UNIVERSITY OF CALIFORNIA,
IRVINE

The Role of Melanin-Concentrating Hormone Neurons in Repetitive Behavior

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Pharmacological Sciences

by

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DEDICATION

I am grateful to my parents for their love and encouragement.

Todo hombre puede ser, si se lo propone, escultor de su propio cerebro

Santiago Ramón y Cajal

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ACKNOWLEDGMENTS

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Nothing is possible or worth doing without the love and support of my partner, Brandon Reilly. Thank you for always inspiring me.

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EDUCATION

University of California, Irvine — Irvine, CA
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California State University, Long Beach — Long Beach, CA
MS, Biology — 2010

University of California, San Diego
BS, Molecular Biology — La Jolla, CA
BS, Psychology — 2005

RESEARCH EXPERIENCE

University of California, Irvine — Apr. 2011 – present
Graduate Student, Graduate Advisor: Olivier Civelli, PhD

- Developed an in vivo method to activate/inactivate MCH neurons to determine their role in neuropsychiatric disorders.
- Investigated the MCH system's neuroanatomical connections.
- Established an MCH neuron specific oxytocin receptor knockout line.
- Developed a genetically targeted technique to label MCH neuronal inputs using a rabies virus mediated approach.
- Determined the changes in the MCH system during the estrus cycle.

California State University, Long Beach Graduate Student, — Sept. 2007 – Aug 2010
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- Investigated the neurocircuitry controlling sexual receptivity in female rats via dual label fluorescent in situ hybridization and immunohistochemistry.
- Carried out tract tracing study of axonal projections from the arcuate nucleus to the medial preoptic area via Fluorogold pressure injection.
- Behavioral study investigating the lordosis quotient under various hormonal conditions and third ventricle applications of antagonists.

The Scripps Research Institute, La Jolla, CA Sept. 2005 – Aug. 2007
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- Conducted research on positive selection of thymocytes.
- Cloned plasmid vectors for analysis via cell transfection assays.
- Developed a protocol for multiplex ligation assay to distinguish between heterozygous and homozygous mice.

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Research Assistant I, Supervisor: Joan Vaughan

- Assisted with antisera production including preparation of injections, processing of serum, scheduling, and record keeping.
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- Behavioral testing and analysis of transgenic mice and preparation of tissues for immunohistochemical analysis.

Salk Institute for Biological Studies, La Jolla, CA Aug. 2002 – Mar. 2003
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- Organize a friendly forum in which UCI researchers can practice engaging a general audience in their work by providing a short TED-style talk about their research to the local science-loving public.

School of Medicine Graduate Student Advisory Council, University of California, Irvine — Nov. 2014-present
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- Assist the SOM Associate Dean and staff in organizing events aimed to improve graduate student life ranging from social events to professional development seminars.

Diverse Educational Community and Doctoral Experience (DECADE), University of California, Irvine — 2014-present
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Graduate Student Representative, Department of Pharmacology, University of California, Irvine — 2012-2013

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Graduate InterConnect program, University of California, Irvine — 2011-2012
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Sanathara NM, Sun Y, Xu X, Civelli O. Direct monosynaptic inputs to melanin-concentrating hormone neurons of the lateral hypothalamus.
Manuscript in preparation for resubmission to the Journal of Comparative Neurology.

Wang L, Alachkar A, **Sanathara N**, Belluzzi JD, Wang Z, Civelli O. A Methionine-Induced Animal Model of Schizophrenia: Face and Predictive Validity. Int J Neuropsychopharmacol. 2015.

Sanathara NM, Moraes J, Mahavongtrakul M, Sinchak K. (May, 2014). Estradiol upregulates progesterone receptor and orphanin FQ colocalization in arcuate nucleus neurons and opioid receptor-like receptor-1 expression in proopiomelanocortin neurons that project to the medial preoptic nucleus in the female rat. Neuroendocrinology 2014;100:103-118.

Parks GS, Olivas ND, Ikrar T, **Sanathara NM**, Wang L, Wang Z, Civelli O, Xu X. (May, 2014). Histamine inhibits the melanin-concentrating hormone system: implications for sleep and arousal. J Physiol. 592(Pt 10):2183-96.

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BOOK CHAPTERS

Sinchak K, Dalhousay L, **Sanathara N**. Chapter 7 - Orphanin FQ-ORL-1 Regulation of Reproduction and Reproductive Behavior in the Female. Vitamins & Hormones: Academic Press.

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Sinchak, K., Garcia, B. L., Bowlby, R., Charukulvanich, P., Garcia, M.P., **Sanathara, N.M.** (November, 2010). Mu-opioid receptor neurons and opioid receptor-like receptor neurons in the medial preoptic nucleus project to the region of the ventromedial nucleus of the hypothalamus. Poster presented at the Society for Neuroscience, San Diego, CA.

Sanathara, N.M. (August, 2010). Colocalization of melanin-concentrating hormone receptor-1 and dopamine receptor 1and 2 in the shell of the nucleus accumbens. Talk given at the University of California, Irvine 2010 Summer Research Program Symposium. Irvine, CA.

Sanathara, N.M. (May 2010). Orphanin FQ acts through opioid receptor-Like receptor-1 in β-Endorphin neurons in the arcuate nucleus of the hypothalamus that project to the medial preoptic nucleus to facilitate lordosis. Dissertation talk given at California State University, Long Beach Masters Program, Long Beach, CA.

Sanathara, N.M. (January, 2010). Estradiol upregulates proopiomelanocortin and opioid receptor-like receptor-1 mRNA in arcuate neurons that may project to the medial preoptic nucleus. Talk given at the California State University Biotechnology Symposium. Santa Clara, CA.

Sanathara, N.M., Bowlby, R., Charukulvanich, P., Sinchak K. (January, 2010). Estradiol upregulates proopiomelanocortin and opioid receptor-like receptor-1 mRNA in arcuate neurons that may project to the medial preoptic nucleus. Poster presented at the California State

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Garcia, B.L., Garcia, T., **Sanathara, N.M.**, Sinchak, K. (January 2010). Mu-opioid receptor neurons in the medial preoptic nucleus project to the region of the ventromedial hypothalamus and the arcuate nucleus of the hypothalamus. Poster presented at the California State University Biotechnology Symposium. Santa Clara, CA.

Sanathara, N.M., Vignovich, M., Sinchak, K. (October 2009). Proopiomelanocortin neurons in the arcuate nucleus project to the medial preoptic nucleus and express opioid receptor-like receptor-1 mRNA. Poster presented at the Society for Neuroscience, Chicago, IL.

Sinchak, K., Phillips, J., Charukulvanich, P., **Sanathara, N.M.** (October, 2009). Opioid receptor-like receptor-1 antagonism blocks orphanin FQ facilitation of lordosis and inhibition of mu-opioid receptor internalization in the medial preoptic nucleus of estradiol primed rats. Poster presented at the Society for Neuroscience, Chicago, IL.

Sanathara, N.M., Bowlby, R., Phillips, J., Charukulvanich, P., Sinchak, K. (October, 2009). UFP-101 antagonism of opioid receptor-like receptor-1 blocks orphanin FQ facilitation of lordosis and mu opioid receptor internalization in the medial preoptic nucleus of estradiol primed rats. Poster presented at the College of Natural Sciences and Mathematics Student Research Symposium, Long Beach, CA.

Sanathara, N.M., Charukulvanich, P., Vignovich, M., Sinchak, K. (August, 2009). Arcuate Nucleus Neurons Expressing proopiomelanocortin and opioid receptor-like receptor-1 mRNA project to the medial preoptic nucleus. Poster presented at the College of Natural Sciences and Mathematics Student Research Symposium, Long Beach, CA.

Sanathara, N.M., Vignovich, M., B.L. Garcia, Sinchak, K. (November, 2008). Opioid receptor-like receptor-1 and proopiomelanocortin fluorescent in situ hybridization colocalization in the arcuate nucleus of the hypothalamus. Poster presented at the Society for Neuroscience, Washington D.C.

Sanathara, N.M. (April 2008). Progesterone acts through ORL-1 in β-endorphin neurons in the arcuate nucleus of the hypothalamus that project to the medial preoptic nucleus to facilitate lordosis. Dissertation talk given at California State University, Long Beach Masters Program in order to advance to Masters candidacy.

Sanathara, N.M. (April, 2007). Do beta-endorphin neurons that project to the MPN express the receptor for OFQ (ORL-1)? Talk given at California State University of Long Beach From the Bench Presentation, Long Beach, CA.

ABSTRACT OF THE DISSERTATION

The Role of Melanin-Concentrating Hormone in Repetitive Behavior

By

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Doctor of Philosophy in Pharmacological Sciences

University of California, Irvine, 2016

Professor Olivier Civelli, Chair

Melanin Concentrating Hormone (MCH) is a cyclic neuropeptide that has been evolutionarily conserved as an important sensory integrator. Over 60 years of literature suggests that it plays a central role in modulating a diverse set of behaviors which include feeding, stress, reward, reproduction and sleep in mammals. This broad regulation of functions is suggested by the wide distribution of its receptor, MCHR1, throughout the brain. However, little is known about what inputs directly regulate MCH neurons. Identifying these presynaptic inputs would provide a more nuanced and thorough understanding of the regulatory role of the MCH system. The work presented in this thesis aims to characterize the direct presynaptic inputs received by MCH neurons and identify the neurochemical composition of these regulatory inputs. We identified several brain nuclei that provide direct innervation to MCH neurons using rabies mediated circuit mapping technique in a cre-dependent MCH transgenic line. We further evaluated the neurochemical composition of the presynaptic cell population through immunofluorescent analysis. The second part of this work investigates the functional role of one of the identified presynaptic subpopulations, the oxytocin-MCH neural circuit. Using neuroanatomical, behavioral, and pharmacological tools we identify a novel role for the MCH

system and a previously undefined neurocircuit by which oxytocin regulates repetitive behavior through the MCH system. The behavioral and anatomical findings reported in our study warrant further investigation of the role of this circuit in disorders symptomatic of perseverative behaviors.

Chapter 1

Introduction to the Melanin-Concentrating Hormone System

The MCH system

Melanin-concentrating hormone (MCH) is a 19 amino acid peptide that was first discovered from the pituitary extracts of chum salmon (Kawauchi et al., 1983). In fish, MCH serves as a hypophysiotropic factor that is released from the pituitary to instantly affect pigmentation in response to changing environmental stimuli (Baker, 1994). It alerts the animal and coordinates its response to external stimuli. In mammals, MCH is not made in the pituitary but instead in two hypothalamic nuclei, the lateral hypothalamus area (LHA) and the zona incerta (ZI) (Bittencourt et al., 1992; Knigge et al., 1996). The MCH gene is expressed in very high levels in the hypothalamus just below the levels of oxytocin (OT) and arginine vasopressin (AVP) (Bittencourt et al., 1992). Although its synthesis is restricted mainly to these two nuclei, MCH immunoreactivity is found widely throughout the CNS and concentrated in the prefrontal cortex, nucleus accumbens, hippocampal formation, basal ganglia, diencephalon, and brainstem (Figure 1.1) (Bittencourt et al., 1992). MCH is classically known for its role in feeding and energy metabolism, but more recent studies demonstrate its role in a wide range of functions including sleep, reward, learning and memory, and psychiatric disorders (Adamantidis and de Lecea, 2009; Chung et al., 2009; Chung et al., 2011; Sherwood et al., 2012; Konadhode et al., 2014).

The ability to investigate the physiological functions of the MCH system became feasible when five groups independently reported the discovery of its G protein coupled receptor, melanin-concentrating hormone receptor 1, MCH1R (Bachner et al., 1999; Chambers et al., 1999; Lembo et al., 1999; Saito et al., 1999; Shimomura et al., 1999). A second receptor, MCHR2 (Sailer et al., 2001), was reported but in rodents only MCH1R is expressed (Tan et al., 2002). MCHR1 is a G protein coupled receptor that shares 40% homology to somatostatin receptors (Kolakowski et al., 1996) and belongs to the rhodopsin family class A receptors (Fredriksson et al., 2003). MCH binds to MCHR1 with ~1 nM affinity in vitro and binding results in the pertussis toxin sensitive dose-dependent inhibition of forskolin-elevated levels of intracellular cAMP, demonstrating that MCHR1 signals through G i/o (Bachner et al., 1999; Gao and van den Pol, 2001; Pissios et al., 2003). At higher doses, MCH binding to MCHR1 leads to transient increase in calcium concentration suggesting coupling to Gq in vitro (Saito et al., 2001; Shimomura et al., 1999, (Lembo et al., 1999). Electrophysiological studies show that MCHR1 is primarily inhibitory in vivo (Gao and van den Pol, 2002).

MCHR1 distribution throughout the rat brain follows the pattern of MCH immunoreactivity and is expressed in high levels within the nucleus accumbens shell, hippocampus, caudate putamen, amygdala, locus coeruleus, dorsal raphe, thalamus, hypothalamus and central cortex (Hervieu et al., 2000; Saito et al., 2001, Bittencourt et al., 1992). Both MCH immunoreactivity and MCHR1 expression throughout the CNS implicate the involvement of the MCH system in mediating a number of diverse physiological functions.

The MCH System Regulates a Diverse Set of Behaviors

MCH Regulation of Feeding

The best characterized role for the MCH system is its involvement in food intake and energy metabolism. Central administration of MCH increases food intake (Qu et al., 1996; Rossi et al., 1997), and overexpression of the peptide leads to obesity and insulin resistance (Ludwig et al., 2001). MCH precursor knockout mice display a hypophagic and lean phenotype with an absence of fat deposits and altered metabolism (Shimada et al., 1998). MCH1R knockout mice have reduced body fat and show resistance to diet induced obesity (Chen et al., 2002; Marsh et al., 2002). These data suggest an important role of the MCH system in coordinating food intake to regulate energy homeostasis.

MCH Regulation of Stress

MCH1R is highly expressed in the limbic system and is shown to modulate stress, anxiety, depression. MCH1R knockout mice display reduced anxiety (Roy et al., 2006), and MCH1R knockout female mice show decreased depressive behavior (Roy et al., 2007). However, central MCH injections have shown to be both anxiolytic (Monzon and De Barioglio, 1999; Monzon et al., 2001; Kela et al., 2003) and anxiogenic (Smith et al., 2006). Similarly, while some MCH1R antagonist have shown anxiolytic effects (Borowsky et al., 2002; Millan et al., 2008), other antagonist that have shown decreases in food intake behavior have shown no difference in anxiety in comparison to controls (Basso et al., 2006). It should be noted that MCH1R knockout animals display hyperlocomotion making the interpretation of results in these studies of anxiety and depression difficult. In addition, high doses of selective MCH1R

antagonist have also been shown to act on other GPCRs (Chaki et al., 2005). These studies suggest a complex role of the MCH system in the regulation of stress and anxiety.

MCH Regulation of Reward

MCH1R is highly expressed in the nucleus accumbens shell (NAcSh), an area involved in reward processing. MCH1R is co-expressed in dopamine receptor (D1 and D2) positive cells in the NAcSh (Chung et al., 2009). Both dopamine induced neuronal firing in the NAcSh and cocaine induced locomotion are potentiated by the MCH system. Acute injections of MCH1R antagonist, TPI1361-17, into the NAcSh reduce cocaine self-administration and inhibit reinstatement (Chung et al., 2009) indicating a role for the MCH system in reward processing. Disruption of the MCH system through either pharmacological agents or genetic deletion of the MCHR1 impairs conditioned reinforcement, suggesting that MCHR1 is important for conditioned incentive learning (Sherwood et al., 2012).

MCH Regulation of Reproduction

MCH has also been shown to be a critical link between energy expenditure and reproduction. In ovariectomized estrogen primed female rats MCH microinfusion into the medial preoptic area facilitates a luteinizing hormone surge whereas microinfusion directed into the ZI inhibits the luteinizing hormone surge (Murray et al., 2006). MCH also attenuates the excitatory effect of the neuropeptide kisspeptin on GnRH cells (Wu et al., 2009). Since both kisspeptin and kisspeptin receptor knockout mice fail to enter puberty (Lapatto et al., 2007),

MCH regulation of kisspeptin responsive GnRH neurons may prove to be an important link between energy expenditure and the onset of puberty.

MCH Regulation of Sleep

Recent studies demonstrate an important role of the MCH system in sleep. Dense MCH immunoreactivity has been found in brain areas important in regulating sleep-wake cycle such as the ventrolateral preoptic area, peduncolopontine area, and tuberomammillary nucleus (Elias et al., 2008). In vivo recordings of LHA MCH neurons during the onset of REM sleep show an increased firing rate in head-fixed animals (Hassani et al., 2009). Corroborating these findings, lateral ventricle infusion of MCH in rats markedly increased the number of REM periods at the onset of the dark phase (Verret et al., 2003). Optogenetic stimulation of MCH neurons both increased the period of REM (Jego et al., 2013) and transition from NREM to REM (Tsunematsu et al., 2014). These studies highlight MCH as an important neuromodulator in the sleep-wake cycle, particularly in REM sleep.

