapter 3, we defined a projection-specific role for NAc-projecting MCH neurons in enhancing the reward value of food associated with stimulation. Following this finding, we were left with questions regarding the nature of our observed effect, which could not be answered using the paradigm we designed. MCH neurons have widespread projections to a variety of downstream areas, and we hypothesized that these other projections may also play projection-specific roles in MCH-mediated feeding and reward. Additionally, MCH neurons release

³ Mouse surgeries for experiments shown in this chapter were performed by Lorelei Baron.

multiple neurotransmitters. We were interested in whether the contributions of these specific neurotransmitters (including glutamate, GABA, and MCH peptide) to the projection-specific behavioral effects could be isolated.

In the ORCA paradigm we developed in Chapter 3, mice lived continuously in the behavior chamber, and data collection was continuous for a period of two weeks. This was a limitation on the throughput of the experiment, as a single mouse took two weeks to undergo the full experiment. In this chapter, we aimed to further develop the paradigm to make it higher-throughput, with the capacity to run more mice through the paradigm in a shorter amount of time. There are multiple other reasons why this would be advantageous in the long term, as a higher-throughput behavioral experiment where mice spend less time in the chamber allows for pharmacological and other interventions which are not possible in the continuous version of the paradigm.

In addition to modifying the behavioral paradigm to accommodate different experimental goals, we also aimed to test the behavioral role of different downstream projections of MCH neurons using this updated behavior paradigm. Specifically, we had interest in investigating the MCH projection to the locus coeruleus (LC). The LC is known to be involved with attention/arousal state, as well as regulation of sleep/wake state (Poe et al., 2020). Given that we saw no effect of NAc MCH terminal stimulation on sleep/wake architecture in Chapter 2, we understood that individual MCH projections may be responsible for divergent behavioral roles. Therefore, we hypothesized that the MCH projection to LC might, conversely to the NAc projection, have a role in sleep/wake architecture but not in feeding. After successfully developing a more acute ORCA paradigm, we aimed to test mice with MCH optogenetic stimulation of MCH terminals in LC in order to understand whether this projection had a role in nonhomeostatic feeding.

4.3 Methods

4.3.1 Mice

All mice were housed in a University of Michigan vivarium in a temperature-controlled environment (12 h light and 12 h dark cycle; lights on at 2 AM) with *ad libitum* access to food and water. All animal protocols were approved by the University of Michigan's Institutional

Animal Care and Use Committee and are in accordance with NIH guidelines for the use and care of laboratory mice. *Pmch-iCre* positive mice ($n = 32$) were used (RRID: IMSR_JAX:014099, (Bethany G. Beekly et al., 2020)). Both male and female mice were used in all experiments. Overall health of experimental mice was monitored daily and any mice that displayed obvious signs of distress or weakness were removed from the study.

4.3.2 Surgery

Pmch-iCre positive mice were deeply anesthetized by inhalation of 2% isoflurane and placed on a Kopf stereotaxic apparatus (Tujunga, CA). Following standard disinfection procedure, the skin was retracted to expose the skull and a small hole was drilled into the skull unilaterally over the LH. A pulled glass pipette ($\sim 20\ \mu\text{m}$ tip diameter, Friedrich & Dimmock) was inserted into the brain, and pAAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-hghPA ($1 \times 10^{13}\text{gc/ml}$, addgene 20297) was injected using a picospritzer. The pipette was kept in place for 10 min before withdrawal. Injections targeted the LH (A/P: $-1.4\ \text{mm}$, M/L: $+1.1\ \text{mm}$, D/V: -5.2 , A/P: $-1.5\ \text{mm}$, M/L: $+0.9\ \text{mm}$, D/V: -5.0 , A/P: $-1.65\ \text{mm}$, M/L: $+1.0\ \text{mm}$, D/V: -5.1). An optic fiber ($400\text{-}\mu\text{m}$ diameter core; NA 0.5; RWD) was implanted ipsilaterally over NAc (A/P: $1.0\ \text{mm}$, M/L: $-0.8\ \text{mm}$, D/V: $-4.2\ \text{mm}$), LH (A/P: $-1.4\ \text{mm}$, M/L: $-1.0\ \text{mm}$, D/V: $-4.7\ \text{mm}$), or LC (A/P: -5.4mm , M/L: 0.85mm , D/V: -3.1mm). Fibers were fixed to the skull using dental acrylic.

Following surgery, mice were kept on a warming pad until fully ambulatory and fed a regular chow diet throughout the experimental period unless otherwise noted. All mice were given analgesics ($5\ \text{mg/kg}$ Meloxicam) prior to the end of surgery and 24 hours after surgery. Mice were given a minimum of 3 weeks for recovery before being used in any experiments. After the completion of the experiments, mice were euthanized and the locations of optic fiber tips were verified histologically.

4.3.3 Experimental Design and Statistical Analysis

Acute Optogenetics-Reinforced Consumption Assay

Animals' home cages were outfitted with two FED3 feeding devices (Matikainen-Ankney et al., 2021). FED3s were programmed to release a food pellet (BioServ Dustless Precision Pellets, Rodent Purified Diet, 20mg) following appropriate nose pokes. In order to pair

optogenetic stimulation and pellet consumption, FED3s also acted as a closed-loop optogenetics trigger, activating optogenetic stimulation (20Hz, 10ms pulse width for 15s) upon appropriate pokes in the nose ports. While in the modified home cage, mice could move freely while attached to an optic fiber patch cable.

This paradigm has three phases: Habituation, StimON, and MCHR1 Antagonism. The five-day Habituation phase allows mice to habituate to the optic fiber and demonstrate any baseline preference among food ports. During Habituation, a poke in any available port results in the same outcome: food pellet delivery. However, throughout the Habituation phase, port availability changes, as a way to force mice to sample from each port before the StimON phase. Only one FED3 device (with two ports) is available on days 1 and 3 of Habituation, and the other FED3 device (and its two ports) is available on days 2 and 4 of Habituation. On day 5 of Habituation, both FED3s and all four of their ports are available, and the mouse's baseline preference is used to assign port identities during the StimON phase. The mouse's most-preferred port becomes the No Reward port, and the most-preferred port on the other FED3 device becomes the Stim Only port.

During the StimON phase, the FED3s were programmed to allow all-pairwise combinations of food and optogenetic stimulation at the four different ports. Ports were identified as

1. A No Reward port, which does not result in either pellet delivery or optogenetic stimulation. This is the port which was most preferred by the mouse during the Habituation phase.
2. A Pellet Only port, which delivers a food pellet. This is the other port on the same FED3 as the No Reward port.
3. A Stimulation Only port, which triggers optogenetic stimulation (20Hz for 15s). This is the port which the mouse most preferred on the opposite FED3 device from the one with the No Reward port.
4. A Pellet+Stim port, which triggers both pellet delivery and optogenetic stimulation (20Hz for 15s). This is the port on the other side of the same FED3 device as the Stim Only port.

The StimON phase of the experiment lasted 5 days, a length we experimentally validated as it is long enough for the mouse to develop and demonstrate consistent preference. Similarly to the

Habituation phase, port availability changes throughout the experiment. On Day 1 of StimON, both FED3s are available (now with their new outcome identities), and mice can freely poke. On day 2, only one FED3 is available (either the Stimulated FED3, or the Unstimulated FED3, with the order counterbalanced by mouse). On day 3, the opposite FED3 is available. And on days 4 and 5, both FED3s are once again available for mice to poke freely. This structure forces mice to sample from all four ports, while still assessing their preference via free choice between all four.

The last phase of the experiment, the MCHR1 antagonism phase, is one day immediately following the end of the StimON phase. On this day, all ports are identical to their identities during StimON, and the mouse receives an injection of SNAP-94847 (vehicle: 20% B-cyclodextrin in saline, dose 25mg/kg), an MCHR1 antagonist.

The FED3 data collection includes data regarding both quantity and timing of pokes at each port. Using this data, the number of pokes in each of the four ports was counted on each day, and port preference was calculated as a percentage of total pokes that day. We also collected data of the cumulative pokes over time, to measure changes in port preference within-days by comparing the rate of increase across shorter durations.

Food Restriction

Mice were food restricted for the duration of the Acute ORCA experiment described above. Food restriction followed a similar protocol through each different iteration of the experiment. Regular chow was removed from mice's cages 3-4 days before the first day of the Acute ORCA paradigm. Prior to starting the Acute ORCA paradigm, mice were fed 2-3 grams of 20mg BioServ Dustless Precision pellets in order to maintain their body weight at approximately 85% of their starting weight. Following the start of the Acute ORCA paradigm, mice had access to the same BioServ pellets from the FED3s for one hour per day during their time in the behavior chamber. Mice underwent the behavior in the same order every day. Mice were fed between 1.5-2.5g of BioServ pellets one hour following conclusion of all behavioral testing for the day. The amount they were fed was determined by the amount they had eaten in the behavior chamber that day, such that the total food consumed in one day was between 2.5-3.0 grams. Mice were weighed daily throughout the experiment to ensure that their weight stayed between 80-90% of their starting body weight, with the goal of staying at 85%. If their weight fluctuated significantly, the amount of food available was altered to compensate.

4.3.4 Immunohistochemistry

Following the conclusion of all experiments, mice were anesthetized with pentobarbital and transcardially perfused with phosphate-buffered saline (PBS) followed by formalin. Once removed, brains were post-fixed in 10% formalin overnight then transferred to 20% PBS-sucrose for ~48 hours. Brains were then frozen and 30µm coronal sections were cut using a freezing microtome. Location of optic fibers was confirmed by histology, and mice in which the fiber did not hit the intended target area were excluded.