The Neuroantomy and Neurochemistry of the MCH System

The ontogenesis of the MCH system in the rat occurs very early on in the brain between embryonic day 10 and 16 within the tuberal hypothalamic lateral zone (Brischoux et al., 2001). As the lateral hypothalamic neuropil develops, MCH neurons are pushed out leading to the differentiation of more medial and lateral subpopulations. Parcellation of the MCH system reveals that the most dense population of MCH neurons reside in the lateral tier of the LHA within the dorsal, medial, parvicellular, and magnocellular regions of the LHA (Swanson et al., 2005). In the rodent brain MCH immunoreactive fibers can be found throughout the CNS in the

cortex, hippocampal formation, basal ganglia, diencephalon, brainstem, pons, and reticular formation (Bittencourt et al., 1992).

MCH neurons express several neurotransmitters and neuropeptides. These cells are thought to be mainly GABAergic and co-release GABA following stimulation (Jego et al., 2013). The expression of GABA-synthesizing enzymes, GAD65 and GAD67, colocalize with MCH cells (Elias et al., 2001; Harthoorn et al., 2005). MCH neurons also express glutamate transporters, vGLUT1 and vGLUT2 (Del Cid-Pellitero and Jones, 2012), and the excitatory amino-acid transporter EAAT1 (Collin et al., 2003). Recent work suggests that MCH neurons are not solely GABAergic and photostimulation of these cells led to the release glutamate into the lateral septum (Chee et al., 2015).

Previous work in our lab has focused on the analysis of a number of neuropeptide receptors colocalized within MCH neurons to gain insight into the regulatory circuitry of the MCH system. This work identified several receptors that show high co-expression with MCH neurons including orphanin FQ/N, orexin, MCH and histamine receptor 3 (Table 1.1) (Parks et al., 2014a). Coexpression of several receptors that were previously not linked to the MCH system: kisspeptin receptor 1, neurotensin receptor 1, somatostatin receptor 1, somatostatin receptor 2, neuropeptide S receptor, and cholestocystokinin receptor A also showed co-expression with MCH perikarya (Parks et al., 2014b). Some of these identified receptors regulate similar physiological functions as the MCH system. Further functional studies may reveal that these receptors mediate their effects through the MCH system.

The LHA is one of many regions regulating energy metabolism and feeding but the best positioned to respond to endogenous and environmental cues by communicating with nuclei such

as the nucleus accumbens which regulate motivated behaviors. Therefore, understanding the chemoarchitecture of the LHA, and in particular the MCH system, facilitates the identification of particular neural circuits that could potentially regulate a number of disorders that involve motivated behaviors.

Objective of the Present Study

The aim of the present studies is to provide new information concerning the organization of direct presynaptic inputs to MCH-expressing neurons of the LHA. By defining the organization of these projections and elucidating their identity, we can better understand the regulation and functional organization of MCH neurons within the LHA which regulate a diverse set of physiological behaviors. The present work outlined in Chapter 2 of this thesis identifies several neuropeptides that provide direct input to MCH neurons may modulate the activity of the MCH neuropeptide system. The studies reported in Chapter 3 investigate the role of the MCH system in repetitive behavior and define a novel neural circuit, the oxytocin-MCH, by which MCH modulates OT mediated decrease in repetitive behaviors.

Figure 1.1

A schematic of melanin-concentrating hormone projections in the rat. Brain regions: olfactory bulb, amygdala (Amyg), cortex, hippocampal formation (HF), thalamus, locus coeruleus, pons, brain stem.

Figure 1.1

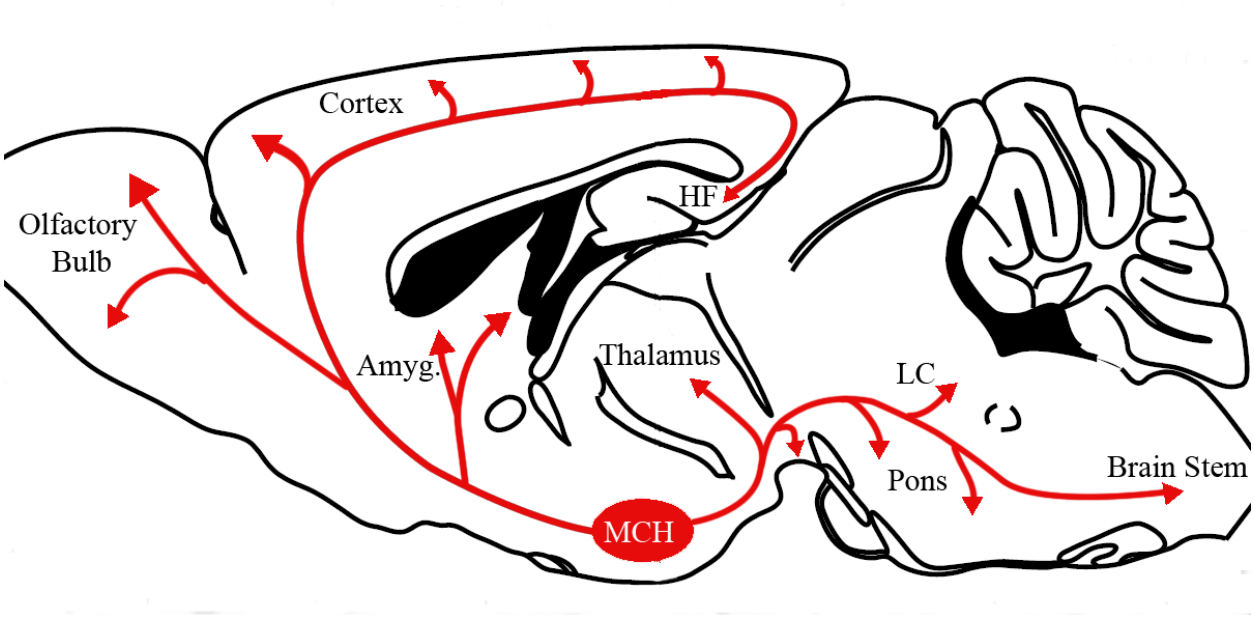
Cortex
HF
Olfactory
Bulb
Amyg.
Thalamus
LC
Pons
Brain Stem
MCH

Table 1.1

Percentage of MCH Neurons Found To Express Each Neuropeptide Receptor

Receptor	Probe Percentage colocalization
OFQ/N	87.50%
MCHR1	64.80%
OrexR1	36.20%
OrexR2	51.40%
H3R	70.00%

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Chapter 2

Direct Monosynaptic Inputs to Melanin-Concentrating Hormone Neurons of the Lateral Hypothalamus

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ABSTRACT

The melanin-concentrating hormone (MCH) system is a neuropeptide system that plays an important regulatory role in food intake, energy balance, glucose homeostasis, sleep, and reward-related behaviors. MCH expression is restricted to the lateral hypothalamus and the zona incerta, while its receptor, MCH1R, has widespread distribution. Although much is known about where MCH neurons project, very little is known about the presynaptic inputs received by these neurons. To reveal the direct monosynaptic inputs to lateral hypothalamic MCH neurons we used a Cre dependent, genetically modified rabies-mediated circuit mapping technique in the pMCHCre transgenic line. We analyzed the distribution of presynaptic neurons from each projection area and found that MCH neurons received the strongest inputs from within the hypothalamus including the paraventricular nucleus, lateral hypothalamus, supramammillary nuclei, and mammillary body. We further investigated the nature of these presynaptic inputs and found that MCH neurons receive direct presynaptic input from oxytocin, arginine vasopressin, MCH, orexin, histamine, and orphanin FQ neurons elucidating novel neural circuits. This study lays the groundwork for further functional studies to investigate these neural circuits.

INTRODUCTION

Melanin-concentrating hormone (MCH) is part of a neuronal system that has been shown to regulate various responses related to energy homeostasis, reproduction, sleep/wake cycle and reward (Shimada et al., 1998; Ludwig et al., 2001; Griffond and Baker, 2002; Chung et al., 2009; Tsunematsu et al., 2014). In rodents this system relies on the actions of one neuropeptide, MCH, acting through a single receptor, MCH1R (Saito et al., 1999). MCH is uniquely expressed in only two brain nuclei, the lateral hypothalamus (LH) and zona incerta (ZI). The MCH receptor, MCH1R, has widespread distribution in the brain. MCH-expressing neurons project widely to several areas in the brain, most prominently to the nucleus accumbens, the hypothalamus, the olfactory tubercle and the prefrontal cortex. Central administration of MCH increases food intake (Qu et al., 1996; Rossi et al., 1997), and MCH overexpression leads to obesity and insulin resistance (Ludwig et al., 2001). MCH precursor knockout mice display a hypophagic and lean phenotype with no fat deposits and altered metabolism (Shimada et al., 1998). MCH1R knockout mice have reduced body fat and show resistance to diet induced obesity (Chen et al., 2002; Marsh et al., 2002). MCH1R is highly expressed in the nucleus accumbens shell (NAcSh), an area involved in reward processing. MCH1R is co-expressed in dopamine receptor (D1 and D2) positive cells in the NAcSh (Chung et al., 2009). These studies suggest a complex role of the MCH system in the regulation of food intake, energy expenditure, and reward processing. Despite the important role of the MCH system in a number of central functions, very little is known about the neurocircuitry regulating the system. Many G protein coupled receptors have been colocalized with MCH synthesizing neurons and have been shown to modulate the system. Electrophysiology studies have shown that MCH neurons are excited by oxytocin (OT), arginine vasopressin (AVP) (Yao et al., 2012), and inhibited by orphanin FQ/N (OFQ/N) (Parsons and

Hirasawa, 2011), and histamine (Parks et al., 2014a) through their cognate receptors expressed by MCH cells. However, many of these GPCR ligands have also been shown to act on other cells within the LHA. Thus, it is difficult to assess whether these peptides are affecting the general tone in this nucleus through volume transmission or acting directly on MCH neurons. In order to understand the functional implications of the MCH system we need to understand how the MCH system is regulated and thus define the neuronal pathways that regulate it. This can be achieved by identifying the presynaptic inputs that impinge on MCH synthesizing neurons. To reveal the direct monosynaptic inputs to lateral hypothalamic MCH neurons we used rabies-mediated circuit mapping technique in the pMCHCre transgenic line. Although recent studies using rabies mediated circuit mapping have revealed nuclei that show direct presynaptic inputs to MCH neurons, the chemoarchitecture of these presynaptic neurons remains uncharacterized. We identify distinct presynaptic neurons that connect directly to MCH neurons in the LHA. The mapping of these novel connections lays the groundwork for further functional examination of these neural circuits which may reveal novel functions of MCH system.

MATERIALS AND METHODS

Animals

Tg(Pmch-cre)1Lowl/J mice (Jackson Laboratories, Bar Harbor, Maine, USA) that express Cre-recombinase (Cre) in MCH neurons were maintained on a C57BL/6 genetic background by intercross breeding. Tg(Pmch-cre) adult males were used for rabies mediated circuit mapping experiments. Littermate wildtype mice were used for control experiments.

Adult male animals were group housed under controlled conditions (temperature 21°C ± 2°C; 12 hr light–dark cycle, lights on at 7:00 AM) with free access to water and food.

Stereotaxic Viral Injection

All stereotaxic surgery was conducted in Dr. Xiangmin Xu's lab using a stereotaxic machine (MyNeuroLab two arm stereotaxic with Angle Two software) in accordance with University of California, Irvine Institutional Animal Care and Use Committee. Under isoflurane anesthesia, 6 week aged Pmch-cre mice were injected with 0.2 ul of the AAV8 helper virus encoding the B19G glycoprotein, TVA (an avian virus envelope protein receptor) and GFP (AAV-DIO-HTB-GFP, ~ 2 x 1011 genome units/ml) was injected unilaterally into the LHA (flat skull coordinates from bregma: anteroposterior (AP), −1.82 mm; mediolateral (ML), +.91 mm; and dorsoventral (DV), −5.25 (Paxinos and Franklin, 2001)). Virus delivery was performed using a pulled glass pipette (tip diameter, ~30 μm) loaded with virus, lowered into the brain and pulsed into the brain at a rate of 20 - 30 nl/min, with 10 ms pulse duration using the Picospritzer (General Valve, Hollis, NH). The pipette was left at the injection site for 5 min after completion of the injection to prevent backflow of virus. The injection pipette was then slowly withdrawn and the mouse was removed from the stereotaxic frame and the incision closed with either wound clips or sutures. Following surgery, mice were individually housed and allowed to recover for three weeks in rooms with controlled humidity and temperature and access to regular lab chow and water ad libitum to allow the helper virus infection to take (n = 4/group). The mice then underwent a similar surgery (same coordinates) for the delivery of the G-deleted EnvA pseudotyped rabies virus (EnvA-SADDGmCherry, 0.1 ul, ~2 x 109 infectious units/ml). Mice

were housed separately in BSL-2 level suites with regular lab chow and water ad libitum for 10 days prior to transcardial perfusion.

Histology and Immunostaining

Mice were anesthetized with isoflurance and perfused transcardially with PBS and chilled 4% paraformaldehyde. The brains were removed rapidly from the cranium and postfixed overnight in 4% paraformaldehyde. Brains were then transferred to 30% sucrose in a 0.1M phosphate buffer solution, pH 7.5 and stored at 4°C until sectioning. Coronal brain sections 30 μm thick were collected using a freezing microtome (Leica SM2010R, Germany) and stored in cryoprotectant (sodium phosphate buffer, pH 7.4, with 0.9% saline, 30% sucrose, and 30% ethylene glycol) at 20°C until use. Every one out of 4 sections was counter-stained with 10 μM DAPI and mounted for examination and quantification of starter cells and their presynaptic cells in different brain structures between Bregma 3.56 to -6.12 as described in the mouse brain atlas (Paxinos and Watson, 2001). Sections were stained with a GFP antibody to amplify GFP signal resulting from the helper AAV expression to identify starter cells. Due to the robust mCherry expression no immunostaining against mCherry was performed. For GFP staining, a chicken anti-GFP primary antibody (Aves Labs, 1:500 dilution) followed with an Alexa Fluor (AF) 488-conjugated donkey anti-chicken secondary antibody (Jackson ImmunoResearch, 1:200 dilution) applied to the sections. Selected sections showing strong synaptic labeling were also immunostained with various antibodies for neurochemical characterization of these presynaptic cells. To immunochemically identify presynaptic cells the following antibodies were used: rabbit anti-oxytocin primary antibody (Millipore, 1:2000), rabbit anti-Vasopressin primary antibody (Millipore, 1:1500) followed with Alexa 488-conjugated donkey anti-rabbit secondary

antibody (Jackson ImmunoResearch, 1:200); rabbit polyclonal anti-MCH antibody (antibody courtesy of W. Vale, Salk Institute, La Jolla, CA, USA, 1:150,000) followed with Dylight 647-conjugated donkey anti-rabbit secondary antibody (dilution, Jackson ImmunoResearch, 1:200); goat anti-orexin A antibody (Santa Cruz, 1:15,000) followed with Alexa 488-conjugated donkey anti-goat secondary antibody Jackson ImmunoResearch, 1:200), polyclonal guinea pig anti-OFQ/N antibody (Neuromics Inc., 1:500) followed by Alexa Fluor 488 conjugated goat anti-guinea pig secondary antibody (Jackson, 1:200); Histidine Decarboxylase (Progen, 1:1000) followed with Dylight 647-conjugated donkey anti-rabbit secondary antibody (Jackson ImmunoResearch, 1:200). Stained sections were mounted on glass slides and coverslipped using ProLong Gold Antifade Reagent (Life Technologies, Carlsbad, CA, USA) and allowed to dry in the dark. Slides were stored at −20°C until imaging with a fluorescence microscope (Biorevo BZ-9000; Keyence) or a confocal microscope (LSM700; Carl Zeiss). Image stitching was completed by using BZ-II Analyzer (Keyence) and images were adjusted for brightness and contrast using Adobe Photoshop CS5. Quantitative analyses of labeled direct synaptic connections was examined across a series of sections and labeled cells and brain nuclei were manually defined using the mouse brain atlas (Paxinos and Franklin, 2001).

Results

Transsynaptic Labeling of Direct Monosynaptic Inputs to MCH Neurons using Rabies Mediated Circuit Mapping

To demonstrate monosynaptic inputs to LHA MCH neurons, we used a retrograde transsynaptic tracing system based on a modified rabies virus, SADΔG-EGFP (EnvA)

(Wickersham et al., 2007). We used pMCHCre transgenic mice in which only MCH cells express Cre-recombinase (Parks et al., 2014a)(Supplementary Figure 2.1). We targeted LHA MCH neurons by injecting the helper virus, AAV-DIO-HTB-GFP, which encodes: 1) the rabies envelope glycoprotein (G) necessary for trans-synaptic spreading of the rabies virus, 2) the TVA receptor, an avian receptor protein that confers infection capability to rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein (EnvA) and 3) a GFP reporter (Figure 2.1 A-B). Four weeks following the injection of the helper virus, we injected mice in the same site with a pseudotyped G glycoprotein deleted rabies viral SADΔGmCherry strain that contains an mCherry reporter, to infect TVA expressing MCH cells. Ten days later, allowing time for the pseudotyped rabies virus to monosynaptically spread to cells that are directly presynaptic to MCH cells, we collected tissue for histological examination. Helper virus signal detected by GFP expression was colocalized with MCH neurons within the LHA and infected "starter cells" were identified by the expression of GFP and mCherry ($n = 52.8 \pm 4.2$ per animal across four cases; Figure 2.1 C). As anticipated, in control wild-type litter-mates (pMCHCre -/-) injected with the same viruses, we did not detect any mCherry-positive neurons (Figure 2.1 D) indicating viral specificity for cre expressing cells.

Input Patterns of the LHA MCH Neurons

Presynaptic inputs to LHA MCH starter cells were detected by mCherry signal in several nuclei spanning the rostrocaudal extent of the brain including the nucleus accumbens (ACb), lateral and medial preoptics areas (LPO/MPA), paraventricular nucleus (PVH), supraoptic nucleus (SO), dorsomedial and ventromedial hypothalamus (DMH/VMH), lateral hypothalamus (LHA), arcuate nucleus of the hypothalamus (ARH), zona incerta (ZI), ventral tegmental area

(VTA), supramammillary nucleus (SuM), and mammillary bodies (MMB) (Figure 2.2 A). The presynaptic inputs were primarily ipsilateral to the starter neurons, however, a few contralateral inputs coming from the hypothalamus and mainly from the PVH were detected (data not shown). The number of direct presynaptic input cells was measured across the rostrocaudal extent of the brain (Figure 2.2 B) and a total of 3076.5 ± 320.0 direct presynaptic neurons projected to LHA MCH starter neurons. The majority of inputs came from within hypothalamic nuclei, with the PVH, LHA, MMB and SuM providing the main inputs to MCH starter cells (Figure 2.3 A). To account for variability between animals, we normalized the amount of inputs from each brain nuclei to the number of MCH starter cells generating a connection strength index (CSI), defined as the ratio of the number of presynaptic neurons versus the number of starter neurons) (Figure 2.3 B, Table 2.1). The LHA, SuM, MMB, and PVH provided the strongest input to MCH starter cells with CSIs of 35.34. ± 5.19, 5.14 ± 0.51, 3.74 ± 0.63, and 2.08 ± 0.48, respectively.

Neurochemical Characterization of Hypothalamic Inputs to MCH Neurons

We further characterized a subpopulation of presynaptic neurons within the hypothalamus to gain further insight on which neuropeptides directly communicate to MCH cells. To characterize the identity of PVH presynaptic neurons we performed immunohistochemical analysis of rabies labeled presynaptic cells for OT and AVP, which were both reported to depolarize LHA MCH neurons (Yao et al., 2012). We evaluated the percentage of presynaptic cells from the PVH that express OT and AVP and also measured the total percentage of PVH OT and AVP neurons that formed direct presynaptic contact with MCH starter cells. The population of mcherry labeled presynaptic neurons in the PVH that express OT is 1.86% ± 1.4% (Figure 2.4 A-C, 2.4 G) and AVP is 5.15% ± 2.6% (Figure 2.4 D-F, 2.4 G).

The total percentage of OT immunoreactive cells that show presynaptic labeling is 0.70% ± 0.5% (Figure 2.4 F). The total percentage of AVP immunoreactive cells that show presynaptic labeling is 1.53% ± 0.6% (Figure 2.4 F).

LHA presynaptic neurons showed a significant input to MCH starter cells. To characterize the identity of LHA presynaptic neurons we performed immunohistochemical analysis of rabies labeled presynaptic cells for MCH and orexin, since our group previously identified significant colocalization of MCHR1, OXR1, and OXR2 in MCH neurons (Parks et al., 2014b). The population of mcherry labeled presynaptic neurons that express MCH is 40.3% ± 6.7% (Figure 2.5 A-C, 2.5 G) and orexin is 61.0% ± 6.3% (Figure 2.5 D-F, 2.5 G). The total percentage of MCH immunoreactive cells that show presynaptic labeling is 52.9% ± 3.3% (Figure 2.5 F). The total percentage of orexin immunoreactive cells that show presynaptic labeling is 84.0% ± 3.3% (Figure 2.5 F).

Our previous work identified a functional connection between MCH neurons and histamine (Parks et al., 2014a). To assess what percentage of presynaptic neurons were histaminergic, we used antibody against histidine decarboxylase, a marker for cells synthesizing histamine (Schwartz et al., 1970). The population of mcherry labeled presynaptic neurons that express HDC is 32.4% ± 2.9% (Figure 2.6 A-C, 2.6 G) and the total percentage of HDC positive immunoreactive cells that show presynaptic labeling is 27.4% ± 3.4% (Figure 2.6 F).

The highest reported colocalization of a neuropeptide reporter expressed in MCH neurons is opioid receptor like receptor 1 (ORL-1), the receptor for the neuropeptide OFQ/N. We

therefore investigated the colocalization of OFQ/N in presynaptically labeled neurons. The population of mcherry labeled presynaptic neurons that express OFQ/N is 79.6% ± 3.1% (Figure 2.6 A-C, 2.6 G) and the total percentage of OFQ/N positive immunoreactive cells that show presynaptic labeling is 21.1% ± 3.3% (Figure 2.6 F).

Discussion

MCH neurons in the LHA are excellent homeostatic sensors and receive many innervations from cells within the hypothalamus as well as other nuclei. In the present study, rabies mediated transsynaptic tracing was used to identify the major presynaptic nuclei that project to LHA MCH neurons and neurochemically characterize these inputs providing an important first step in elucidating the neural circuitry regulating the MCH system. In particular, this data represents a systematic study of the direct presynaptic populations projecting to MCH starter cells located in the perifornical region of the LHA. The primary inputs received by this population were from the PVH, LHA, SuM and MMB. Based on previous studies within our lab and the broader literature available on MCH inputs we focused our immunohistochemical analysis on presynaptic inputs on OT, AVP, MCH, orexin, HDC, and OFQ/N.

The neuropeptide OFQ/N showed the highest colocalization within the population of presynaptic input neurons to MCH starter cells. Nearly 80% of OFQ/N was found colocalized with presynaptically labeled cells. However, this only represented a little over 20% of the total OFQ/N neuron population. OFQ/N is expressed throughout the CNS with dense expression within hypothalamic nuclei (Neal et al., 1999). The greatest reported receptor colocalization in MCH neurons is of ORL-1 (90%), the cognate receptor for OFQ/N, demonstrating that most

MCH neurons are regulated by the OFQ neuropeptide system. Electrophysiological data show that OFQ/N potently inhibits the MCH system by activating GIRK channels (Parsons and Hirasawa, 2011). Interestingly, OFQ/N infusions directed specifically into the perifornical region, the site of MCH starter neurons in this study, leads to hypophagia leading to a decrease in both regular chow and sugar in rats (Parsons et al., 2012). This effect is not seen when the injection is outside the perifornical region of the LHA. Whether this effect is mediated by MCH neurons or another perifornical subtype remains to be investigated.

The neuropeptide showing the second highest colocalization within the population of presynaptic input neurons to MCH starter cells is the neuropeptide orexin and a significant amount of the orexin cell population (84%) provides direct synaptic input to MCH neurons. Our group previously showed that both Orexin R1 and R2 mRNA colocalized with MCH expressing neurons (Parks et al., 2014b). Ultrastructural studies investigating the relationship between orexin and MCH reported dense orexin innervation of MCH neurons within the rat hypothalamus (Guan et al., 2002). These data along with our present results provide a strong anatomical link between MCH and orexin neurons. Functional connectivity is supported by electrophysiological experiments showing that orexin neurons excite MCH cells (van den Pol et al., 2004), while MCH neurons inhibit orexin neurons (Rao et al., 2008). Recent studies have elucidated the complex role of MCH and orexin neurons in sleep (Tsunematsu et al., 2014). These neuropeptides have reciprocal actions on sleep-arousal circuitry in rodents in which MCH promotes sleep and orexin promotes arousal (Konadhode et al., 2014).

A significant amount of the presynaptic population is immuopositive for the MCH neuropeptide. An even higher amount of the total population of MCH neurons provide presynaptic input to MCH starter cells providing further support for autoregulation of the MCH system. A high level of MCHR1 colocalization in MCH neurons was reported by our group (Parks et al., 2014b). Another group also reported that the application of MCH on MCH neurons in the LHA showed a significant decrease in calcium current suggesting that the MCH system exerts inhibitory feedback on itself (Gao et al., 2003).

A moderate number of the presynaptic population in the medial mammillary and tuberomammillary nucleus are histaminergic, as measured by immunoreactivity to HDC. Less than 30% of the total histaminergic population is colocalized with the presynaptic population. We previously demonstrated that 70% of MCH neurons express histamine 3 receptor mRNA and that histamine exerts an inhibitory effect on MCH neurons via GIRK channels (Parks et al., 2014a). Retrograde tract tracing studies show that medial mammillary neurons received strong innervation from MCH neurons demonstrating a reciprocal innervation (Casatti et al., 2002). This interaction between histamine and MCH neurons was further confirmed by an optogenetic study in which stimulation of MCH terminals inhibited the histamine system in the TMN (Jego et al., 2013). The functional significance of this interaction is still unknown but it has been suggested that MCH and histamine interaction may regulate sleep arousal circuitry.

The neuropeptides OT and AVP were both previously reported to excite MCH neurons (Yao et al., 2012). These peptides are found predominantly in the PVH. Our results show that although a few presynaptic neurons from the PVH coexpress OT and AVP, the majority of these

cells do not. However, our data are representative of the perifornical subpopulation of MCH neurons. Both OT and AVP send axons to the LHA and may modulate a different subpopulation of MCH neurons. Both of these peptides regulate social interaction (Bachner-Melman and Ebstein, 2014), and further studies of these systems may reveal the nature of its regulation of the MCH system.

A recent report of MCH presynaptic inputs was published during the writing of this manuscript (Gonzalez et al., 2016). Although presynaptic input from the PVH and LHA were reported in both studies, some notable differences in the presynaptic nuclei exist. Our study did not find similar patterns of presynaptic strength and we did not find significant presynaptic inputs from cortical regions. However, these discrepancies may be the results of some important distinctions between the studies. The greatest difference may be that the MCH starter cells targeted in the former study were more rostral in the LHA and thus represent a different MCH subpopulation. Another important distinction is that in analyzing the data, we normalized our cell counts to the number of MCH starter cells whereas the former study normalized cell counts to the volume of brain areas. An important similarity is that both studies show direct presynaptic input from OT and AVP within the MCH presynaptic population and in our study we quantified the percentage of inputs.

LHA MCH neurons have been differentiated into several subpopulations that coexpress a diverse set of neuropeptides and neurotransmitters (Swanson et al., 2005; Parks et al., 2014b). Taking into account the unique chemoarchitecture of MCH subpopulations and the functional diversity of MCH neurons (Saito et al., 2000; Hervieu et al., 2002; Adamantidis and de Lecea,

2009; Macneil, 2013; Monti et al., 2013; Presse et al., 2014), these distinct populations of MCH neurons may regulate specific neural circuits and thus mediate distinct behavioral and physiological functions. Our findings provide insight on the direct inputs received by LHA MCH neurons and lay the groundwork for further functional studies to investigate these neural circuits.

Figure 2.1: Strategy for identifying direct presynaptic inputs to LHA MCH using Rabies Tracing Approach in pMCHCre mice.

(A) Schematic representation of MCHCre mouse line and tracing strategy of monosynaptic inputs to MCH neurons in vivo. We used MCHCre transgenic mice in which only MCH cells express Cre-recombinase. First, we injected the helper virus AAV8 which encodes the rabies envelope glycoprotein (G) important for trans-synaptic spreading of the rabies virus and the TVA receptor, an avian receptor protein that confers infection capability to rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA. Second, we injected rabies viral SADΔGmCherry strain to infect TVA expressing MCH cells. This strain has been genetically engineered to have the G glycoprotein deleted and replaced with a mCherry reporter. N, P, M and L respectively represent the viral genomic sequences encoding the wild-type nucleoprotein, phosphoprotein, matrix protein and polymerase required for rabies virus expression. The pseudotyped rabies virus can thus infect MCH cells and monosynaptically spread to cells which are directly presynaptic to MCH cells. The virus is then trapped in these presynaptic partner cells and cannot spread further due to the deficiency of G in these presynaptic cells.

(B) Experiment design and time line.

(C) AAV helper virus infected MCH neurons specifically and "starter cells" were verified by RV and GFP co-expression.

(D) Representative presynaptic labeling (red) observed in a pMCHCre-transgenic mouse but not a wildtype control littermate.