For MCH immunohistochemistry, coronal sections were washed with 1x PBS and then incubated in 10% NDS (Normal Donkey Serum) blocking solution for 1 hour at room temperature. Once blocked, sections were incubated in MCH primary antibody solution (Rabbit anti-MCH Polyclonal Antibody (Catalog #H-070-47, Phoenix Pharmaceuticals) (1:8000)) overnight at RT. Following primary antibody staining, sections were washed with PBS-Tween then incubated in secondary antibody solution (Donkey anti-Rabbit Alexa Fluor 647 Conjugated Antibody (Catalog #A-31573, Invitrogen) (1:1250)) for 1 hour at RT. Slices went through a final PBS wash and then mounted onto glass slides using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA). Images were then captured by a fluorescence microscope (Olympus IX83/Keyence BZ-X800) at 10x magnification.

4.3.5 Statistical analysis

Statistical analyses were performed using Prism 9.0 (GraphPad), IBM SPSS Statistics, or Matlab software. Details of statistical tests employed can be found in the relevant figure legends. In all statistical tests, normal distribution and equal variance were established. The data presented met the assumptions of the statistical test employed. Exclusion criteria for experimental mice were (i) sickness or death during the testing period (ii) if histological validation of the injection site demonstrated an absence of reporter gene expression (iii) or if histological validation demonstrated a mistargeted fiber placement. These criteria were established before data analysis. N numbers represent final numbers of healthy/validated mice.

4.4 Results

4.4.1 Development of the Acute ORCA behavioral paradigm

In order to adapt the ORCA paradigm to be higher throughput, we ran a series of pilot experiments meant to fine-tune the experimental timeline and determine the best way to recapitulate our ORCA result in a shorter training period. For each of these experiments, time in the experimental chamber was one hour per day, and mice were food-restricted outside the behavior chamber (see Methods for details). The behavior timeline for our first iteration of Acute ORCA is shown in Figure 4.1A. This experimental design was very similar to that which we used in Chapter 3. Mice first underwent a four-day Habituation phase, during which they had access to two FED3s and all four nose ports, each of which delivered a food pellet following a nose poke. All four ports were available during all four days of the Habituation phase, which allowed mice to habituate to the behavior chamber and display any innate preference they may have had between the ports. Following this, there was a three-day StimON phase during which one port on each FED3 triggered food pellet delivery, and both ports on one FED3 triggered a brief optogenetic stimulation. Following three days of StimON, there was a two-day StimSwap period where the FED3 triggering optogenetic stimulation was swapped to the opposite side. This would allow us to observe whether mice had developed a spatial preference for a port, or a preference for the outcome associated with that port.

We observed that mice were not making an attempt to poke at every available port during the one hour they were in the behavior chamber. Instead, they tended to prefer one port throughout the paradigm, regardless of the outcome of that port. In example data from individual mice, both a mouse with the fiber placed in the NAc (Figure 4.1B) and a mouse with the fiber placed in the LH (Figure 4.1C) preferred the same port regardless of whether that port triggered optogenetic stimulation. This led us to understand that mice did not adequately sample all available ports during either the Habituation phase or the StimON phase, and subsequent iterations of the experiment must implement a way to force mice to try each available port.

This change resulted in the second iteration of the Acute ORCA experiment, shown in Figure 4.2A. While the first and last days of Habituation were the same as the first iteration, the middle two days of the Habituation phase were altered such that only a single FED3 was available. Since mice were food-restricted during the 23 hours of the day that they were not in the behavior chamber (see Methods for details), this change was meant to add motivation for them to try every available port, rather than sticking to one the whole time. The same change was made to the StimON phase, such that only a single FED3 was available on the middle two days.

This way, once stimulation became available, mice were required to sample by poking from both sides over the course of the StimON phase.

The above changes worked as intended; mice sampled from all ports, and their preference changed once optogenetic stimulation became available. Two individual mice with the optic fiber placed over the NAc (Figure 4.2 B,C) showed an effect similar to that which we saw in our original ORCA behavior paradigm in Chapter 3. While they had no obvious preference for any port during the Habituation phase (Figure 4.2 B,C, left), both mice developed a much stronger preference for the Pellet+Stim port during the StimON phase (Figure 4.2 B,C, right). In contrast, two individual mice with the optic fiber placed over the LH showed a strong preference for the Pellet Only port during the StimON phase (Figure 4.2 D,E, right), despite one of these mice having a very strong preference for the Pellet Only port during Habituation as well. From these data, we concluded that forcing pokes on one FED3 or the other on certain days was an effective way to encourage greater sampling from all four ports on days when they are available.

The final iteration of the Acute ORCA experiment made one additional change which led to more consistent results. In previous iterations, optogenetic activation was assigned to the FED3 which was less preferred by the most mice. This worked well in the continuous ORCA paradigm, because mice had all 24 hours per day to explore and develop new preferences. However, we chose to make the counterbalancing port-specific, rather than just FED3-specific, in the final iteration of Acute ORCA. To this end, the port identities were assigned differently for every mouse that underwent the experiment. The port which was most preferred during the Habituation phase was assigned as the No Reward port during the StimON phase, and the other port on that side was the Food Only port. Of the ports on the other FED3, the port which was most preferred during Habituation was assigned as the Stim Only port during StimON, and the other port on that side was the Pellet+Stim port. This is outlined in Figure 4.3. After the StimON phase of the final iteration of Acute ORCA, there was one additional day in which the port identities were kept the same as they were on the previous day, and mice received an injection of MCHR1 antagonist SNAP-94847 or vehicle (20% B-cyclodextrin in saline) to assess whether the effect remained in the absence of MCH binding to MCHR1.

4.4.2 Changes in total pokes and pellets throughout Acute ORCA

The overall poking and pellet consumption behavior for each group of mice is shown in Figure 4.4. Interestingly, mice with the fiber placed in the LC seemed to have an increase in total poking behavior on the StimON days compared to the Habituation day (Figure 4.4A, left), while the other groups showed a decrease in poking during the StimON days (Figure 4.4 B,C,D, left). All groups showed a decrease in pellet consumption on the StimON days compared to the Habituation day (Figure 4.4, right), however MCH antagonism with SNAP-94847 seemed to further decrease both poking behavior and pellet consumption (Figure 4.4).

4.4.3 Differential effects of MCH stimulation in LC, NAc, and LH during Acute ORCA

In developing the Acute ORCA paradigm, we aimed both to increase throughput and to test the effect of stimulating a new MCH projection: that which terminates in the locus coeruleus (LC). This preliminary experiment included low sample sizes, with three mice per group. Despite this, we observed that the effect of MCH stimulation on the reward value of paired food was different in all three areas tested (the LC, NAc, and LH; Figure 4.5). All ports delivered a food pellet during the Habituation phase, and while all groups showed a preference for the No Reward port over all other ports during Habituation (Figure 4.5 A,B,C,D, left), this is a side effect of the fact that StimON port identities were decided with Habituation preferences in mind, meaning that the No Reward port was always assigned to the port which was most preferred during Habituation.

During the StimON phase, mice with the fiber placed in the LC showed a preference for the two ports triggering optogenetic stimulation over the two other ports (Figure 4.5A, right). Mice with the fiber placed in the NAc showed a preference for the Pellet+Stim and Pellet Only ports (Figure 4.5B, right). Mice with the fiber placed in the LH showed a preference for all four ports relatively equally (Figure 4.5C, right). Control mice, who were Cre- and thus did not express the Chr2, showed a preference for the ports which delivered a food pellet (Figure 4.5D, right). The timescale at which these preferences emerged is shown in Figure 4.6. Again, the early emergence of a preference for the No Reward port in all groups during Habituation is expected due to the rationale behind the assignment of StimON port identities (Figure 4.6 B,C,D,E, left). For mice with the fiber placed in the LC, a preference for both the Pellet+Stim and No Reward ports emerges quickly during the First StimON day (Figure 4.6B, middle) – the No Reward

preference likely lingering from the previous day before the port identities were changed. By the Last StimON day, these mice quickly demonstrate a preference for the Pellet+Stim port, followed by a subsequent preference for the Stim Only port (Figure 4.6B, right). Mice with the fiber placed in the NAc quickly develop a preference for the Pellet+Stim port on the First StimON day (Figure 4.6C, middle), which interestingly diminishes by the Last StimON day (Figure 4.6C, right). Mice with the fiber placed in the LH do not show an emerging preference on the First StimON day (Figure 4.6D, middle), but have a preference quickly emerge for the Pellet Only and Pellet+Stim ports on the Last StimON day (Figure 4.6D, right). Control mice show a pattern similar to those mice with the fiber placed in the LH (Figure 4.6 E).

4.4.4 MCHR1 Antagonism with SNAP-94847

The final day of the Acute ORCA paradigm included treatment with SNAP-94847, an MCHR1 antagonist. Unexpectedly, mice with the fiber placed in the LC showed a preference for the Stim Only port after SNAP-94847 antagonism, and decreased preference for the Pellet+Stim port relative to the previous day (Figure 4.7A). All other groups of mice, including those with the fiber placed in the NAc (Figure 4.7B), those with the fiber placed in the LH (Figure 4.7C), and the Cre- control mice (Figure 4.7D) showed the same trend following SNAP-94847 antagonism: a preference for the two ports which delivered food pellets. Expectedly, SNAP-94847 did decrease the number of pellets consumed for all groups observed (Figure 4.4, right).

4.5 Discussion

In this chapter, we demonstrated that the ORCA paradigm detailed in Chapter 3 can be shortened in order to increase throughput. Subsequently, we used the Acute ORCA paradigm to investigate the role of an additional MCH projection: the MCH projection to the LC. Importantly, these experiments are preliminary, and proper interpretation of these findings must acknowledge that the sample sizes are small, with only three mice per group. However, these findings begin to suggest that MCH has different roles in regulating feeding and reward at different efferent sites. We have shown that the MCH projection to the NAc is likely involved in attributing reward value to paired food. However, somewhat unexpectedly, we have also observed an effect of the MCH projection to LC on reward. Knowing that the LC is involved in sleep, as is the MCH system, we expected the MCH projection to the LC not to have a role in

feeding or reward. Instead, we saw that mice with the optic fiber placed in the LC showed a preference for ports delivering optogenetic stimulation. This led us to conclude that MCH → LC stimulation is reinforcing.