Figure 2.1

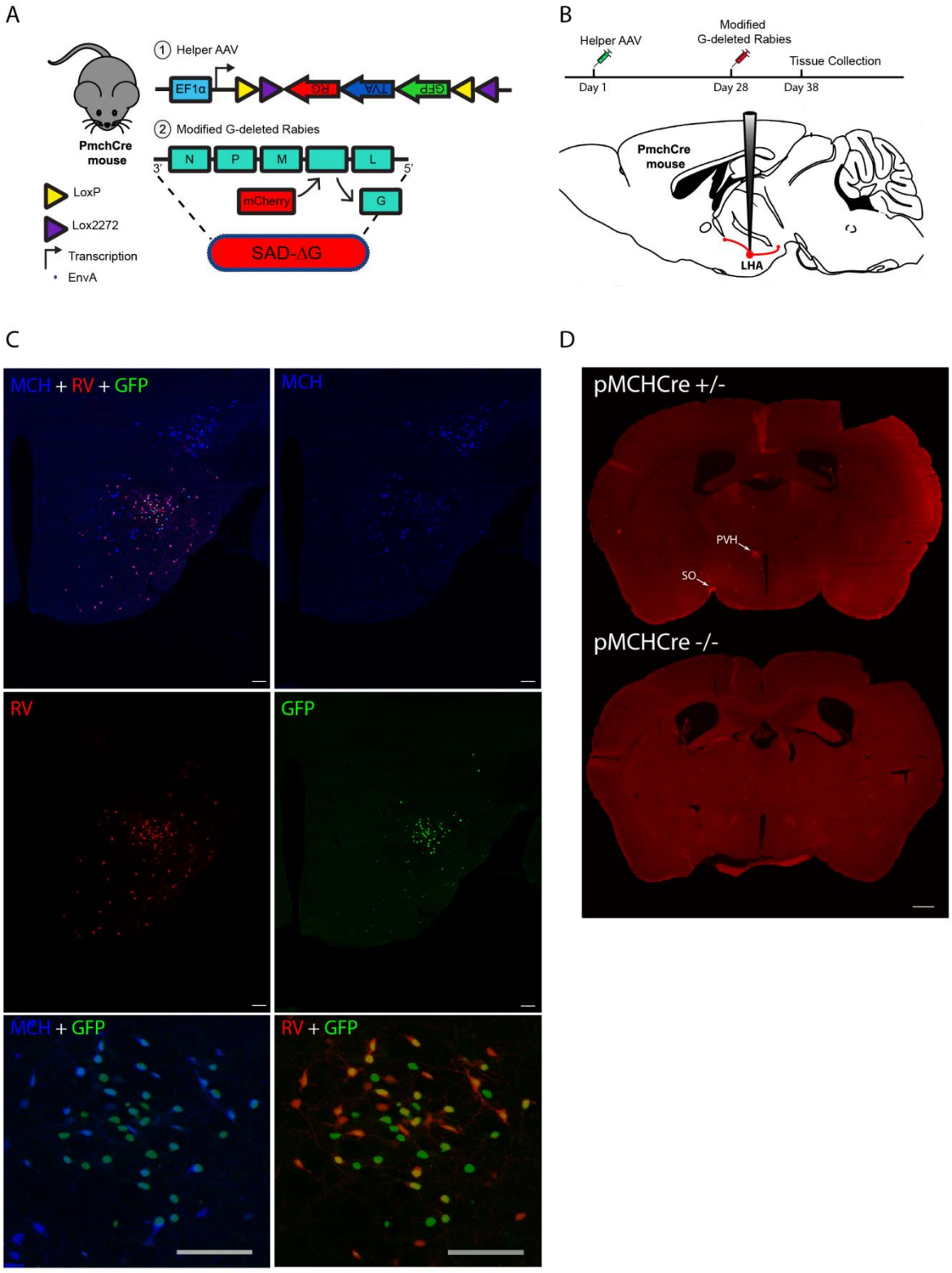
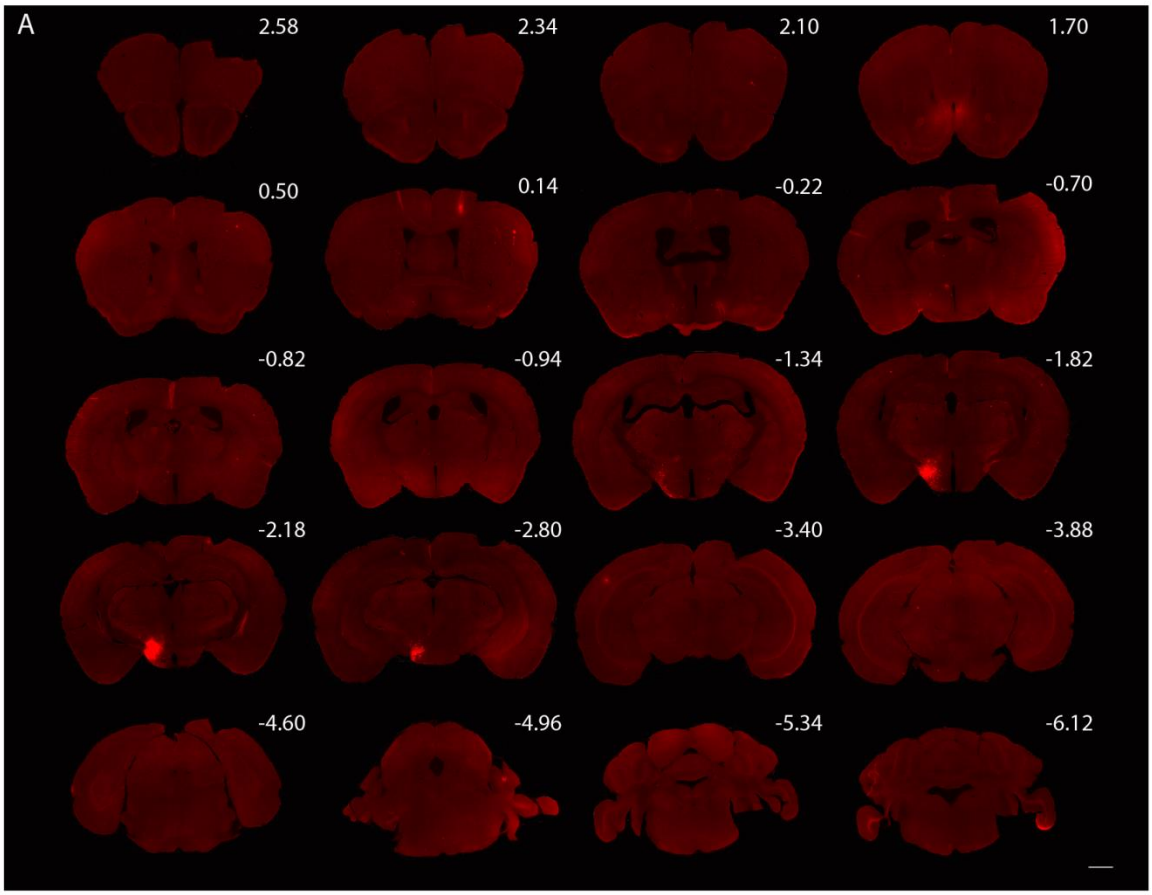

A
1 Helper AAV
EF1α
RG
TVA
GFP
PmchCre mouse
2 Modified G-deleted Rabies
N
P
M
L
3'
5'
mCherry
G
SAD-ΔG
LoxP
Lox2272
Transcription
EnvA
B
Helper AAV
Modified G-deleted Rabies
Tissue Collection
Day 1
Day 28
Day 38
PmchCre mouse
LHA
C
MCH + RV + GFP
MCH
RV
GFP
MCH + GFP
RV + GFP
D
pMCHCre +/-
PVH
SO
pMCHCre -/-

Figure 2.2: Rabies mediated direct monosynaptic inputs to LHA MCH.

(A) Representative series of coronal sections showing the pattern of presynaptic labeling in a pMCHcre mouse following unilateral rabies virus injection.

(B) The distribution of presynaptic cell numbers in a 1:4 series of sections along the rostrocaudal extent of a pMCHCre mouse. Scale bar 500um.

Figure 2.2


A
2.58
2.34
2.10
1.70
0.50
0.14
-0.22
-0.70
-0.82
-0.94
-1.34
-1.82
-2.18
-2.80
-3.40
-3.88
-4.60
-4.96
-5.34
-6.12

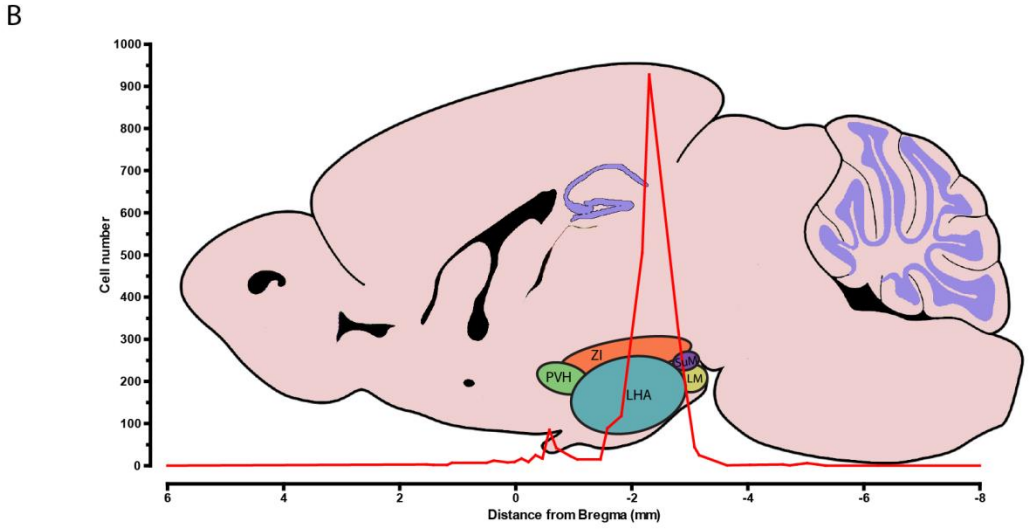

B
Cell number
1000
900
800
700
600
500
400
300
200
100
0
ZI
SuM
PVH
LM
LHA
6
4
2
0
-2
-4
-6
-8
Distance from Bregma (mm)

Figure 2.3: Brain nuclei that show the highest presynaptic input strength to MCH neurons in the LHA.

Coronal sections from rabies virus injected pMCHCre mice showing presynaptic neuronal inputs from the

(A) Paraventricular nucleus (PVH) of the hypothalamus and supraoptic nucleus (SO)

(B) Lateral hypothalamus (LHA) including the parasubthalamic nucleus (PSTh)

(C) Mammillary body (LM,ML,MM) and

(D) Supramammillary nuclei (SuM). Scale bar, 50 μm.

(E) The input connection strength index is the ratio of the number of presynaptic neurons versus the number of starter neurons within the lateral hypothalamus averaged across four animals.

LHA, lateral hypothalamic area; SuM, supramammillary area; MMB, mammillary bodies; PVH, paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus; MPA, medial preoptic area; VTA, ventral tegmental area; ARH, arcuate nucleus of the hypothalamus; VMH, ventromedial hypothalamus; LPO, lateral preoptic area; ZI, zona incerta; Acb, nucleus accumbens.

Figure 2.3

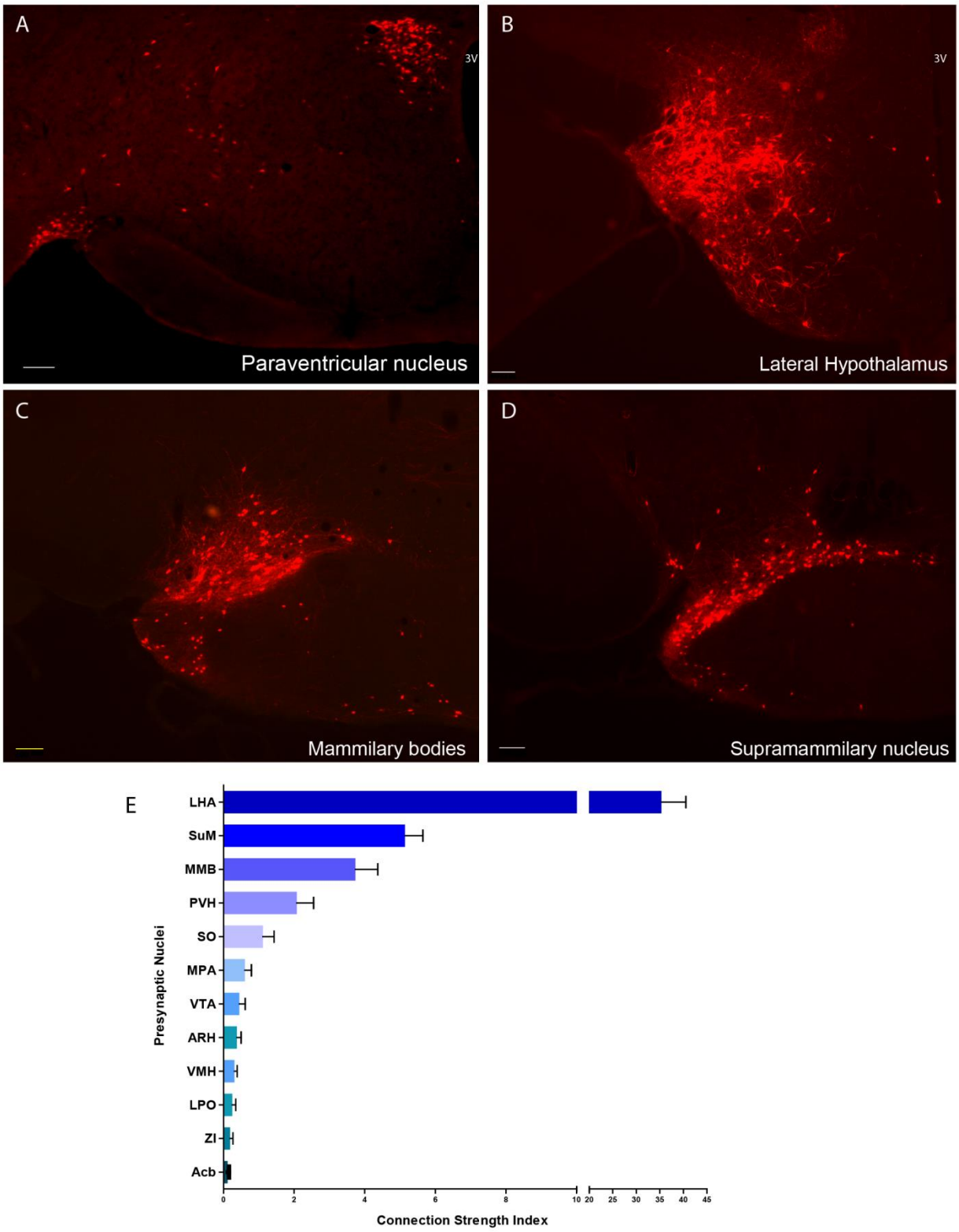

A
3V
Paraventricular nucleus
B
3V
Lateral Hypothalamus
C
Mammilary bodies
D
Supramammilary nucleus
E
LHA
SuM
MMB
PVH
SO
MPA
VTA
ARH
VMH
LPO
ZI
Acb
Presynaptic Nuclei
0
2
4
6
8
10
20
25
30
35
40
45
Connection Strength Index

Figure 2.4: PVH OT and AVP presynaptic inputs to LHA MCH neurons.

(A) Presynaptic inputs in the PVH (red fluorescence).

(B) Oxytocin (green fluorescence) immunoreactive neurons in the same PVH section.

(C) Merged image showing colocalization of oxytocin and presynaptic cells.

(D) Presynaptic inputs in the PVH (red fluorescence).

(E) AVP (green fluorescence) immunoreactive neurons in the same PVH section.

(F) Merged image showing colocalization of AVP and presynaptic cells. Scale bar = 5 0um.

(G) The percentage of PVN rabies labeled cells colocalized with OT or AVP.

(H) The percentage of the OT and AVP neuropeptide population in the PVN colocalized with presynaptically labeled cells.

Figure 2.4

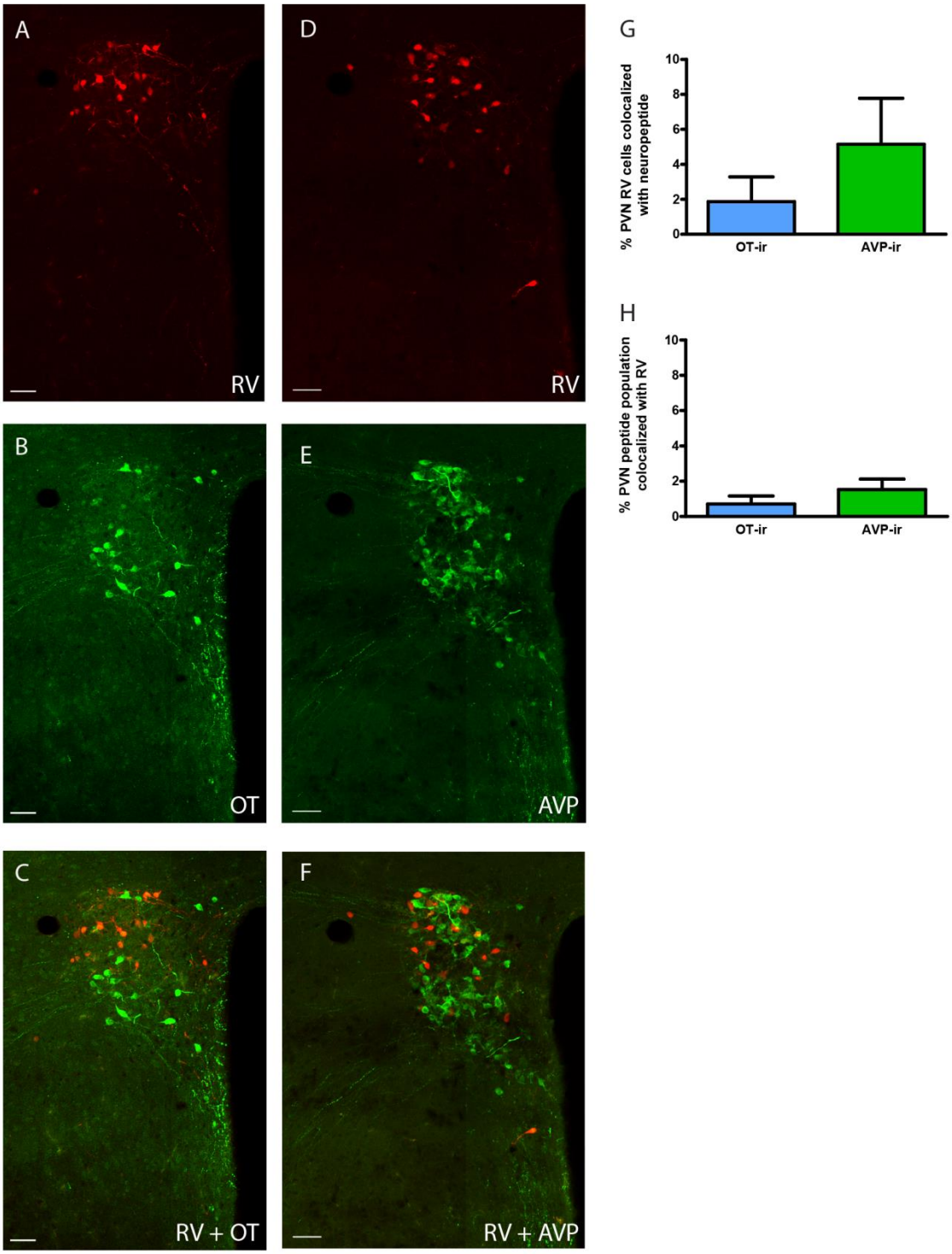

A
RV
D
RV
B
OT
E
AVP
C
RV + OT
F
RV + AVP
G
% PVN RV cells colocalized with neuropeptide
10
8
6
4
2
0
OT-ir
AVP-ir
H
% PVN peptide population colocalized with RV
10
8
6
4
2
0
OT-ir
AVP-ir

Figure 2.5: MCH and orexin presynaptic inputs to LHA MCH neurons.

(A) Presynaptic inputs in the LHA (red fluorescence).

(B) MCH (blue fluorescence) immunoreactive neurons in the same LHA section.

(C) Merged image showing colocalization of MCH and presynaptic cells.

(D) Presynaptic inputs in the LHA (red fluorescence).

(E) Orexin (green fluorescence) immunoreactive neurons in the same LHA section.

(F) Merged image showing colocalization of orexin and presynaptic cells. Scale bar = 100um.

(G) The percentage of total rabies labeled cells colocalized with MCH or orexin.

(H) The total percentage of the MCH and orexin neuropeptide population colocalized with presynaptically labeled cells.

Figure 2.5

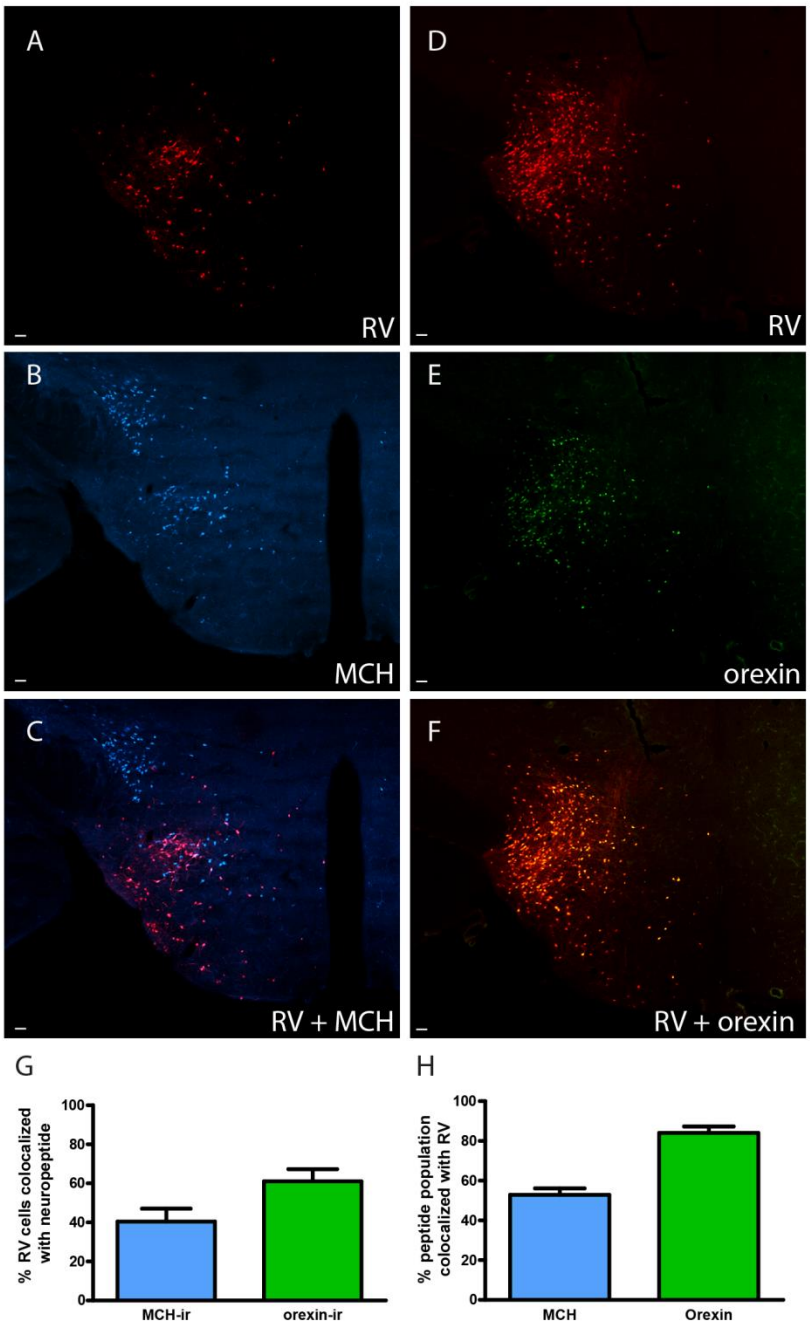

A
RV
D
RV
B
MCH
E
orexin
C
RV + MCH
F
RV + orexin
G
% RV cells colocalized with neuropeptide
100
80
60
40
20
0
MCH-ir
orexin-ir
H
% peptide population colocalized with RV
100
80
60
40
20
0
MCH
Orexin

Figure 2.6: HDC and OFQ/N presynaptic inputs to LHA MCH neurons.

(A) Presynaptic inputs in the TMN (red fluorescence).

(B) HDC (blue fluorescence) immunoreactive neurons in the same TMN section.

(C) Merged image showing colocalization of HDC and presynaptic cells.

(D) Presynaptic inputs in the SuM (red fluorescence).

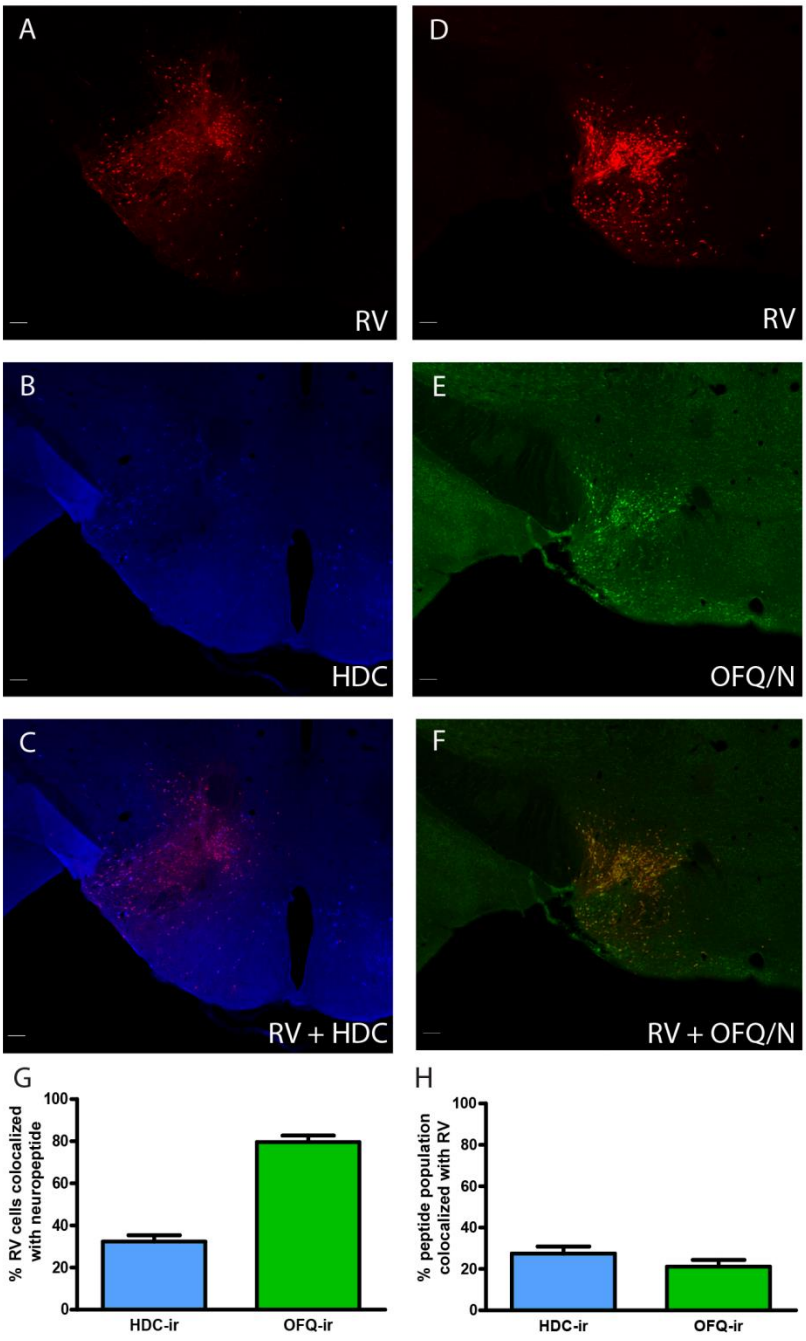
(E) OFQ/N (green fluorescence) immunoreactive neurons in the same SuM section.

(F) Merged image showing colocalization of OFQ/N and presynaptic cells. Scale bar = 100um.

(G) The percentage of total rabies labeled cells colocalized with HDC or OFQ/N.

(H) The total percentage of the HDC and OFQ/N neuropeptide population colocalized with presynaptically labeled cells.

Figure 2.6


A
RV
D
RV
B
HDC
E
OFQ/N
C
RV + HDC
F
RV + OFQ/N
G
% RV cells colocalized with neuropeptide
100
80
60
40
20
0
HDC-ir
OFQ-ir
H
% peptide population colocalized with RV
100
80
60
40
20
0
HDC-ir
OFQ-ir

Supplementary Figure 2.1: Immunohistochemistry of MCH neurons in the lateral hypothalamus of pMCHCre-zsgreen a mouse.

A) The lateral hypothalamus as presented in the Paxinos & Franklin Mouse Atlas (Paxinos and Franklin, 2001).

B) A grayscale photomicrograph of MCH immunoreactivity in the lateral hypothalamus and zona incerta.

C) Red fluorescence of MCH immunoreactivity at higher magnification.

D) Green fluorescence of the zsgreen reporter protein

E) Merged image of MCH immunoreactivity and zsgreen reporter protein. Bar scale on panels B-E represent 100 um.

Supplementary Figure 2.1

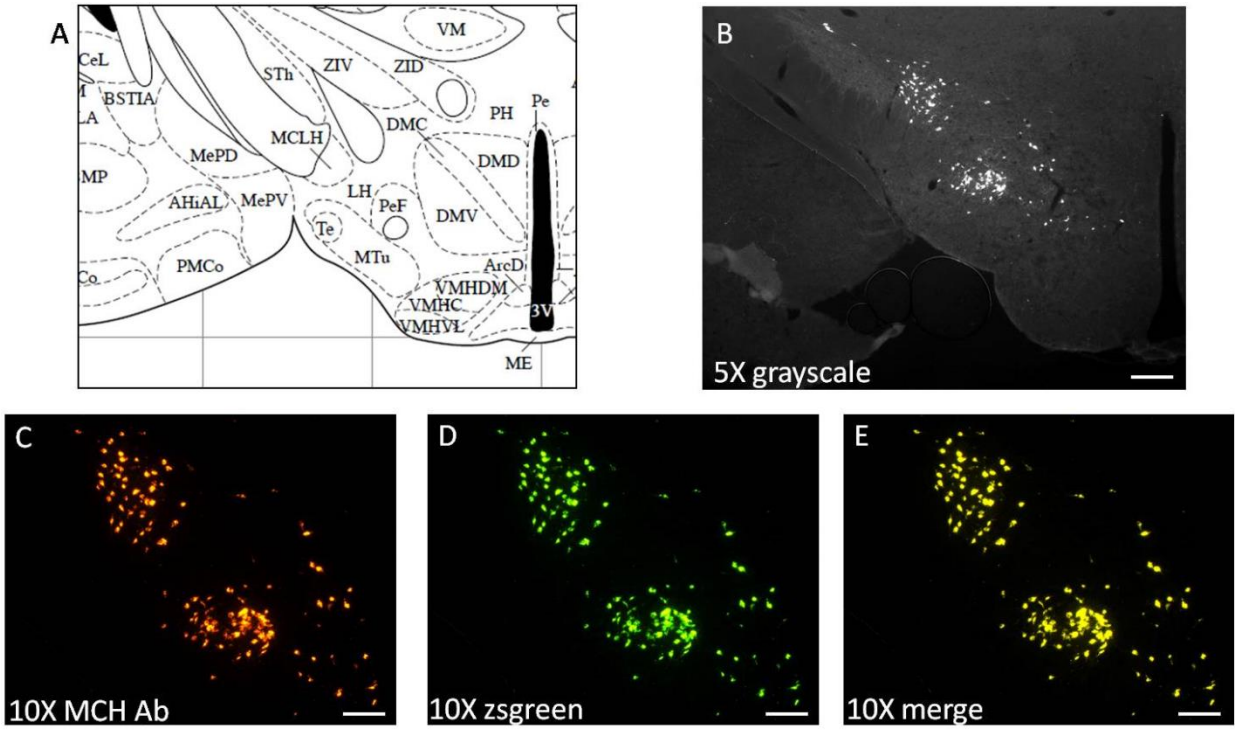


Table 2.1

Brain Nuclei	Abbreviation	CSI	sem
lateral hypothalamic area	LHA	35.34182	5.187955
supramammillary area	SuM	5.139675	0.506152
mammillary body	MMB	3.735889	0.631086
paraventricular nucleus of the hypothalamus	PVH	2.076922	0.474645
supraoptic nucleus	SO	1.117936	0.315224
medial preoptic area	MPA	0.6052278	0.184524
ventral tegmental area	VTA	0.4488627	0.166212
arcuate nucleus of the hypothalamus	ARH	0.3799834	0.11743
ventromedial hypothalamus	VMH	0.3103543	0.076297
lateral preoptic area	LPO	0.2466918	0.101846
zona incerta	ZI	0.1865899	0.08295
nucleus accumbens	Acb	0.1169891	0.03794

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Chapter 3

Melanin-Concentrating Hormone Modulates Oxytocin Mediated Repetitive Behavior

Nayna M. Sanathara, Celia Garau, Lien Wang, Amal Alachkar, Zhiwei Wang, Katsuhiko Nishimori, Xiangmin Xu, Olivier Civelli

ABSTRACT

Repetitive and perseverative behaviors are common features of a number of neuropsychiatric diseases such as Angelman's syndrome, Tourette's syndrome, obsessive-compulsive disorder, and autism spectrum disorders. We report that disruption of the melanin concentrating hormone (MCH) system increases repetitive behavior while its activation decreases it. Melanin concentrating receptor knockout (MCHR1KO) mice and MCH ablated animals show increased repetitive behavior while central MCH infusion decreases it. Regulation of repetitive behavior in both animal models and human trials has also been linked to the oxytocin system. We report that nearly 60% of MCH neurons express oxytocin receptors, and demonstrate that MCH neurons receive direct presynaptic input from oxytocin neurons. We further demonstrate the existence of an interaction between the MCH and oxytocin systems by showing that central infusions of MCH and oxytocin alone or together reduce repetitive behavior while antagonizing the MCH system blocks oxytocin-mediated reduction of repetitive behavior. Our findings reveal a novel role for the MCH system as a mediator of the role of oxytocin in regulating repetitive behavior.

Introduction

Repetitive behaviors are commonly observed in certain neuropsychiatric disorders such as obsessive–compulsive disorder (OCD), autism spectrum disorders (ASD), Angelman's syndrome, and Tourette's syndrome. Repetitive behaviors are linked to the dysregulation of the cortico-striato-thalamo-cortical circuitry (CSTC). Although many regions are involved in regulating the CSTC circuit, several studies have shown dysregulation of the orbitofrontal cortex (OFC) to be involved in repetitive behaviors (Chamberlain et al., 2008; Ahmari et al., 2013; Burguiere et al., 2013). The OFC has been shown to be connected both directly and indirectly with the hypothalamus (Ongur and Price, 2000; Barbas, 2007), with the lateral OFC showing the greatest connectivity to the lateral hypothalamus (Hirose et al., 2016). One of the best characterized neuropeptide systems in the lateral hypothalamus that has been implicated in the involvement of disorders symptomatic of repetitive behaviors is the melanin-concentrating hormone (MCH) system.