There is limited existing research investigating the role of MCH projections to the LC, most of which suggests that MCH actions in LC induce sleep or depressive-like behaviors, rather than reward/pleasure as we seem to observe. Microinjection of MCH peptide into rat LC increases the time spent in REM sleep (Monti et al., 2015), as well as increases depressive-like behavior in a variety of rodent behavioral assays for depression/anxiety (Kurban et al., 2024; Urbanavicius et al., 2019; Ye et al., 2018). These results invite a potential caveat: the observation of a depressive-like phenotype after intra-LC injection of MCH may be due to an increase in REM sleep rather than an increase in depressive-like behavior, as a majority of depression/anxiety assays in rodents measure locomotion and mobility, which would necessarily be affected by more time spent sleeping. The depressive-like phenotype is reversed with antagonism of the MCHR1 via SNAP-94847 injection (Ye et al., 2018). MCH effects in LC are likely due to effects on LC-norepinephrine neurons, as a significant decrease in prefrontal cortical levels of norepinephrine was observed following intra-LC injection of MCH (Urbanavicius et al., 2019).

Importantly, the above results are all pharmacological, involving injection of MCH peptide itself, rather than evoked activity of MCH neurons. Our optogenetic stimulation of MCH neurons, however, is likely leading to release of multiple neurotransmitters from MCH neurons. Therefore, it is possible that the effect of MCH peptide itself in LC is anxiogenic, while the effect of other neurotransmitters is rewarding. Interestingly, our mice with the optic fiber placed in LC show slightly different behaviors on the final StimON day (Figure 4.5A, right) compared the SNAP-94847 injection day (Figure 4.7A, left). In the absence of MCHR1 antagonism, mice receiving optogenetic stimulation of MCH terminals in LC show a preference for both the Food+Stim port and the Stim Only port, however following MCHR1 antagonism with SNAP-94847, they show a preference specifically for the Stim Only port. One way to interpret this is that the effect of fast neurotransmitter release from MCH terminals in LC is rewarding, but MCH neuron release of MCH peptide is responsible for attributing that rewarding value to paired food. Although a more parsimonious explanation is that MCHR1 antagonism with SNAP-94847 reduces feeding generally, with no effect on the reinforcing properties we observe in this task.

More work remains to be done in order to fully assess the role of individual neurotransmitters in LC.

One study used fiber photometry to show that MCH neurons are preferentially activated during exploratory rearing compared to other behaviors (Concetti et al., 2024). They go on to show that optogenetic activation of MCH neurons in LH leads to increased rearing behavior, and that SNAP-94847 injection leads to decreased rearing behavior (Concetti et al., 2024). This effect may be governed by LC norepinephrine activity, as optogenetic excitation of LC led to decreased LH MCH activity as measured by fiber photometry, and optogenetic inhibition of LC led to increased LH MCH activity (Concetti et al., 2024). While this work implicates both MCH peptide activity and LC neuron activity in one behavior, they do not physiologically show that the behavior is due to activity of MCH neurons terminating in LC, but rather imply an LC → MCH directionality of the effect. It may be that LC activity and MCH activity interact to increase an animal's propensity for exploration. This exploratory drive may be a positive stimulus which could explain our observation that mice voluntarily nose poke for LC MCH stimulation.

This work is the first to show a potential relationship between LC MCH terminal activation and reward value of feeding. More work must be done to further examine this relationship, first and foremost by increasing the sample size of mice in this group. Following this, later experiments should examine the neurotransmitter release involved in this effect in order to fully understand the behavioral role of the MCH projection to LC.

4.6 Figures

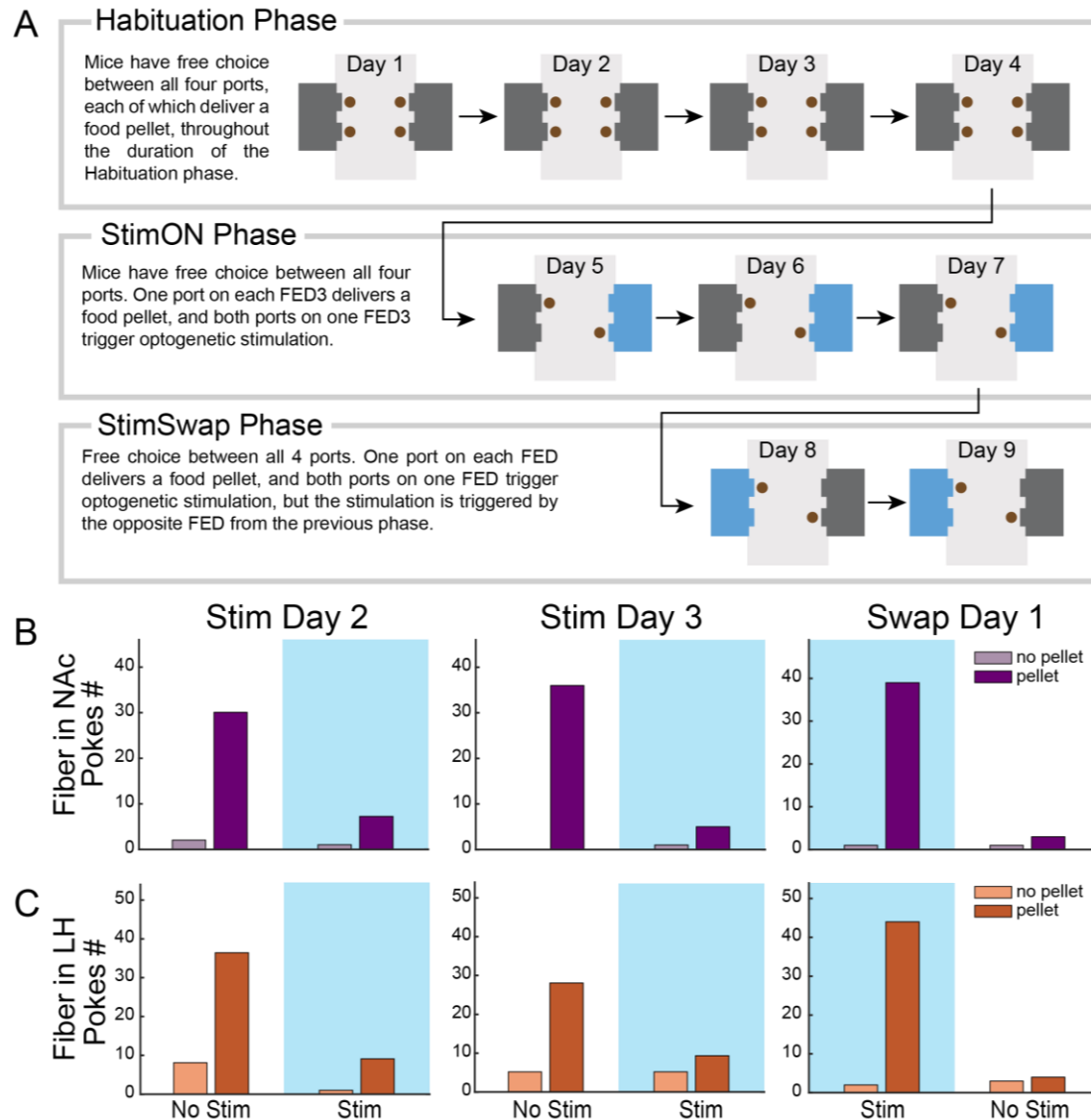


Figure 4.1 Description of Acute ORCA paradigm: First Iteration

A. Behavior timeline/description for the first iteration of the Acute ORCA paradigm. The Habituation phase lasted four days total; during this time, mice had free choice between all four food ports on both FED3s, each of which delivered a food pellet. The StimON phase lasted three days; during this time, one port on each FED3 delivered a food pellet (denoted by brown circle) and both ports on one FED3 (shown in blue) triggered optogenetic stimulation. The StimON phase was followed by a two-day StimSwap phase, during which the stimulation was switched to be triggered by the opposite FED3, while the ports which trigger optogenetic stimulation remain the same. Mice were placed in the behavior box for one hour per day and food restricted when outside of the box.

B. Data from an individual mouse, with optic fiber placed over NAc, which underwent the Acute ORCA paradigm in its first iteration. This mouse preferred the Pellet Only port during StimON days, and preferred the Pellet+Stim port on the Swap day. The mouse had a preference for a particular port regardless of stimulation availability.

C. Data from an individual mouse, with optic fiber placed over LH, which underwent the Acute ORCA paradigm in its first iteration. This mouse preferred the Pellet Only port during the StimON days, and preferred the Pellet+Stim port on the first Swap day, meaning that the mouse had a preference for a particular port regardless of stimulation availability.

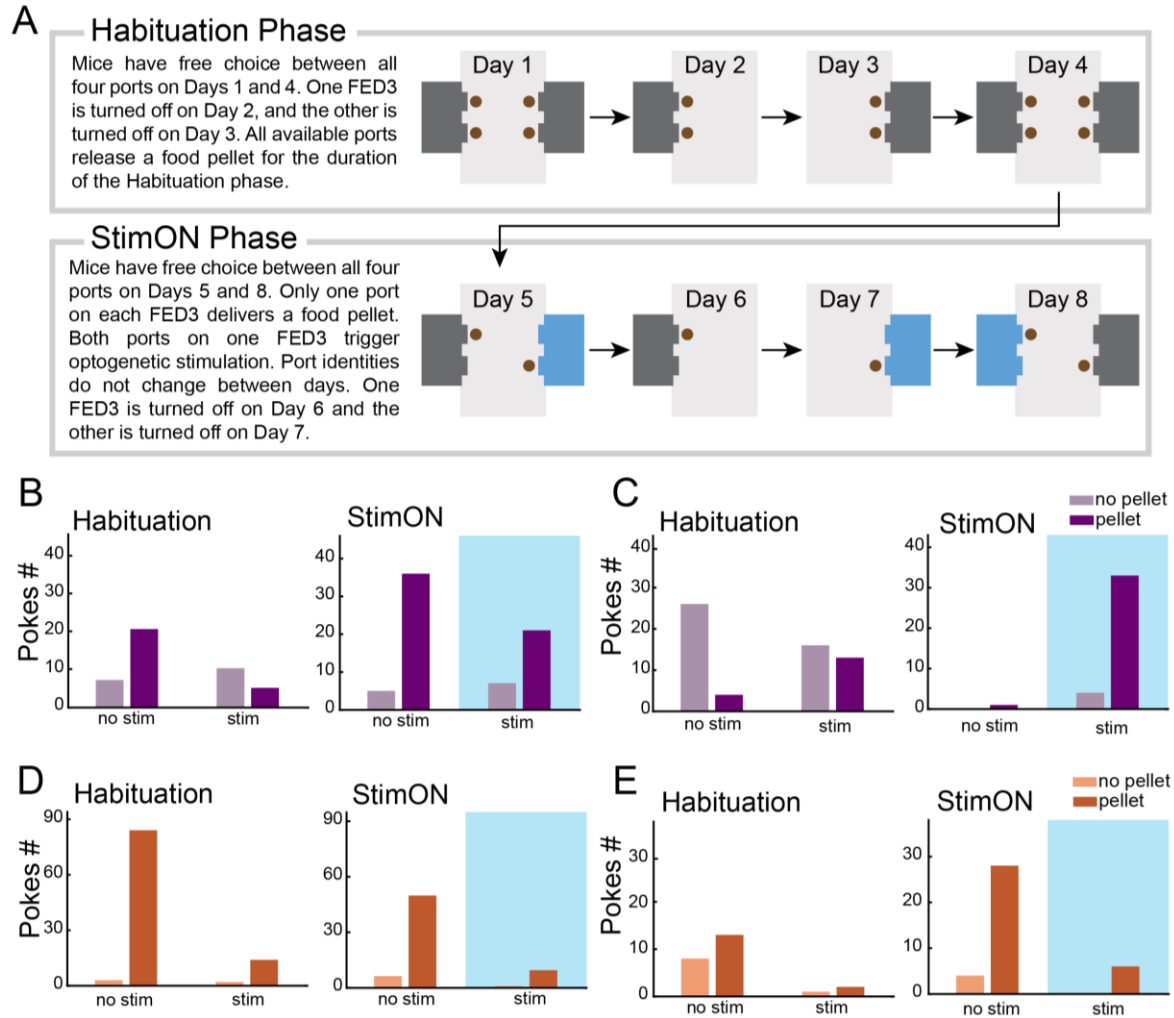


Figure 4.2 Description of Acute ORCA paradigm: Second Iteration

A. Behavior timeline/description for the first iteration of the Acute ORCA paradigm. The Habituation phase lasted four days total; on Days 1 and 4, both FED3s were available and all four ports released a food pellet. However, on Days 2 and 3, one FED3 was unavailable on each day, as a way to force mice to sample from each port. The StimON phase lasted 4 days; during which one port on each FED3 released a food pellet (denoted with brown circle), and both ports on one FED3 (shaded in blue) triggered optogenetic stimulation. On Days 5 and 8, both FED3s were available. However, on Days 6 and 7, one FED3 was unavailable on each day, forcing mice to sample from all ports. Port identities remained the same throughout the StimON phase, and only their availability changed. Mice were placed in the behavior box for one hour per day and food restricted when outside of the box.

B. Data from an individual mouse, with optic fiber placed over NAc, which underwent the Acute ORCA paradigm in its second iteration. This mouse preferred the Pellet Only port throughout, but showed an increase in preference for the Pellet+Stim port when the stimulation was available.

C. Data from an individual mouse, with optic fiber placed over NAc, which underwent the Acute ORCA paradigm in its second iteration. This mouse showed a substantial increase in preference for the Pellet+Stim port when stimulation became available.

D. Data from an individual mouse, with the optic fiber placed over the LH, which underwent the Acute ORCA paradigm in its second iteration. This mouse preferred the Pellet Only port throughout, but showed a relative increase in preference for the Pellet+Stim port when the stimulation was available.

E. Data from an individual mouse, with the optic fiber placed over the LH, which underwent the Acute ORCA paradigm in its second iteration. This mouse showed an increase in preference for the Pellet Only port when stimulation became available.

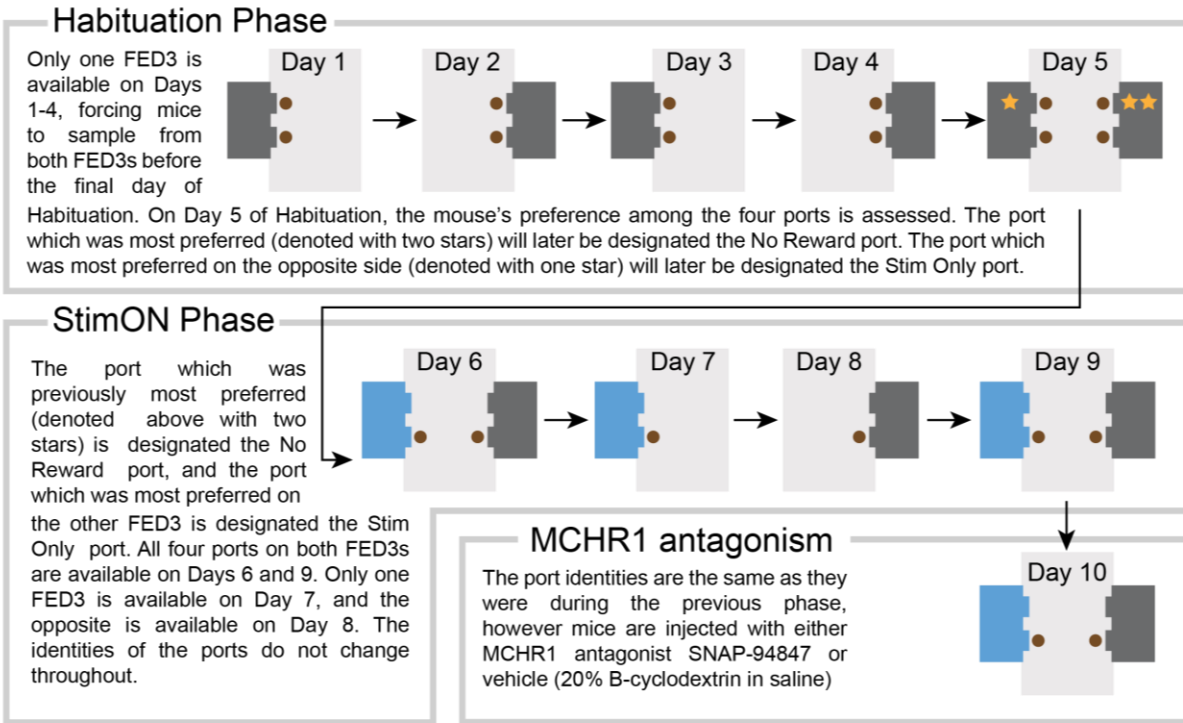


Figure 4.3 Description of Acute ORCA paradigm: Final Iteration

This is the Acute ORCA paradigm which most accurately recapitulated the ORCA results in Chapter 3. The Habituation phase was 5 days long, and only one FED3 was available for Days 1-4, with the available FED3 alternating sides for those four days. On Day 5, both FED3s, and all four ports were available. On this final day of Habituation, the mouse's preference between all four ports was assessed. The most-preferred port (denoted with two stars) would later be designated the No Reward port. The most-preferred port on the opposite FED3 (denoted with one star) would later be designated the Stim Only port. Throughout the Habituation phase, all available ports delivered a food pellet. The StimON phase lasted 4 days; during which one port on each FED3 released a food pellet (denoted with brown circle), and both ports on one FED3 (shaded in blue) triggered optogenetic stimulation. On Days 6 and 9, both FED3s were available. However, on Days 7 and 8, one FED3 was unavailable on each day, forcing mice to sample from all ports. Port identities remained the same throughout the StimON phase, and only their availability changed. On Day 10, immediately following StimON, mice were injected with MCHR1 antagonist SNAP-94847 (or vehicle; 20% B-cyclodextrin in saline) while port identities remained the same as the final StimON day.