Melanin-concentrating hormone (MCH) is a 19 amino acid peptide made in two specific hypothalamic nuclei, the lateral hypothalamus (LH) and the zona incerta (ZI) (Bittencourt et al., 1992; Knigge et al., 1996). MCH immunoreactivity is found widely throughout the CNS but is concentrated in the prefrontal cortex, nucleus accumbens, hippocampal formation, basal ganglia, diencephalon, and brainstem (Bittencourt et al., 1992). MCH acts by activating its receptor, MCHR1, which is highly expressed in the limbic system. The MCH system is known not only for its involvement in food intake and energy metabolism (Qu et al., 1996; Rossi et al., 1997; Ludwig et al., 2001), but also for modulating stress, anxiety, and depression (Hervieu et al., 2000; Chung et al., 2011a; Chung et al., 2011b). Its importance in mediating neuropsychiatric

disorders is supported by the discovery of MCHR1 single nucleotide polymorphisms in patients with bipolar disorder and schizophrenia (Severinsen et al., 2006). Evidence from animal studies suggests a role for MCH in regulating repetitive behavior. MCH infusions were reported to rescue melanocyte-stimulating hormone (MSH) mediated compulsive behavior in rats (Sanchez et al., 1997). However, administration of the MCHR1 antagonist, GW3430, led to reduction in repetitive behavior as measured through marble-burying task (Millan et al., 2008; Gehlert et al., 2009). In the latter studies, the antagonist doses at which reduction in repetitive behavior was reported grossly exceeded the amount required for 90% binding to MCHR1 and could represent off-target effects. More recently, a genome wide association study on autism related genes upregulated in the lateral septum in postpartum mice identified increased expression of MCH1R gene implicating it as a possible regulator of sociability in dams within the context of psychiatric illness (Eisinger et al., 2013). The MCH gene is also upregulated in postpartum in the medial preoptic nucleus (Rondini et al., 2010; Driessen et al., 2014). This area is associated with mental disorders displaying low sociability, such as autism spectrum disorders (ASD), a core symptom of which is repetitive behavior.

A neuropeptide system that is gaining increasing importance in mediating repetitive behaviors is the oxytocin (OT) system. OT is classically known for its role in parturition and maternal behavior (Anacker and Beery, 2013). But it is also known to affect social behaviors (Pedersen, 1997; Carter, 1998; Uvnas-Moberg, 1998; Young et al., 2002). OT has been implicated as a potential therapeutic for autism (Ho and Blevins, 2013; Lukas and Neumann, 2013; Salmina et al., 2013), since intranasal OT administrations in ASD patients increases social communication and improves the timing of social responses (Watanabe et al., 2014). Acute

administration of OT has been shown to decrease repetitive stereotypical behaviors in inbred mouse models of ASD (Teng et al., 2013). In Asperger's Syndrome clinical trials, OT treatment, improved social cooperation and social aptitude (Andari et al., 2010) and reduced repetitive behaviors (Hollander et al., 2003). In a recent genotype-phenotype investigation in children diagnosed with ASD, two oxytocin receptor single nucleotide polymorphisms were linked to greater impairment on the repetitive behavior scale (Harrison et al., 2015).

The interaction between the OT and MCH systems was first shown when OT was found to depolarize MCH cells in the LH and not other LH GABAergic neurons, suggesting a selective innervation of the MCH system (Yao et al., 2012). This implies that OT is not involved in general excitation or setting a general tone in the LH, but rather selectively targets MCH neurons to facilitate MCH neurotransmission.

The main purpose of the current study was to identify the precise role of the MCH system in repetitive behavior and to determine whether there is an interaction between the MCH and OT system in regulating this behavior. We studied repetitive behavior in MCHR1 KO mice, MCH ablated animals, and in wildtype mice infused with OT, MCH, OT and MCH in the absence and presence of MCHR1 antagonist, GW803034. We also tested these animals in anxiety assays, which can be comorbid with repetitive behavior.

We report that disruption of the MCH system increases repetitive behavior, while its activation relieves repetitive behavior. Furthermore, we show that a majority of MCH neurons express oxytocin receptor and OT neurons from the PVN form direct presynaptic contact with

MCH neurons in the lateral hypothalamus. This study provides evidence for the hypothesis that MCH receives input from OT to regulate repetitive behavior by showing that the OT and MCH systems interact in the lateral hypothalamus, and that OT, MCH, and OT+MCH administration reduces repetitive behavior. Blockade of the MCH system attenuates OT reduction in repetitive behavior. Our results suggest that OT acts on the MCH system to reduce repetitive behavior, which may provide a new rationale for treating preservative behaviors.

MATERIALS AND METHODS

Animals:

All animal experiments were done in accordance with the University of California, Irvine's Animal Institutional Animal Care and Use Committee.

PmchCre/+;R26iDTR/+ were generated from crossing hemizygous Tg(Pmch-cre)1Lowl/J (Jackson Laboratories, Bar Harbor, Maine, USA) mice that express Cre-recombinase (Cre) in MCH neurons (Kong et al., 2010), with homozygous R26iDTR/+ mice obtained from Dr. Satchidinanda Panda and originally generated in the lab of Dr. Ari Waisman (Buch et al., 2005). Adult male animals were group housed under controlled conditions (temperature 21°C ± 2°C; 12 hr light–dark cycle, lights on at 7:00 AM) with free access to water and food. All operant procedures were performed during the light phase (between 10 AM and 4 PM). A schematic diagram for generating iDTRPmchCre is shown in Supplementary Figure 3.1 A.

Heterozygous Tg(Pmch-cre)1Lowl/J mice were maintained on a C57BL/6 genetic background by intercross breeding. Tg(Pmch-cre) adult males were used for rabies mediated circuit mapping experiments.

Male CD1 mice (Charles Rivers, Wilmington, MA, USA) 7 to 11 weeks were used for all behavioral pharmacology experiments involving intracerebroventricular (i.c.v.) infusions of peptides. Mice were single-housed following cannulation surgery in 12/12 hr light/dark cycle, with food and water ad lib. OXTR-Venus knock-in male mice brain tissue was acquired from the lab of Dr. Katsuhiko Nishimori from Tohoku University, Miyagi, Japan.

Surgery:

All stereotaxic surgery was conducted in Dr. Xiangmin Xu's lab using a stereotaxic machine (MyNeuroLab two arm stereotaxic with Angle Two software) in accordance with UCI IACUC. Under isoflurane anesthesia, 6 week aged Pmch-cre mice were injected with 0.2 ul of the AAV8 helper virus encoding the B19G glycoprotein, TVA (an avian virus envelope protein receptor) and GFP unilaterally into the LH (flat skull coordinates from bregma: anteroposterior (AP), −1.82 mm; mediolateral (ML), +.91 mm; and dorsoventral (DV), −5.25 (Paxinos and Franklin, 2001)). Following surgery, mice were individually housed and allowed to recover for three weeks in rooms with controlled humidity and temperature and access to regular lab chow and water ad libitum to allow the helper virus infection to take (n = 4/group). The mice were then underwent a similar surgery (same coordinates) for the delivery of the G-deleted EnvA pseudotyped rabies virus. The animals were housed separately in BSL-2 level suites with regular lab chow and water ad libitum for 10 days prior to transcardial perfusion.

For i.c.v. injections, a stainless-steel guide cannula (23-gauge, 6 mm length) was directed at the lateral ventricle. CD1 Male mice were anesthetized by intraperitoneal (i.p.) administration of 0.1ml/10 grams of a mixture of ketamine and xylazine (Ketamine 100mg/kg, Xylazine, 10 mg/kg, Western Medical Supply, Arcadia, CA). Mice were secured in a Kopf stereotaxic instrument (Tujunga, CA, USA) and guide cannula were implanted at .5 mm posterior to bregma, 1.0 mm lateral, and 2.0 mm below the skull surface (Paxinos and Franklin, 2001). Animals were allowed to recover for one week before the start of experiments. Upon completion of the experiments animals were perfused with chilled 0.9% saline and 4% PFA and brain sections were mounted on slides and Nissl stained to verify the cannula placements.

Drugs

DT Injection:

DT (RK-01-517, MBL International Corp., Woburn, MA) was dissolved in sterile saline (1 mg/ml) and stored at -80°C until use. Freshly thawed DT stock solution was diluted in sterile saline and injected intraperitoneally (16ng/g body weight) to 8–12 weeks old iDTR+PmchCre+ and iDTR+PmchCre- (control) littermate mice. The dose was repeated 2 days later. Daily body weight measurements were taken for 12 days after the initial DT injection.

Drug administration:

Both MCH (1 nmol) and OT (10 pmol) were dissolved individually in phosphate-buffered saline (pH 7.4) with 0.2% bovine serum albumin. The dose of each drug was determined by previously reported findings for MCH (Chaffer and Morris, 2002; Chung et al.,

2009) and OT (Argiolas et al., 1988; Amico et al., 2004; Neumann and Landgraf, 2008). The injection cannula was connected via PE50 tubing to a 50 µl Hamilton microsyringe fitted to a microinjection pump (KDS 200, KD Scientific). Infusions were administered in a volume of 2 µl over 2 min., and an additional 2 min. was allowed for diffusion before the infusion cannulas are removed. 3mg/kg MCH1R antagonist, GW803430, was administered 30 min. i.p. before i.c.v. injections. We selected this dose based on previously reported receptor occupancy studies demonstrating that near complete blockade of the MCH system is achieved following i.p. administration at the 3mg/kg dose (Gehlert et al., 2009).

Statistical Analysis:

Prism software version 5.01 (GraphPad) was used for statistical analysis. Data expressed as mean ± SEM. Results were analyzed by t test or ANOVA followed by the appropriate post hoc comparisons, and $P < 0.05$ was considered statistically significant.

Behavioral Testing

Locomotion Activity and Open Field: Locomotion was monitored in an open field test chamber (40 x 40 cm, Med Associates, inc.). Two weeks following DT injection mice were acclimated to the behavior room for 30 min. and placed directly into the activity monitor for 60 min. The distance traveled was measured by infrared beam arrays and recorded, analyzed and calculated by Activity Monitor 5 software (Med Associates, Inc.). To evaluate open field activity, a center-to-periphery exploration ratio was assessed on the time spent by the animal in

the center area of the chamber (33.75 x 33.75) vs. the peripheral area defined as the 6.25 cm strip surrounding the center area.

Self-Grooming:

MCHR1KO and iDTRpMCHcre mice were scored for spontaneous grooming behaviors as described earlier (Silverman et al., 2010). Each mouse was placed individually into a standard mouse cage (46 cm length × 23.5 cm wide × 20 cm high; illuminated at ~ 40 lux) with a thin layer of bedding. Scoring proceeded after a 5-min habituation period in the test cage. Each mouse was scored for 10 min to measure cumulative time spent grooming all body regions. CD1 mice that were cannulated for icv infusions were given drug treatments, acclimated to the behavior room for 30 min. and placed directly into the activity monitor and observed by a person blind to the treatment. The time spent grooming was recorded every 5 min. in a 30-min session. Behavioral observations were initiated 5 min. after the start of the test and recorded for the first min. of every 5 min. A single observer, blind to the treatment, recorded behavioral measurements. The time spent grooming was recorded for each animal in every 1 min. observation period.

Genital Grooming:

CD1 mice were given drug treatments, acclimated to the behavior room for 30 min. and placed directly into the activity monitor and observed by a person blind to the treatment. The observer recorded genital grooming bouts for 1 min. every 5 min. within a 30 min. period. A

genital grooming bout is defined as the initiation of genital-specific grooming behavior for a minimum of 5 s.

Marble Burying Test:

Mice were habituated to the behavior room for 30 min. before being placed into a polypropylene cage (30 × 18 × 12 cm) containing 24 glass marbles (1.5 cm diameter) evenly spaced on 3 cm deep rodent bedding (bed-o'cob, The Andersons Inc., Maumee, OH). No food or water was present during the observation period. The sessions were videotaped and the number of marbles covered at least two-thirds were counted as buried after 30 min. and analyzed by two observers blind to the treatment.

Elevated Plus Maze:

A standard elevated plus-maze made of grey Plexiglas was placed in a sound-proof observation room with controlled light (200 Lux on the central platform of the maze). Animals were habituated to the behavior room for 30 min. before being placed in the center facing an open arm and allowed to explore for 5 min. The behavior was recorded and scored by two independent observers that were blind to animal treatments. The time spent in the closed and open arm and the number of entries to the open and closed arms was scored.

The Light Dark Box:

The light/dark testing apparatus was a 11.5 × 19 × 11 inches (outer dimensions) Plexiglas box, consisting of a light compartment (11 × 11 inches) and a smaller dark compartment (11 × 7

inches) connected by a door in the center of the wall separating the two compartments. A 60-W bulb located 16 inches above the center of the light compartment provided bright illumination. Mice were placed in the center of the dark compartment for 30 seconds before gaining access to the light compartment and were allowed to explore the box freely for 10 min. Time spent in the light and dark compartments was recorded with a video camera and scored by two independent observers blind to animal treatments.

The Three Chamber Social Interaction Test:

The Plexiglas apparatus used for the social interaction test consists of three rectangular chambers (manufactured to specification at University of California, Irvine machine shop). Each chamber is 20×40×20 cm and the dividing walls are made with a movable door with a 5 cm opening centrally positioned allowing free access to each chamber. Two empty wire-mesh containment cups (9 cm diameter × 10 cm height) were placed in the center of the right and left chamber (one per each side). Test mice were allowed to explore the center chamber for 5 min. with the dividing doors closed. An unfamiliar mouse of the same strain, gender, and age was placed inside the containment cup located in one of the side chambers. The placement of the unfamiliar mouse in the side chambers was counter-balanced between trials. After habituation, the dividing doors were removed between compartments to allow free access for the test mouse to explore the 3 chambers for 10 min. The time spent in direct contact between the test mouse with the empty cup and the unfamiliar mouse was measured. Tests were video recorded and analyzed by ANY-MAZE software (Stoelting Co.).

Olfactory Habituation/Dishabituation Test:

The ability to discriminate social smells was measured using modifications of the olfactory habituation/dishabituation task, as previously described (Yang and Crawley, 2009). Animals were individually tested for time spent sniffing cotton tipped swabs (6 inches length, Fisherbrand) suspended from the cage lid in a clean mouse cage with fresh litter. Sequences of three identical swabs were presented to each animal to measure habituation to the same scent. Dishabituation was evaluated by presentation of a new odor. Swabs were presented in sequences of three identical scents for 2 min. periods each in the following order: distilled water, almond extract (McCormick, Hunt Valley, MD; 1:100 dilution), banana extract (McCormick, Hunt Valley, MD; 1:100 dilution), scent of unfamiliar mouse of the same strain and sex, scent of a second unfamiliar mouse of the same strain and sex. Time spent sniffing the swab was measured by an observer uninformed about the genotype of the test mouse.

Immunohistochemistry

At the end of all behavioral experiments iDTRPmchCre animals were perfused transcardially with 0.9% saline and chilled 4% paraformaldehyde in 0.2 M Sorensen's phosphate buffer. The brains were removed rapidly from the cranium and postfixed overnight in 4% paraformaldehyde. Brains were then transferred to 30% sucrose in a 0.1M phosphate buffer solution, pH 7.5 and stored at 4°C until sectioning (Sinchak & Micevych, 2001). Coronal brain sections (30 μm) through the lateral hypothalamus were cut on a microtome and collected into wells containing phosphate-buffered saline (PBS, pH 7.5). To evaluate the extent of MCH-neuron ablation MCH neurons were visualized using rabbit polyclonal anti-MCH antibody

(antibody courtesy of W. Vale, Salk Institute, La Jolla, CA, USA) as previously described (Parks et al., 2014). Every one out of four lateral hypothalamic sections was immunostained with rabbit polyclonal anti-MCH antibody (antibody courtesy of W. Vale, Salk Institute, La Jolla, CA, USA; diluted 1:150,000). A goat anti-rabbit AlexaFluor 555 (1:500; Jackson Immuno) was used to visualize MCH immunoreactivity. The number of MCH immunoreactive neurons from section was compared between DT-injected iDTR+PmchCre+ mice and iDTR+PmchCre- littermates. For c-Fos immunohistochemistry, mice were anesthetized 90 min. after marble-burying and perfused transcardially with 0.9% saline followed by 4% paraformaldehyde. Brain sections were incubated with rabbit anti-c-Fos antibody (1:5,000; PC38T, Calbiochem, La Jolla, CA) and visualized with goat anti-rabbit AlexaFluor 488 (1:500; Jackson Immuno).

PmchCre transgenic animals used for rabies mediated circuit mapping were processed for tissue collection same as mentioned above. 30 um sections of brains were stored in cryoprotectant at 20°C until use. Every fourth section was processed for immunohistochemistry. Starter cells were identified by enhancing the GFP signal using chicken anti-GFP (Aves Lab, 1:250) and visualized using goat anti chicken Alexa 488 (Invitrogen, 1:200). Every fourth section of the paraventricular nucleus presynaptically labeled mCherry neurons were assessed for OT immunoreactivity (1:2000; EMD Millipore) and visualized with donkey anti-rabbit Alexafluor 488 (1:200; Jackson Immuno). Slides were mounted and stored in -20˚C until analysis.

OXTR-Venus knock-in mice brains were collected as previously described by Dr. Nishimori's lab (Yoshida et al., 2009). Tissue was shipped on ice to Irvine, California from Miyagi, Japan)

and stored at 4 °C until sectioning. 30 um sections of brains were stored in cryoprotectant (sodium phosphate buffer, pH 7.4, with 0.9% saline, 30% sucrose, and 30% ethylene glycol) at 20°C until use. A 48 hr incubation at 4 °C with chicken anti-GFP antibody (1:500, Aves) and rabbit anti-MCH antibody were used to label Venus and MCH immunoreactive neurons, respectively. A goat anti-chicken Alexafluor 488 (1:200, Jackson Immuno) and goat anti-rabbit AlexaFluor 594 (1:500; Jackson Immuno) were used to visualize GFP and MCH immunoreactivity.