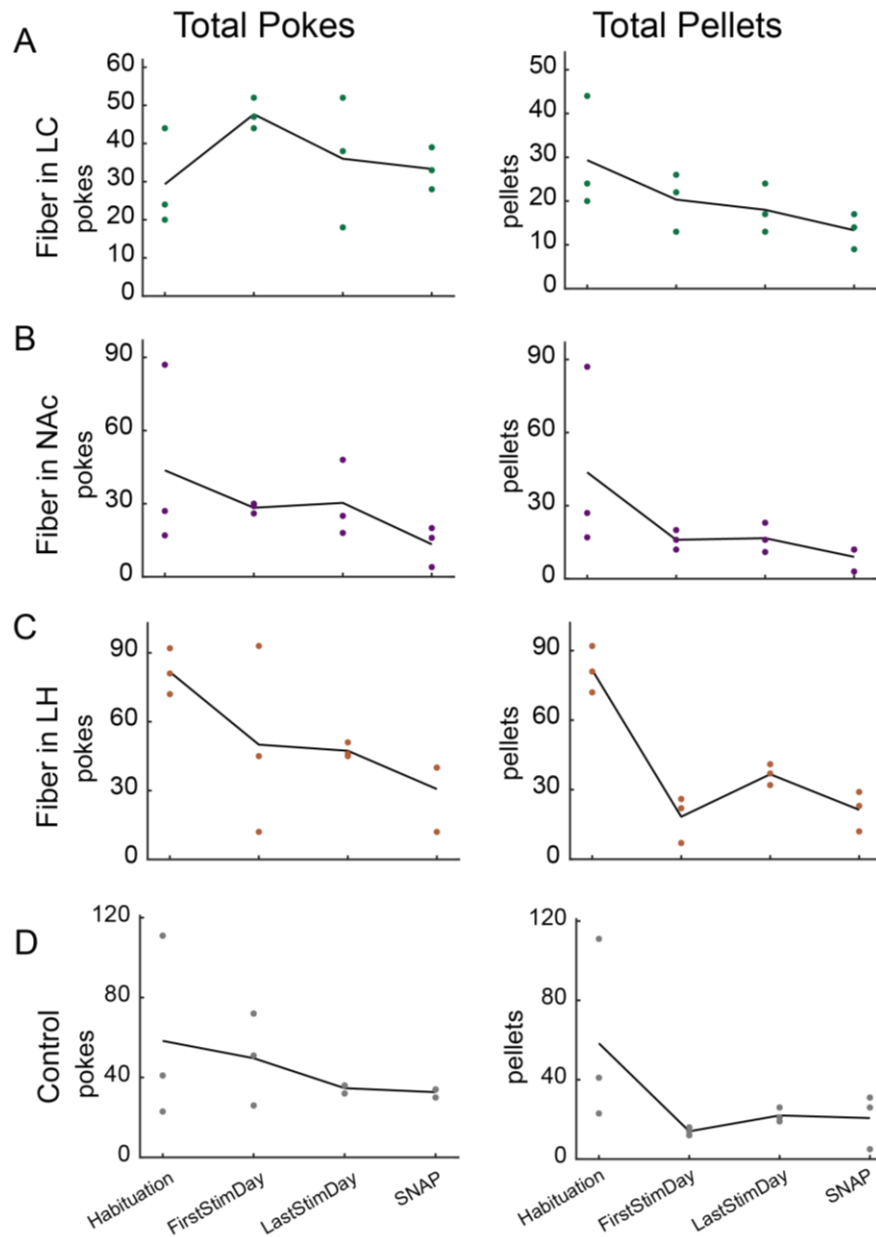


Figure 4.4 Total pokes and pellets consumed in Acute ORCA paradigm

A. Total number of pokes at all ports combined (left) and total number of pellets eaten at all pellet-delivering ports combined across the Habituation, First StimON, Last StimON, and SNAP day of the experiment for the group of mice (n=3) with optic fiber placed over the LC.

B. Total number of pokes at all ports combined (left) and total number of pellets eaten at all pellet-delivering ports combined across the Habituation, First StimON, Last StimON, and SNAP day of the experiment for the group of mice (n=3) with optic fiber placed over the NAc.

C. Total number of pokes at all ports combined (left) and total number of pellets eaten at all pellet-delivering ports combined across the Habituation, First StimON, Last StimON, and SNAP day of the experiment for the group of mice (n=3) with optic fiber placed over the LH.

D. Total number of pokes at all ports combined (left) and total number of pellets eaten at all pellet-delivering ports combined across the Habituation, First StimON, Last StimON, and SNAP day of the experiment for the group of control mice (Cre $-/-$, n=3) with optic fiber placed over either the LC, NAc, or LH.

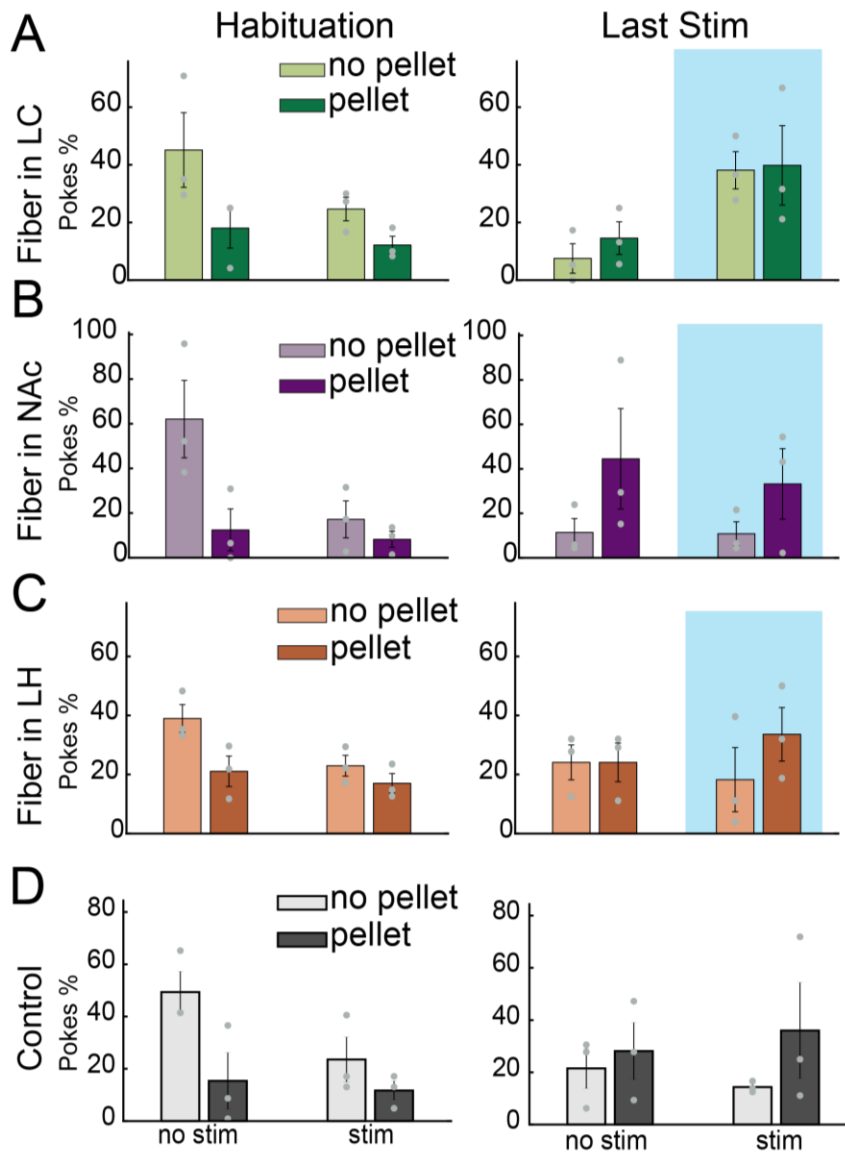


Figure 4.5 Validation of Acute ORCA paradigm: Final Iteration.

A. Group data from mice with optic fiber placed over LC (n=3) which underwent the Acute ORCA paradigm in the final iteration, described in Figure 4.3. When receiving MCH terminal stimulation in LC, mice seem to shift their preference to the two stimulated ports (regardless of pellet availability) once stimulation becomes available.

B. Group data from mice with optic fiber placed over NAc (n=3) which underwent the Acute ORCA paradigm in the final iteration, described in Figure 4.3. When receiving MCH terminal stimulation in NAc, mice seem to shift their preference toward the Pellet+Stim port once stimulation becomes available, consistent with data shown in Chapter 3.

C. Group data from mice with optic fiber placed over LH (n=3) which underwent the Acute ORCA paradigm in the final iteration, described in Figure 4.3. When receiving MCH cell body stimulation in LH, mice seem to prefer the ports which deliver a food pellet, consistent with data shown in Chapter 3.

D. Group data from Control mice (Cre^{-/-}) with optic fiber placed over either LC, NAc, or LH (n=3) which underwent the Acute ORCA paradigm in the final iteration, described in Figure 4.3. Mice prefer the ports which deliver a food pellet during the StimON phase.