Histology:

Lateral ventricle guide cannulae placements in CD1 mice were assessed after the behavioral experiments. Every fourth brain section was mounted onto a Superfrost Plus slide, air dried on a 37° C slide warmer, stained with thionin, dehydrated in an alcohol series followed by xylene, and coverslipped with DPX mounting medium. The injection sites were verified by using a bright-field microscope to visualize the thionin staining.

RESULTS

The MCH system regulates repetitive behavior

To study the role of the MCH system in repetitive behavior we investigated marble-burying behavior, stereotypy, and self-grooming behavior in MCHR1 KO animals, MCH ablated mice, and wild type mice infused centrally with MCH.

MCHR1 KO and diphtheria toxin (DT) treated iDTR+PmchCre+ (MCH-ablated) mice showed a significant increase in repetitive behavior as measured by marble-burying behavior and stereotypy measurement compared to their respective controls (Figure 3.1 A B, D, and E). Consistent with these results, icv infusion of MCH in wildtype mice decreased marble-burying in comparison to vehicle infusion (Figure 3.1 C) but did not affect stereotypy (Figure 3.1 F). Self-grooming behavior was not altered in MCHR1 KO animals, MCH ablated mice, and wild type mice infused centrally with MCH compared to their respective controls (Figure 3.1 G-I). Increased locomotor activity was detected in MCHR1KO as previously reported (Figure 3.1 D, (Marsh et al., 2002; Smith et al., 2005; Zhou et al., 2005; Smith et al., 2008)), but no changes in locomotor activity were detected in adult DT treated iDTR+PmchCre+ vs. DT treated iDTR+PmchCre- control mice (Figure 3.1 E), and wild type mice infused centrally with MCH vs. vehicle (Figure 3.1 F).

We also investigated whether DT MCH-ablated displayed altered exploratory, anxiety and social behaviors. Treatment with DT led to a slight, but significant decrease in weight in iDTR+PmchCre+ compared to iDTR+PmchCre- control mice as an interaction between genotype and time (Figure 3.2 A). This interaction has been previously reported in both MCHR1 KO mice and in MCH ablated animals (Jeon et al., 2006; Whiddon and Palmiter, 2013). No significant change between iDTR+PmchCre+ and Cre- control littermates was seen in exploratory and anxiety behavior as tested by open-field assay, light-dark box, and elevated plus maze (Figure 3.2 B-D). No significant difference between genotypes was found in social interaction and olfactory discrimination (Figure 3.2 E-F). Ablation efficiency was measured following behavioral testing. The average MCH cell count per hypothalamic tissue section was

reduced by 99% in DT injected iDTR+PmchCre+ mice compared with iDTR+PmchCre- littermates (1023 ± 64.71 vs. 5.2 ± 2.185; Supplementary Figure 3.1).

MCH Ablated and MCH Intact Mice show differential c-fos activation following Marble-Burying Task

Mice models of repetitive behavior have shown decreased activity in the lateral orbitofrontal cortex (Ahmari et al., 2013; Burguiere et al., 2013). To investigate whether MCH ablation alters brain activity we measured cFos immunoreactivity in DT treated iDTR+PmchCre+ and iDTR+PmchCre- 90 min following the marble-burying task. DT treated iDTR+PmchCre+ mice showed significantly decreased cFos immunoreactivity in the lateral orbital cortex, compared to iDTR+PmchCre- (Figure 3.3 A). No significant differences were found between groups in the medial or ventral orbital cortex (Figure 3.3 B-D). A significant decrease in cFos immunoreactivity in DT treated iDTR+PmchCre+ and iDTR+PmchCre- mice was found in the lateral septal nucleus intermediate part, retrosplenial agranular cortex, retrosplenial granular cortex, primary somatosensory cortex barrel fields, primary somatosensory cortex trunk region, basolateral amygdaloid nucleus anterior portion (Supplementary Figure 3.2). No significant differences were found in cFos immunoreactivity in DT treated iDTR+PmchCre+ and iDTR+PmchCre- mice in the ventral striatum, piriform cortex, primary and secondary motor cortex, cingulate cortex area 1 and 2, dorsal and ventral part of the lateral septal nucleus, and LH (data not shown).

Colocalization of MCH and OT Receptors

Oxytocin has been shown to depolarize MCH cells but not other GABAergic neurons in the LH, suggesting a selective innervation of this system (Yao et al., 2012). To determine

whether MCH neurons express OXTR, we used OXTR-Venus knock-in mice, which express the GFP variant Venus in all neurons expressing OXTR (Yoshida et al., 2009). Immunohistochemical analysis of Venus expression in OXTR-Venus mice showed 56.8% ± 1.6% overlap with MCH (Figure 3.4) whereas 4% of OT neurons express MCHR1 as reported by Chee et al. (Chee et al., 2013). Based on our data and these previously reported findings, we hypothesized that OT neurons are upstream of MCH neurons.

Rabies-Mediated transsynaptic tracing of MCH neurons

To determine whether OT neurons are upstream of MCH cells and whether they form direct synaptic contact with MCH neurons we used a Cre dependent, genetically modified rabies-mediated circuit mapping technique in an MCH-Cre transgenic line (Callaway, 2008; Sun et al., 2014). We analyzed the distribution of presynaptic neurons from OT projection areas and found that MCH neurons receive OT inputs from within the hypothalamus (Figure 3.5 A-D, Supplementary Figure 3.3). A subpopulation of cells presynaptic to MCH neurons from the PVN express oxytocin (0.7% ± 0.4%) suggesting that most OT neurons indirectly communicate to MCH neurons

MCH mediates OT-induced reduction of repetitive behavior

To test whether OT infusion reduces repetitive behavior in mice, we investigated its effect in marble burying task (Deacon, 2006; Thomas et al., 2009; Greene-Schloesser et al., 2011; Angoa-Perez et al., 2013). We found that i.c.v. infusions of OT (10 pmol) and MCH (1 nmol) administered either individually or together reduced marble burying behavior in male CD1 mice to similar levels, suggesting a non-additive effect. To investigate whether OT reduction of

marble-burying was through its actions on the MCH system, we used an MCHR1 antagonist, GW803430. Pretreatment with 3mg/kg dose of GW803430, significantly reversed the MCH, OT, and MCH+OT mediated reduction in marble burying (Figure 3.6 A). Baseline marble-burying behavior was not significantly different from vehicle at the 3mg/kg i.p dose of GW803430 (Figure 3.6 A).We also tested exploratory and anxiety behaviors in these animals. No significant difference between groups was detected in locomotor activity, open-field assay, light-dark box, or elevated plus maze (Figure 3.6 B-E). i.c.v. infusion of OT has been shown to increase genital grooming in mice (Amico et al., 2004). In order to investigate whether OT mediated genital grooming involves the MCH system, we investigated the effects of i.c.v. administration of MCH, OT, and MCH + OT on genital grooming behavior (Figure 3.6 F). As expected, OT significantly increased bouts of genital grooming in comparison to control as previously reported (Amico et al., 2004); MCH, GW803430, or MCH + GW803430 showed no significant difference from vehicle infusions. Genital grooming in MCH + OT and GW803430 + MCH + OT combined groups did not significantly differ from OT treatment alone group. Self-grooming was also analyzed and no significant differences between treatments were found (Figure 3.6 G).

DISCUSSION

The MCH system has been implicated in regulating diverse sets of behaviors including food intake, energy expenditure, memory and reward (Pissios et al., 2006; Adamantidis and de Lecea, 2009; Chung et al., 2011a). Using a combination of genetic, neuroanatomical, and pharmacological approaches we report a novel role for this system in regulating repetitive

behavior. Our data also show that this role is mediated at least in part by interactions between the MCH and oxytocin systems.

First, our data show that disruption of the MCH system increases repetitive behavior. Both MCHR1 knock out animals and MCH ablated mice display significant increases in marble-burying and stereotypical behavior when compared to controls. On the other hand, MCH icv infusions decrease marble-burying behavior without affecting stereotypic behavior. Thus, more chronic disruption of the MCH system, as modeled through MCHR1 KO and DT mediated MCH ablation, leads to the development of stereotypy. However, acute blockade via MCHR1 antagonist or stimulation via central MCH infusion, does not induce stereotypy. Furthermore, MCH ablated mice show a significant decrease in neuronal activation measured by cFos immunoreactivity in the lateral orbital cortex, an area important in regulating repetitive behavior. Together, our behavioral and anatomical observations complement the finding that optogenetic activation of the lateral orbital cortex in the Sapap3 mutant mice, an animal model that displays pathological repetitive behavior, alleviates this perseverative behavior (Burguiere et al., 2013). The lateral orbital cortex is part of the orbitofrontal cortex; this subsection of the prefrontal cortex is shown to be important in OCD like behaviors (Ahmari et al., 2013; Burguiere et al., 2013), and also expresses MCHR1 in high levels (Hervieu et al., 2000). Thus, long term disruption of the MCH system leads to decreased activation of the lateral orbital cortex contributing to an increase in repetitive behavior.

We also identify an anatomical link between MCH and OT neurons in which OT acts through MCH to regulate repetitive behavior. MCH neurons were previously shown to be depolarized by OT neurons in the LH (Yao et al., 2012). Using rabies-mediated circuit mapping

of MCH neurons we demonstrate retrograde labeling of a subpopulation of OT neurons that form direct presynaptic contact with MCH cells. Both OT fibers and OXTR mRNA have been reported in the LH (Wang et al., 1996; Yoshida et al., 2009; Yao et al., 2012). In support of this observation we show that nearly 60% of MCH neurons colocalize with OT receptor, suggesting a functional relationship between these two neuropeptide systems. OT is classically known for its role in parturition and maternal behavior (Anacker and Beery, 2013). It also has an effect on social behaviors and as a potential treatment for autism (Ho and Blevins, 2013; Lukas and Neumann, 2013; Salmina et al., 2013). Intranasal treatment of OT in ASD patients increases social communication and improves the timing of social responses (Watanabe et al., 2014). In clinical trials, OT treatment in Asperger's Syndrome, an ASD, improves social cooperation and social aptitude (Andari et al., 2010) and reduces repetitive behaviors in these individuals (Hollander et al., 2003). Our data show that OT acts through MCH to regulate repetitive behavior and links the MCH system to the neurocircuitry underlying OCD type behaviors indicating a role for MCH in repetitive behavior.

To determine the interaction between MCH and OT in repetitive behavior we antagonized the MCH system in mice infused with MCH, OT, or MCH+OT. Infusions of MCH, OT, and MCH+OT significantly reduced repetitive behavior in comparison to control. Antagonizing MCHR1 using GW803430 blocked the reduction in repetitive behavior not only in MCH infused animals but also OT and MCH + OT groups demonstrating that OT acts through the MCH system to reduce repetitive behavior. Our data reveal that MCH mediates OT regulation of repetitive behavior. Our data show that OT-MCH relationship did not extend to exploratory or anxiety related behaviors as assayed through open-field, light-dark box, and elevated plus maze assays. This data support previous reports demonstrating that there is no

correlation for marble-burying test and anxiety assays (Thomas et al., 2009). As previously reported, we also found that OT infusions increased genital grooming but MCH infusions did not. MCH antagonist prior to OT infusion did not block genital grooming in OT infused mice demonstrating that the MCH system is not involved in OT mediated genital grooming. In conclusion, we identify a novel role for the MCH system and identify a previously undefined neurocircuit by which OT regulates repetitive behavior through the MCH system.

The colocalization of OXTR and MCH immunoreactivity in the LH and OT immunolabeled cells in the PVN projecting to MCH neurons demonstrate that OT can directly regulate MCH neurotransmission. Our behavioral pharmacology experiments show that OT acts on the downstream MCH neural circuit to reduce repetitive behavior. The behavioral and anatomical findings in our study warrant further investigation of the role of this circuit in disorders symptomatic of perseverative behaviors.

Figure 3.1: MCH system regulates marble-burying behavior.

(A) Number of marbles that were buried in 30-min marble-burying in wildtype vs. MCHR1 KO [unpaired t-test; t(28) = 3.987, *P = 0.0004, n = 14, 16].

(B) Number of marbles that were buried in 30-min marble-burying in DT treated $iDTR^{+}PmchCre^{-}$ (n = 11) and $iDTR^{+}PmchCre^{+}$ (n = 10), [unpaired t-test; t(19) = 4.072, *P = 0.0006].

(C) Number of marbles that were buried in 30-min marble-burying in CD1 mice centrally infused with vehicle vs. MCH [unpaired t-test; t(32) = 3.541, *P = 0.0012, n = 13, 12].

(D) Stereotypic counts in wildtype vs. MCHR1 KO [unpaired t-test; t(10) = 5.659, P = 0.0002, n = 6]

(E) Stereotypic counts in DT treated $iDTR^{+}PmchCre^{-}$ (n = 11) and $iDTR^{+}PmchCre^{+}$ (n = 10) [unpaired t-test; t(19) = 2.106, P = 0.0487]

(F) Stereotypic counts in CD1 mice centrally infused with vehicle vs. MCH [unpaired t-test; t(17) = 0.08685, P = 0.3972, n = 9-10].

(G) Time spent self-grooming by wildtype vs. MCHR1 KO [unpaired t-test; t(16) = 1.488, P = 0.1563, n = 9, 16]

(H) Time spent self-grooming by DT treated $iDTR^{+}PmchCre^{-}$ (n = 11) and $iDTR^{+}PmchCre^{+}$ (n = 10) [unpaired t-test; t(19) = 1.102, P = 0.28]

(I) Time spent self-grooming by CD1 mice centrally infused with vehicle vs. MCH [unpaired t-test; t(21) = 0.6213, P = 0.5411, n = 11,12].

(J) Total distance travelled by wildtype vs. MCHR1 KO [unpaired t-test; t(23) = 2.322, P = 0.0294, n = 12, 13].

(K) Total distance travelled by DT treated iDTR^{+}PmchCre^{-} (n = 11) and iDTR^{+}PmchCre^{+} (n = 10) [unpaired t-test; t(19) = 0.1, P = 0.46].

(L) Total distance travelled by CD1 mice centrally infused with vehicle vs. MCH [unpaired t-test; t(12) = 0.0977, P = 0.9237, n = 7].