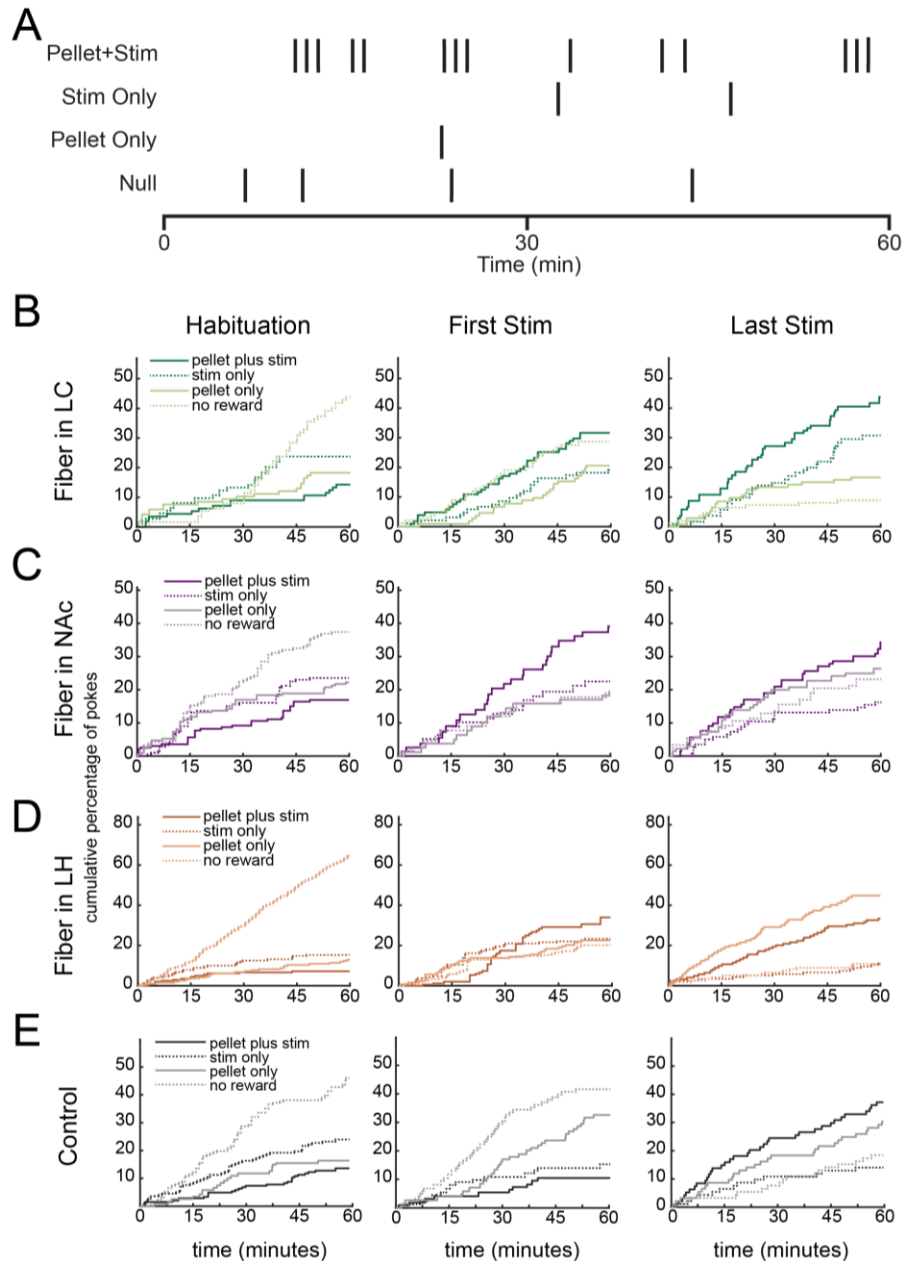


Figure 4.6 Temporal patterns of preference emergence

A. Raster plot of a representative mouse's poking behavior over the hour spent in the behavior chamber. Vertical lines represent pokes at the specified port.

B. Cumulative poking behavior for the group of mice (n=3) with optic fiber implanted over the LC. By the end of the first StimON day (middle) and through the final StimON Day (right) mice prefer the two ports delivering optogenetic stimulation.

C. Cumulative poking behavior for the group of mice (n=3) with optic fiber implanted over the NAc. On the first StimON day (middle) these mice prefer the Pellet+Stim port, however this preference lessens by the second StimON day (right).

D. Cumulative poking behavior for the group of mice (n=3) with optic fiber implanted over the LH. By the final StimON day (right) these mice prefer the two ports which deliver a food pellet.

E. Cumulative poking behavior for the group of control mice (Cre -/-, n=3) with optic fiber implanted over either the NAc, LH, or LC. These mice prefer the ports which deliver a food pellet.

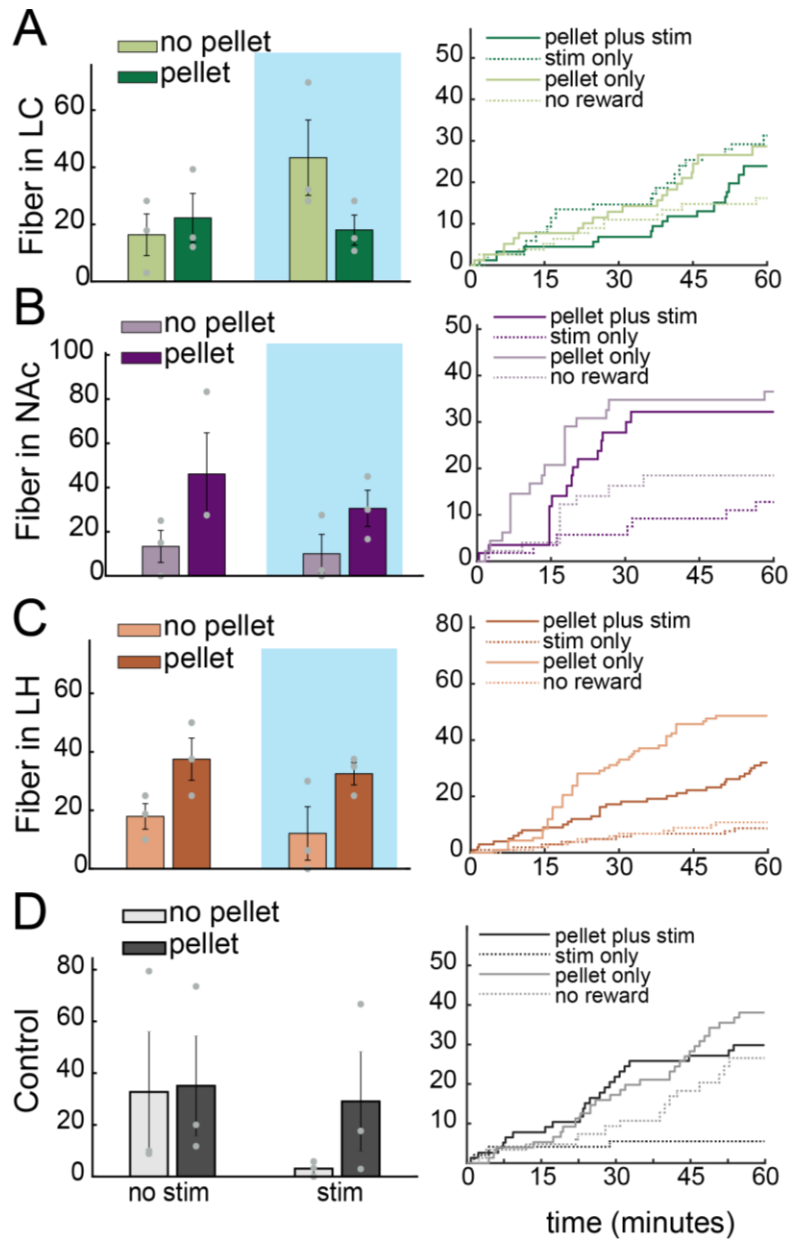


Figure 4.7 MCHR1 Antagonism in the Acute ORCA paradigm

A. Port preference (left) and cumulative poking behavior (right) following injection with MCHR1 antagonist SNAP-94847 for the group of mice ($n=3$) with optic fiber placed over the LC. Following MCH antagonism, these mice seem to show a decrease in preference for food ports delivering a food pellet, yet maintain a preference for the Stim Only port.

B. Port preference (left) and cumulative poking behavior (right) following injection with MCHR1 antagonist SNAP-94847 for the group of mice ($n=3$) with optic fiber placed over the NAc. Following MCH antagonism, these mice seem to prefer the two ports releasing a food pellet, with little-to-no preference for ports delivering stimulation.

C. Port preference (left) and cumulative poking behavior (right) following injection with MCHR1 antagonist SNAP-94847 for the group of mice ($n=3$) with optic fiber placed over the LH. Following MCH antagonism, these mice seem to prefer the two ports releasing a food pellet, with little-to-no preference for ports delivering stimulation.

D. Port preference (left) and cumulative poking behavior (right) following injection with MCHR1 antagonist SNAP-94847 for the group of control mice (Cre $-/-$, $n=3$) with optic fiber placed over either the LC, NAc, or LH. Following MCH antagonism, these mice seem to prefer the two ports releasing a food pellet.

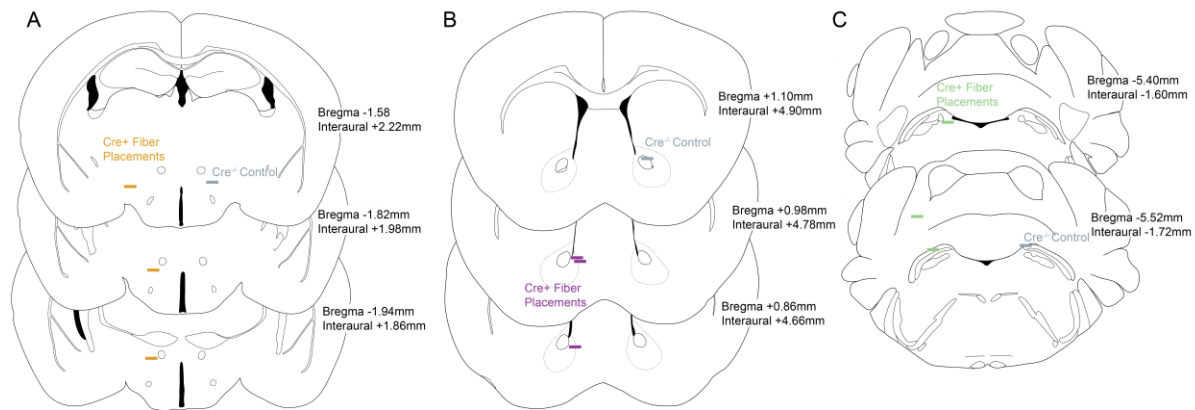


Figure 4.8 Fiber Placements for Acute ORCA Experiments

A. Lateral Hypothalamus (LH) fiber placements for mice shown in Acute ORCA data Figures 4.4 through 4.7. Cre+ Experimental mice are shown on the left hemisphere in orange. Cre- control mouse is shown on the right hemisphere in grey.

B. Nucleus Accumbens (NAc) fiber placements for mice shown in Acute ORCA data Figures 4.4 through 4.7. Cre+ Experimental mice are shown on the left hemisphere in purple. Cre- control mouse is shown on the right hemisphere in grey.

C. Locus Coeruleus (LC) fiber placements for mice shown in Acute ORCA data Figures 4.4 through 4.7. Cre+ Experimental mice are shown on the left hemisphere in green. Cre- control mouse is shown on the right hemisphere in grey.

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Chapter 5 Discussion

5.1 Overview

The earliest investigations into the MCH system demonstrated its role in feeding (Ludwig et al., 2001; Shimada et al., 1998). However, the MCH system plays a number of roles in the mammalian brain. The anatomy of the MCH system is of note here, as this work has hypothesized and subsequently demonstrated a divergence in the behavioral role of MCH neuronal activity based on the area to which the neurons project.

In Chapter 2, we demonstrated that the well-established role for MCH in promoting and maintaining REM sleep is not shared by all MCH neurons. When we optogenetically activated MCH cell bodies in LH continuously for three hours, we observed that mice had an increase in REM sleep bouts. However, when we optogenetically activated MCH terminals in NAc, we saw no effect on REM sleep. Additionally, neither location of optogenetic activation resulted in a change in food intake behavior. This indicates that the behavioral role of MCH neurons is projection-specific, and suggests the existence of distinguishable MCH subpopulations.

In Chapter 3, we characterized the role of the MCH projection to NAc in non-homeostatic feeding through use of the ORCA paradigm. This paradigm allows mice the choice to poke between four different food ports, each resulting in a different outcome: (1) a port which does nothing, (2) a port which delivers a food pellet, (3) a port which triggers a brief optogenetic stimulation, and (4) a port which both delivers a food pellet and triggers a brief optogenetic stimulation. When given this choice, we observed that mice receiving optogenetic stimulation of MCH cell bodies in LH had a preference for ports delivering a food pellet. However, those mice receiving optogenetic stimulation of MCH terminals in NAc had a preference specific to the port delivering both food and optogenetic stimulation. This led us to conclude that MCH projections to NAc play a role in enhancing the reward value of food, but not necessarily in promoting feeding, as they did not do so in Chapter 2.

In Chapter 4, we successfully modified the ORCA paradigm in order to increase throughput and be implemented on a more acute schedule. This involved changes to the structure

of both the Habituation and StimON phases of the experiment, but we were ultimately able to recapitulate the expected results under the acute conditions. Once this was established, we used this acute paradigm to test the involvement of a second projection target, that to the LC, in non-homeostatic feeding behavior. We observed that mice receiving MCH terminal stimulation in LC showed a preference for stimulation-paired ports, and concluded that MCH → LC stimulation may be reinforcing. This experiment must be repeated to increase the sample size.

5.2 MCH in the nucleus accumbens

Our conclusions in all three data chapters of the present thesis serve to further characterize the role of NAc MCH activity. While previous work has done so to a limited extent, the majority of work focuses on MCH peptide rather than MCH neuron activity. It is known that MCH injection into the NAc leads to increased feeding for multiple hours following injection (Guesdon et al., 2009), but this effect is seen regardless of whether the peptide is injected into the NAc or into the third ventricle (Gomori et al., 2003; Guesdon et al., 2009). Further, MCH delivery into the NAc shell increases food intake, and MCHR1 antagonism in NAc shell reduces food intake (Georgescu et al., 2005). Similarly to the effects of MCH peptide delivery into NAc, infusions of either AMPA receptor antagonists (Maldonado-Irizarry et al., 1995; Stratford et al., 1998) or GABA agonists (Reynolds & Berridge, 2001; Stratford & Kelley, 1997, 1999) into the NAc shell also lead to increased food intake. This combination of results suggests that increases in feeding can be driven by overall reduction in NAc shell neural activity.

Indeed, when measuring neuronal firing in the NAc shell during performance of operant behaviors for palatable food, Taha & Fields observed a decrease in action potential firing at a majority of neurons (Taha & Fields, 2006). Our findings here complement earlier findings which suggest that MCH may be involved in this orexigenic decrease in NAc activity. Here we show an increase in preference for a particular food following increased MCH neuron activity in NAc, though we do not have the methodological resolution to understand the NAc-specific mechanism by which this occurs. Assuming that there is release of MCH peptide evoked by our optogenetic stimulation parameters, others have demonstrated the inhibitory effect of MCH peptide in the NAc. In medium spiny neurons (MSNs) of the NAc shell, GluR1 expression is reduced, as is EPSC amplitude, after application of MCH peptide (Sears et al., 2010). Reduction of EPSC

amplitude by MCH peptide is modulated by an MCH-mediated increase in basal K⁺ conductance, leading to reduced action potential firing (Sears et al., 2010).

However, inconsistent with the above result, while we observed an increase in preference for a food associated with NAc MCH stimulation, we still observed a decrease in the raw amount of food mice consumed on days when stimulation was available regardless of the location of stimulation (Figure 3.2). This was also observed by another group which saw a significant decrease in mice's food intake during a time when optogenetic stimulation of MCH neurons was

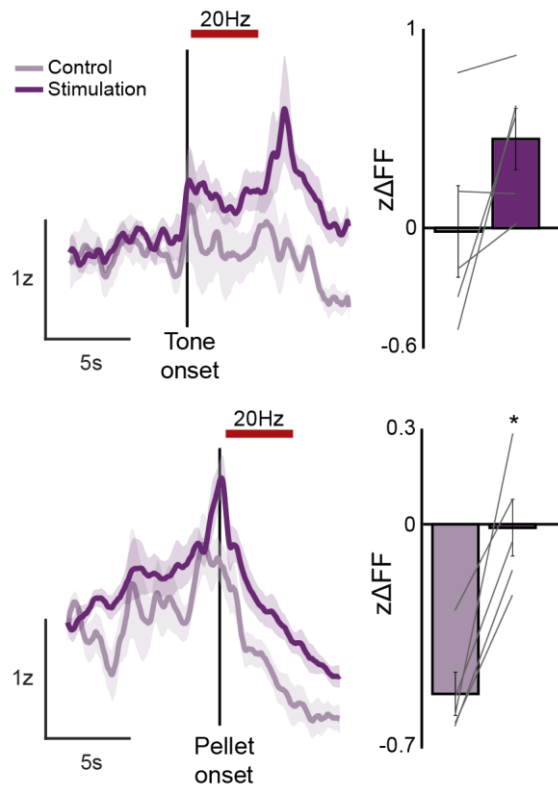


Figure 5.1 Optogenetic stimulation of MCH terminals in NAc leads to increased dopamine release in medial NAc shell in response to food rewards

Unpublished data from Dr. Liam Potter, Burgess Lab. Across-animals (n=5) mean tone-aligned (top) or pellet-aligned (bottom) medial NAc shell dopamine release in MCH-ChrimsonR expressing mice, opto-paired and unpaired trials. Period of opto-excitation in paired trials indicated in red. Quantification inset (Mean zΔFF over stim window, Opto unpaired (OFF) vs. paired (ON) trials, tone two-tailed paired t-test N.S., pellet $**p < 0.0051$, $t = -5.5734$, $df = 4$). Acute opto-excitation of MCH axon terminals in the mNAcSh enhances DA release in response to food-rewards.

delivered (Dilsiz et al., 2019). However, due to differences in methodology, it is difficult to determine whether this finding does, in fact, contradict findings suggesting that MCH infusion increases food intake. Optogenetic stimulation, used here and in Dilsiz et. al., is not limited to just release of MCH peptide, and would instead include release of any relevant neurotransmitters from activated neurons. Additionally, while optogenetic stimulation lasts on the order of seconds, MCH peptide may remain bioavailable for much longer following infusion. Therefore, more work is necessary to understand the specific mechanistic details of NAc-projecting MCH activity.

One probable explanation is that NAc-projecting MCH neurons intersect with the mesolimbic dopamine system. Mesolimbic dopamine circuitry projecting from ventral tegmental area (VTA) to the NAc is involved with responses to both drugs of abuse (Koob & Bloom, 1988; Wise & Bozarth, 1985) and food reinforcers (Hoebel et al., 1989; Kelley & Berridge, 2002), making this a likely target for our observed effect

of NAc-projecting MCH neurons on non-homeostatic feeding. As discussed in Chapter 1, MCHR1 is abundantly expressed in the NAc medium spiny neurons which make up the mesolimbic dopamine pathway (Georgescu et al., 2005). Additional work suggests that MCH may be dependent on dopamine in order to reinforce consumption (Domingos et al., 2013). MCH optogenetic stimulation can induce dopamine release in response to tastants which do not normally induce such dopamine release, and MCH neuron ablation leads to a near complete decrease in dopamine release in response to tastants which normally do induce dopamine release (Domingos et al., 2013). Indeed, data from our own laboratory (Potter et al, unpublished) has demonstrated that MCH terminal stimulation in NAc leads to increased dopamine release in the medial NAc shell (Figure 5.1). However, conflicting data have also been reported, showing that MCH directly inhibits dopaminergic cells (Duncan Spencer et al., 2024), and also that MCH fails to inhibit dopamine release in MCHR1-KO mice (M. J. Chee et al., 2019). The behavioral implications of this dopamine release need to be further characterized in order to fully understand how MCH activity impacts mesolimbic dopamine systems, as well as how this may play a role in reinforcing feeding behavior.

5.3 MCH involvement in a variety of behaviors

The MCH system is, somewhat paradoxically, implicated in many disparate, and seemingly unrelated behaviors. We want to address two main explanations for this disparity: (1) the variety of different projections of MCH neurons to downstream brain areas, and (2) the release of a variety of neurotransmitters and peptides from MCH neurons. In the present work, we focus on the hypothesis that the heterogeneity of MCH behavioral roles can be explained by anatomically distinct subpopulations of MCH neurons projecting to specific downstream areas, with a focus on the MCH projection to the NAc. By focusing on the NAc projection, we have begun to develop a proof of principle, that it is indeed possible for MCH neurons to have specified roles based on their projection target. There are a number of other projections of MCH whose behavioral roles we do not address in this work.

Of particular interest to the project are the projections to the locus coeruleus (LC) and to the arcuate nucleus of the hypothalamus (ARC). While we've begun to explore the MCH projection to the LC in Chapter 4, we recognize that more work needs to be done in order to make complete sense of this projection. The LC is known to be involved with regulation of

sleep/wake states, for example, which is a role we did not investigate in the present work. Conversely, several populations of neurons in the ARC are relevant to energy balance and homeostatic feeding. The MCH projection to the ARC has the potential to be involved with regulation of feeding in a way that differs from the MCH projection to the NAc, which we may be able to resolve in our ORCA paradigm.

Additionally, there is the fact that MCH neurons release multiple neurotransmitters which may play different roles in behavior. We do not account for this here (except for the use of MCHR1 antagonist SNAP-94847 in Chapter 4), but we know that release of different neurotransmitters will lead to different postsynaptic effects. We therefore expect that the type of neurotransmitter release from particular MCH neurons during particular times or contexts is another mechanism by which MCH leads to such diverse effects on behavior. An example of this mechanism has been shown by Schneeberger and colleagues, who observed that when VGLUT2 was genetically knocked out of MCH neurons, mice lost their sucrose preference, but when MCH peptide was knocked out of MCH neurons, this did not happen (Schneeberger et al., 2018). This is one example of MCH neurons' glutamate release having a different behavioral role than MCH neurons' release of MCH peptide, and sets a precedent for more examples to come in the future.

5.4 Clinical and translational potential of the MCH system

Due to its known influence over appetite, energy balance, and metabolism, the MCH system, and MCHR1 specifically, has been a target of drug development with the goal of developing treatments for clinical obesity. Since 1999, there have been hundreds of patents filed related to MCHR1 antagonists, from more than 40 different companies (Szalai et al., 2014), all with the goal of finding a use for MCHR1 antagonists in obesity treatment. None of these drugs have made it to market, however, heavily due to inefficacy observed in clinical trials. The earliest MCHR1 antagonists were peptides, and were used in basic science research to learn more about the MCH system (MacNeil & Bednarek, 2009), but peptides are vulnerable to degradation, and do not easily cross the blood-brain barrier, so small molecule antagonists are favored clinically.

Numerous small molecule MCHR1 antagonists have been developed since then, the first of which was reported in 2001 (Havnes et al., 2001). To outline the full breadth of these

developments would be outside the scope of the current thesis, but the majority of these compounds failed not due to adverse effects, but due simply to lack of desired effects. Pharmaceutical research groups have shown effects of small-molecule MCHR1 antagonists on feeding in rodents (Borowsky et al., 2002; Takekawa et al., 2002), which have not been replicated in human clinical trials. One drug, NGD-4715, entered phase I clinical trials in 2006, and was determined to be safe and well-tolerated, and also displayed the desirable effect on glucose and lipid metabolism, but showed potential for undesirable interactions with statins commonly prescribed for obesity treatment (Tarrant et al., 2017). Another, Alb-127158(a), was also determined to be safe and well-tolerated in 2001, with no major adverse effects, but did not sufficiently reach the CNS at the expected dose (Moore et al., 2014). Notably, about half of patients in this clinical trial reported difficulty sleeping, which is consistent with the known role of MCH in sleep/wake regulation.

The fact that MCHR1 drugs tend not to induce adverse side effects is promising for future drug development, although the lack of efficacy of the drugs tested is a reason for skepticism. There are numerous results in rodents to suggest that these drugs are, in fact, able to produce the intended effect on body weight and metabolism, and the majority of human trials seem to indicate obstacles related primarily to compounds' bioavailability. It may be the case that many of these small molecule MCHR1 antagonist compounds can be structurally modified in order to more easily pass the blood-brain barrier, or to otherwise improve their absorption, distribution, metabolism, and excretion in favor of higher efficacy (Potter & Burgess, 2022). If pharmaceutical research companies are able to dedicate resources to this end, it may be a lucrative addition to the anti-obesity therapeutic market. The leading anti-obesity treatments are Glucagon-like peptide receptor-1 (GLP1R) agonists, which often trigger extreme nausea and vomiting as side effects, causing up to 60% of patients to stop treatment with these drugs (Jastreboff et al., 2022, 2023; Wadden et al., 2021; Wilding et al., 2021). If challenges regarding bioavailability can be overcome, MCHR1 antagonists could be a promising anti-obesity treatment without the negative side effects of GLP1R agonists.

5.5 Limitations and Future Directions

A limitation to the present thesis is the fact that the observed actions of the MCH system cannot be ascribed to MCH peptide itself. Therefore, the results may not be directly comparable

with earlier work on MCH peptide effects. Due to the limitations of optogenetics as a method, we can draw conclusions only about the activity of MCH-expressing neurons. This could be addressed pharmacologically, as we began to do in Chapter 4 using the MCHR1 antagonist SNAP-94847. By antagonizing the MCHR1, we would limit the effect of MCH neuron activity to that elicited only by release of non-MCH neurotransmitters. This was not feasible in Chapters 2 and 3, as those behavioral paradigms required that the mice live continuously in the behavioral setup, with *ad libitum* access to food. Therefore, keeping the concentration of an injected drug consistent over the period of data collection would not have been possible. However, our decision to develop the acute ORCA paradigm used in Chapter 4 was motivated in part by the desire to allow for such pharmacological manipulations to the paradigm.

Pharmacological intervention could also be used to probe the effects of other neurotransmitters, such as glutamate and GABA, in this behavior. Rather than injecting SNAP-94847, we could inject an antagonist for NMDAR, AMPAR, or GABAR. This blockade would have to be local to the particular area of interest in order to limit extraneous side effects of antagonizing such important and widespread receptors. However, a more clever way to probe the effects of these fast neurotransmitters may be via genetic manipulations. Use of vGLUT2-floxed mice to eliminate expression of the vesicular glutamate transporter in MCH neurons could be further illuminating. These mice have previously been used to identify a role of glutamate release from MCH neurons in sleep (Naganuma et al., 2019), and therefore may prove useful in studies of feeding as well.

An additional limitation is the potential for antidromic stimulation which may back-propagate from MCH terminals receiving optogenetic stimulation to the soma and activate MCH collaterals in unintended areas. Our experimental design, having MCH neurons expressing ChR2 and implanting the fiber over the terminal area of interest, cannot rule out this possibility. If there are MCH neurons with collaterals projecting to both the NAc and elsewhere, or the LC and elsewhere (relevant to Chapter 4), then it may be the case that our observed effects are due to MCH activity in other areas of the brain which we cannot account for. Anatomical experiments aiming to map the collateralization of MCH neural circuitry could lead to additional understanding as to whether this is likely to be confounding our behavioral effect.

There are other MCH-mediated behaviors that have the potential to confound our behavioral readouts, which we have not characterized. The role of the MCH system in anxiety is

still debated, with some groups showing anxiogenic (Georgescu et al., 2005; Lagos et al., 2011; Urbanavicius et al., 2019, 2016, 2014; Ye et al., 2018) and other groups showing anxiolytic effects (Monzón & De Barioglio, 1999; Oh et al., 2020) of MCH peptide infusion across various behavioral assays. Anxiety-like behaviors, if promoted by our MCH stimulation, are likely to confound behavioral readouts for both feeding and sleep. If our stimulation serves to either increase or decrease anxiety levels, animals may be more or less comfortable eating and sleeping in the behavioral chamber environment. The lab has explored ways to include analysis of anxiety-like behaviors within the ORCA paradigm using videography. LabGym, a video analysis software developed for quantifying user-defined animal behaviors (Hu et al., 2022), is one method we have investigated. While data are not shown in the present thesis, LabGym has proven to be an effective tool in our experimental setups, and future experiments will integrate it into our existing pipeline. In addition to helping us quantify additional behaviors such as anxiety, LabGym could add a layer of interpretation to our existing results. Where we see changes in food preference, videography could increase behavioral resolution. We may see, for example, that increase in preference for a particular nose port is accompanied by more time spent sniffing and otherwise investigating that port, rather than just poking for reward. We could see, hypothetically, that poking for food and poking for optogenetic stimulation are temporally related – mice may, for example, poke for food immediately following a poke for stimulation. This type of behavioral readout would enhance the conclusions we are able to make about MCH activity as it relates to consummatory behavior.

Presently, we have identified unique roles for two downstream targets of MCH neurons in feeding behavior using our ORCA paradigm. This is but a small portion of the total number of MCH projections which may play unique roles in an organism's behavior (See Figure 1.1). Future experiments must further characterize the role of other MCH projections in feeding behavior before this system can be comprehensively understood. We hypothesize that there are other areas receiving MCH input that will contribute differently to both homeostatic and non-homeostatic feeding behavior; possibly the Arcuate Nucleus, Nucleus of the Solitary Tract, Ventral Tegmental Area, among others. Because we observed an effect of LH MCH cell body stimulation on REM sleep which was not projection-specific, we expect that there will be MCH projections which contribute to REM sleep and not feeding; possibly the Sublateral Dorsal Nucleus, Periaqueductal Gray, or Hippocampal areas. One paper specifically implicates MCH

projections to medial septum, but not to dorsal raphe nuclei, in REM sleep control (Jego et al., 2013). The areas mentioned are just a small portion of MCH projection targets, so there remains much to be discovered about other projection-specific behavioral roles for MCH activity.

Finally, discrepancies in existing literature, including the present work, must be worked out before the role of MCH neurons in behavior can be fully understood. Some work shows sex differences in MCH-mediated behavior (Terrill et al., 2020), while other work shows no sex difference (Dilsiz et al., 2019; Shimada et al., 1998), but the majority of work on the MCH system fails to address sex differences altogether. There are documented differences in the role of MCH in mice versus rats (Chung, Saito, et al., 2009; Croizier et al., 2010). There must be further characterization of this variety of different factors before the field can fully explain either the anatomy or the functional role of the MCH system, and the MCH peptide, across various aspects of behavior.

The work presented in this thesis makes a meaningful contribution to the field's understanding of the MCH system. By isolating MCH subpopulations based on the behavioral role of individual anatomical projections, we can begin to understand the complexities of this system, and how it is able to influence such a wide variety of behaviors. The MCH system remains a promising target for clinical drug development in treatment of obesity and eating disorders, and further dissociation of the different mechanisms by which MCH influences feeding will only improve the field's ability to develop compounds with therapeutic efficacy.

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