Figure 3.1

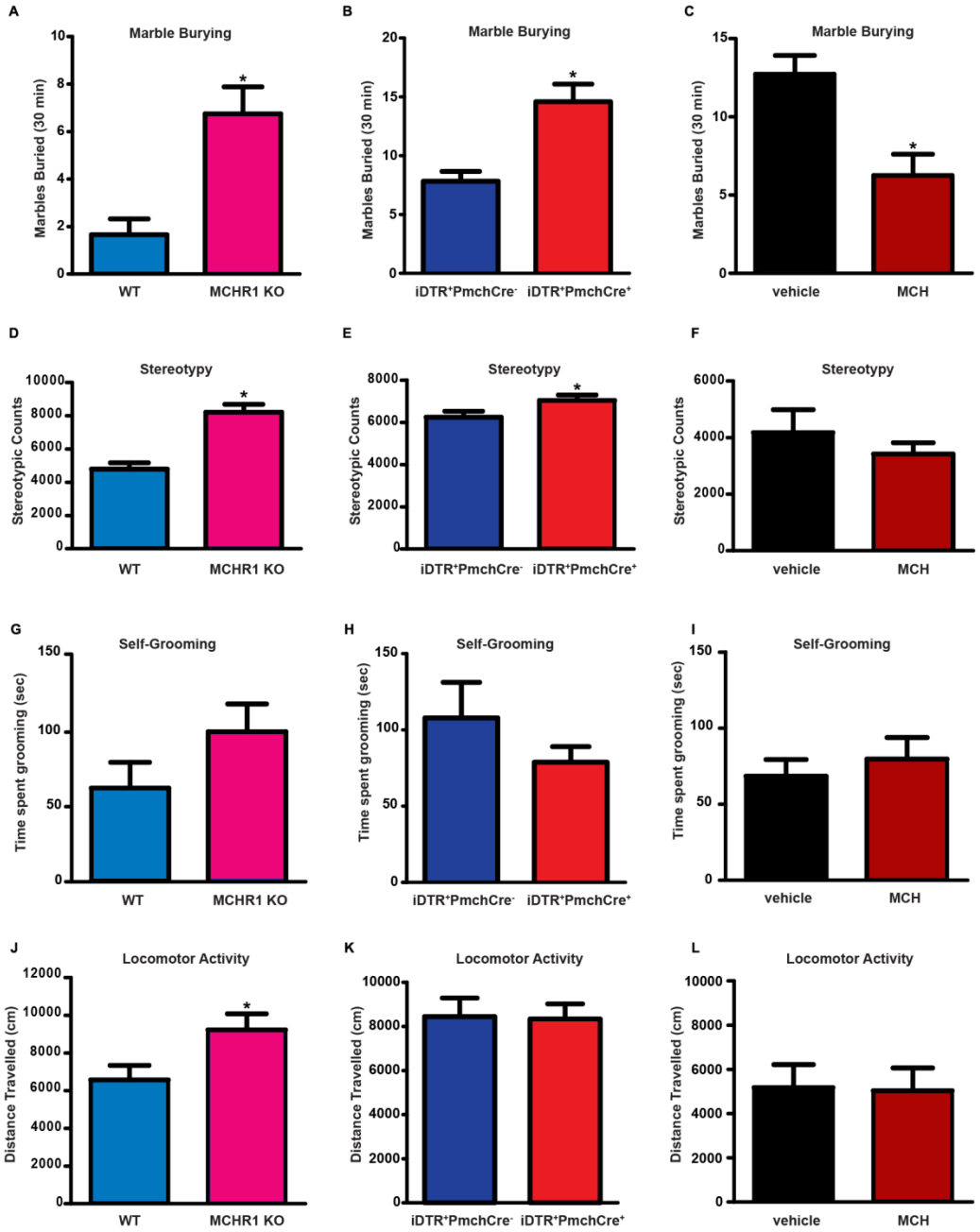

A
Marble Burying
Marbles Buried (30 min)
10
8
6
4
2
0
*
WT
MCHR1 KO
B
Marble Burying
Marbles Buried (30 min)
20
15
10
5
0
*
iDTR+PmchCre-
iDTR+PmchCre+
C
Marble Burying
Marbles Buried (30 min)
15
10
5
0
*
vehicle
MCH
D
Stereotypy
Stereotypic Counts
10000
8000
6000
4000
2000
0
*
WT
MCHR1 KO
E
Stereotypy
Stereotypic Counts
8000
6000
4000
2000
0
*
iDTR+PmchCre-
iDTR+PmchCre+
F
Stereotypy
Stereotypic Counts
6000
4000
2000
0
vehicle
MCH
G
Self-Grooming
Time spent grooming (sec)
150
100
50
0
WT
MCHR1 KO
H
Self-Grooming
Time spent grooming (sec)
150
100
50
0
iDTR+PmchCre-
iDTR+PmchCre+
I
Self-Grooming
Time spent grooming (sec)
150
100
50
0
vehicle
MCH
J
Locomotor Activity
Distance Travelled (cm)
12000
10000
8000
6000
4000
2000
0
*
WT
MCHR1 KO
K
Locomotor Activity
Distance Travelled (cm)
10000
8000
6000
4000
2000
0
iDTR+PmchCre-
iDTR+PmchCre+
L
Locomotor Activity
Distance Travelled (cm)
10000
8000
6000
4000
2000
0
vehicle
MCH

Figure 3.2: Differences between DT treated iDTR^{+}PmchCre^{-} (n = 11) and iDTR^{+}PmchCre^{+} (n = 10) groups in

(A) Body weight over the course of 12d. Two-way ANOVA shows a significant interaction of genotype x time [$F(13, 247) = 12.77$, $P < 0.0001$], time [$F(13, 247) = 17.87$, $P < 0.0001$] but not genotype [$F(1,247) = 0.005$, $P = 0.95$]

(B) Open Field measure of time center:periphery ratio [unpaired t-test; $t(19) = 1.231$, $P = 0.24$].

(C) Light Dark box test in time spent in light compartment [unpaired t-test; $t(19) = 0.6457$, $P = 0.87$].

(D) Elevated Plus Maze measure of percent entry to closed [unpaired t-test; $t(19) = 0.6073$, $P = 0.94$] and open arms [unpaired t-test; $t(19) = 0.2584$, $P = 0.7989$].

(E) the sociability trial (unfamiliar animal versus empty chamber), both groups of mice spent more time in the chamber containing the social partner (unfamiliar animal) than the empty cup, iDTR^{+}PmchCre^{-} [unpaired t-test; $t(20) = 5.353$, $^{*}P < 0.0001$]; iDTR^{+}PmchCre^{+} [unpaired t-test; $t(18) = 4.245$, $^{*}P < 0.0001$].

(F) time spent sniffing nonsocial odors (water, almond, and banana) and social odors (swabs of used cages of unfamiliar mice) during three consecutive presentations of each odor in the Odor discrimination assay. While odor had a significant effect [two-way repeated measures ANOVA with Bonferroni post tests; $F(14, 266) = 16.95$, $^{*}P < 0.0001$], neither genotype [two-way repeated measures ANOVA with Bonferroni post tests; $F (1, 266) = 0.47$, $P = 0.50$, n=10-11] nor genotype x odor [two-way repeated measures ANOVA with Bonferroni post tests; $F (1, 266) = 0.79$, $P = 0.68$] showed significant effect on time spent sniffing. Values represent mean ± SEM.

Figure 3.2

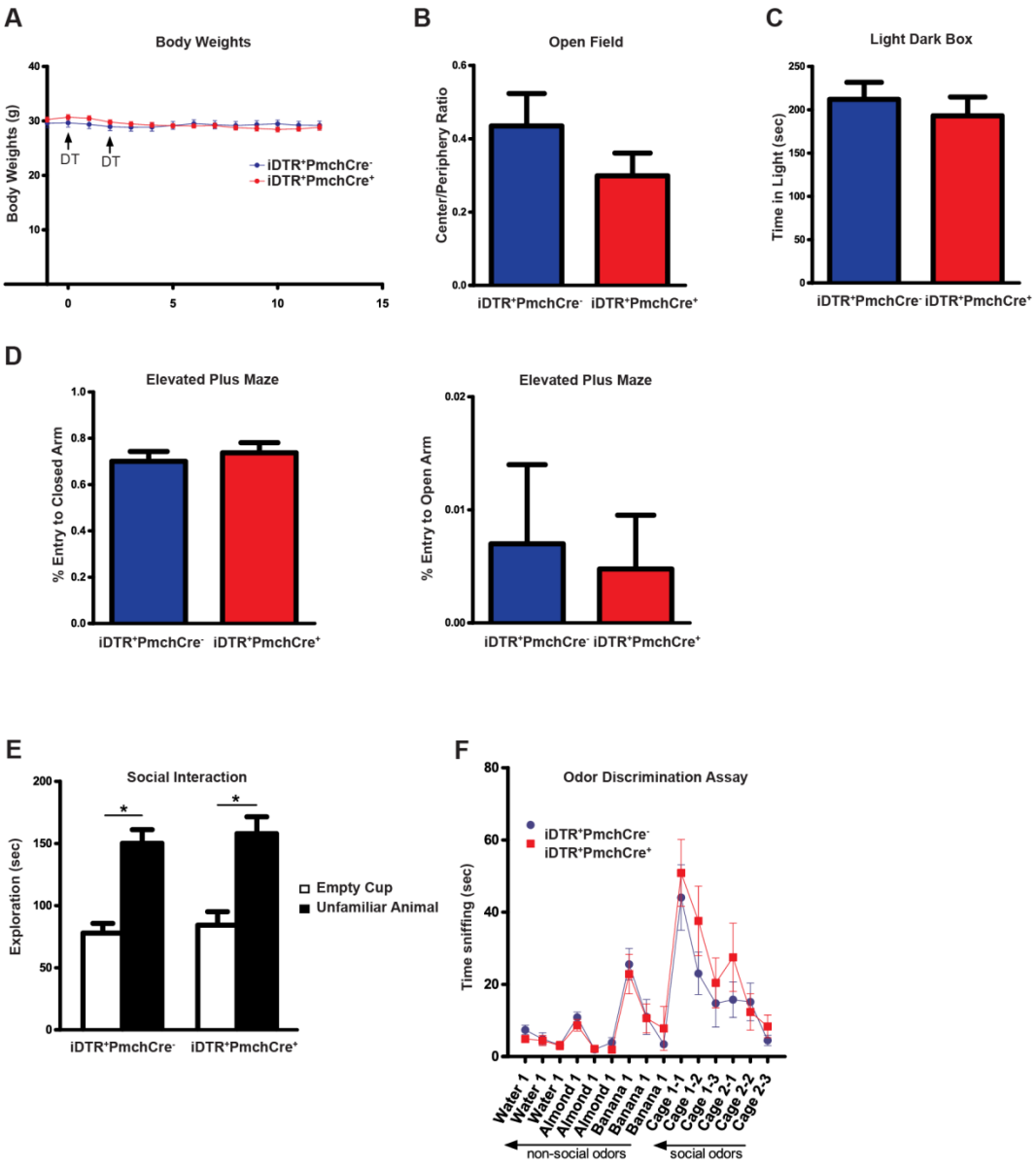

A
Body Weights
Body Weights (g)
DT
DT
iDTR+PmchCre-
iDTR+PmchCre+
B
Open Field
Center/Periphery Ratio
iDTR+PmchCre-
iDTR+PmchCre+
C
Light Dark Box
Time in Light (sec)
iDTR+PmchCre-
iDTR+PmchCre+
D
Elevated Plus Maze
% Entry to Closed Arm
iDTR+PmchCre-
iDTR+PmchCre+
Elevated Plus Maze
% Entry to Open Arm
iDTR+PmchCre-
iDTR+PmchCre+
E
Social Interaction
Exploration (sec)
Empty Cup
Unfamiliar Animal
iDTR+PmchCre-
iDTR+PmchCre+
F
Odor Discrimination Assay
Time sniffing (sec)
iDTR+PmchCre-
iDTR+PmchCre+
Water 1
Water 1
Water 1
Almond 1
Almond 1
Almond 1
Banana 1
Banana 1
Banana 1
Cage 1-1
Cage 1-2
Cage 1-3
Cage 2-1
Cage 2-2
Cage 2-3
non-social odors
social odors

Figure 3.3: cFos immunoreactivity in DT treated iDTR^{+}PmchCre^{-} and iDTR^{+}PmchCre^{+} 90 min. following marble burying in the

(A) Lateral orbital cortex (LO) [unpaired t-test; t(6) = 3.016, *P = 0.02].

(B) Medial orbital cortex (MO) [unpaired t-test; t(6) = 0.7730, P = 0.47].

(C) ventral orbital cortex (VO) [unpaired t-test; t(6) = 2.088, P = 0.08].

(D) Representative images of cFos immunoreactivity (green fluorescence) in the orbitofrontal cortex in DT treated iDTR^{+}PmchCre^{-} and iDTR^{+}PmchCre^{+} 90 min. following Marble Burying. Values represent mean ± SEM (n = 4/group).

Figure 3.3

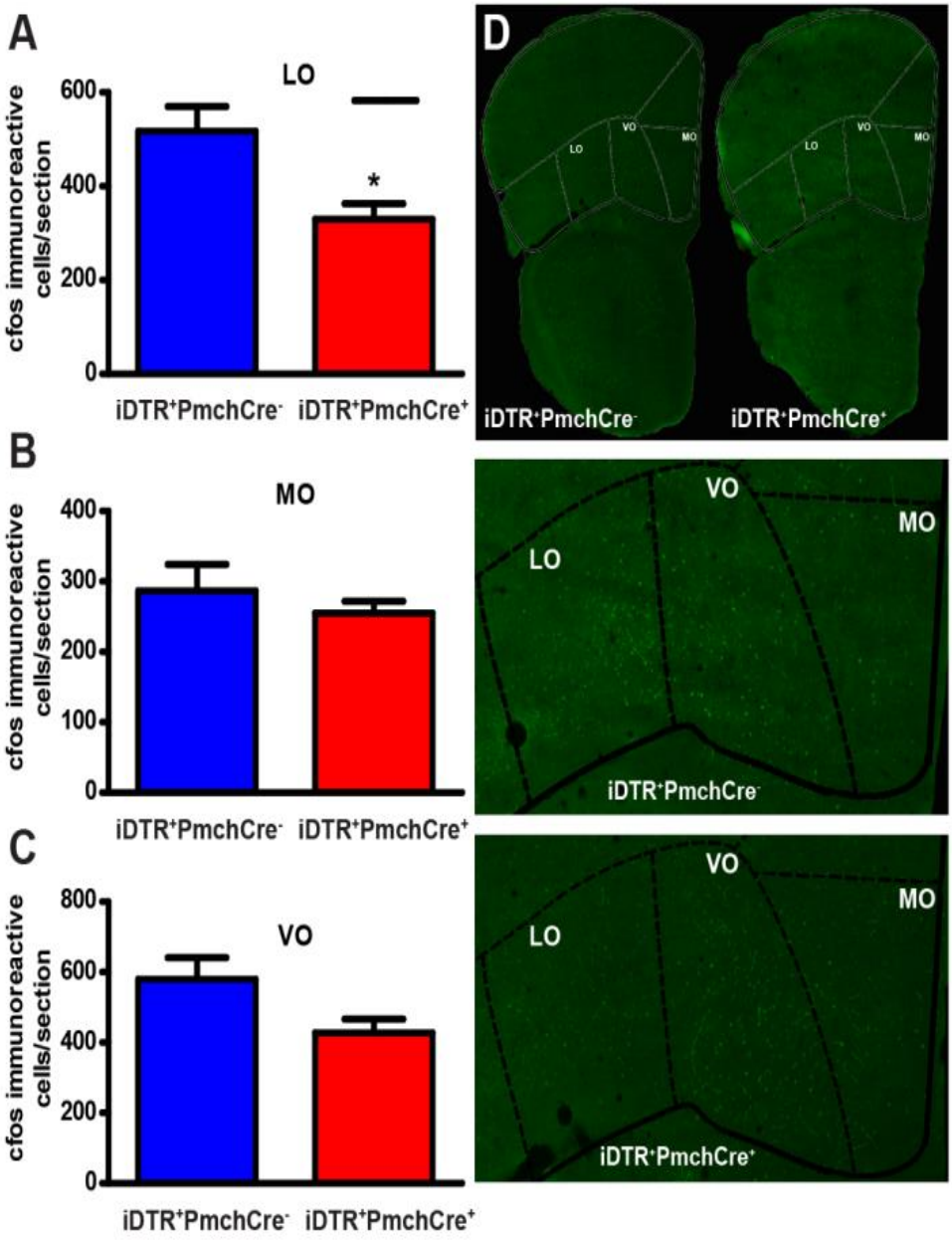

A
LO
cfos immunoreactive cells/section
600
400
200
0
*
iDTR+PmchCre-
iDTR+PmchCre+
D
VO
MO
LO
iDTR+PmchCre-
iDTR+PmchCre+
B
MO
400
300
200
100
0
iDTR+PmchCre-
iDTR+PmchCre+
VO
MO
LO
iDTR+PmchCre-
C
VO
800
600
400
200
0
iDTR+PmchCre-
iDTR+PmchCre+
VO
MO
LO
iDTR+PmchCre+

Figure 3.4: SADdeltaG-mCherry(EnvA) retrogradely labeled cells in PVN expressing oxytocin. A schematic representation of MCHCre mouse line and tracing strategy of monosynaptic inputs to MCH neurons in vivo. We used MCHCre transgenic mice in which only MCH cells express Cre-recombinase. First, we injected the helper virus AAV8 which encodes the rabies envelope glycoprotein (G) important for trans-synaptic spreading of the rabies virus and the TVA receptor, an avian receptor protein that confers infection capability to rabies virus pseudotyped with the avian sarcoma leucosis virus glycoprotein EnvA. The TVA receptor expression in MCH cells allows for the rabies viral SADΔGmCherry strain to infect TVA expressing MCH cells. This strain has been genetically engineered to have the G glycoprotein deleted and replaced with a mCherry reporter. N, P, M and L respectively represent the viral genomic sequences encoding the wild-type nucleoprotein, phosphoprotein, matrix protein and polymerase required for rabies virus expression. The pseudotyped rabies virus can thus infect MCH cells and monosynaptically spread to cells which are directly presynaptic to MCH cells. The virus is then trapped in these presynaptic partner cells and cannot spread further due to the deficiency of G in these presynaptic cells.

(A) PVN oxytocin immunoreactive neurons (green fluorescence) constitute a small subpopulation of direct presynaptic cells (red fluorescence) communicating to MCH cells. Scale bar = 50um.

(B) Monosynaptic rabies labeled neurons in the PVN showing red fluorescence (open arrow).

(C) Oxytocin immunoreactive neurons in the PVN (closed arrow)

(D) Merged image showing colocalization of oxytocin and presynaptic cells (closed arrowhead). B-D. Scale bar = 10um.

Figure 3.4

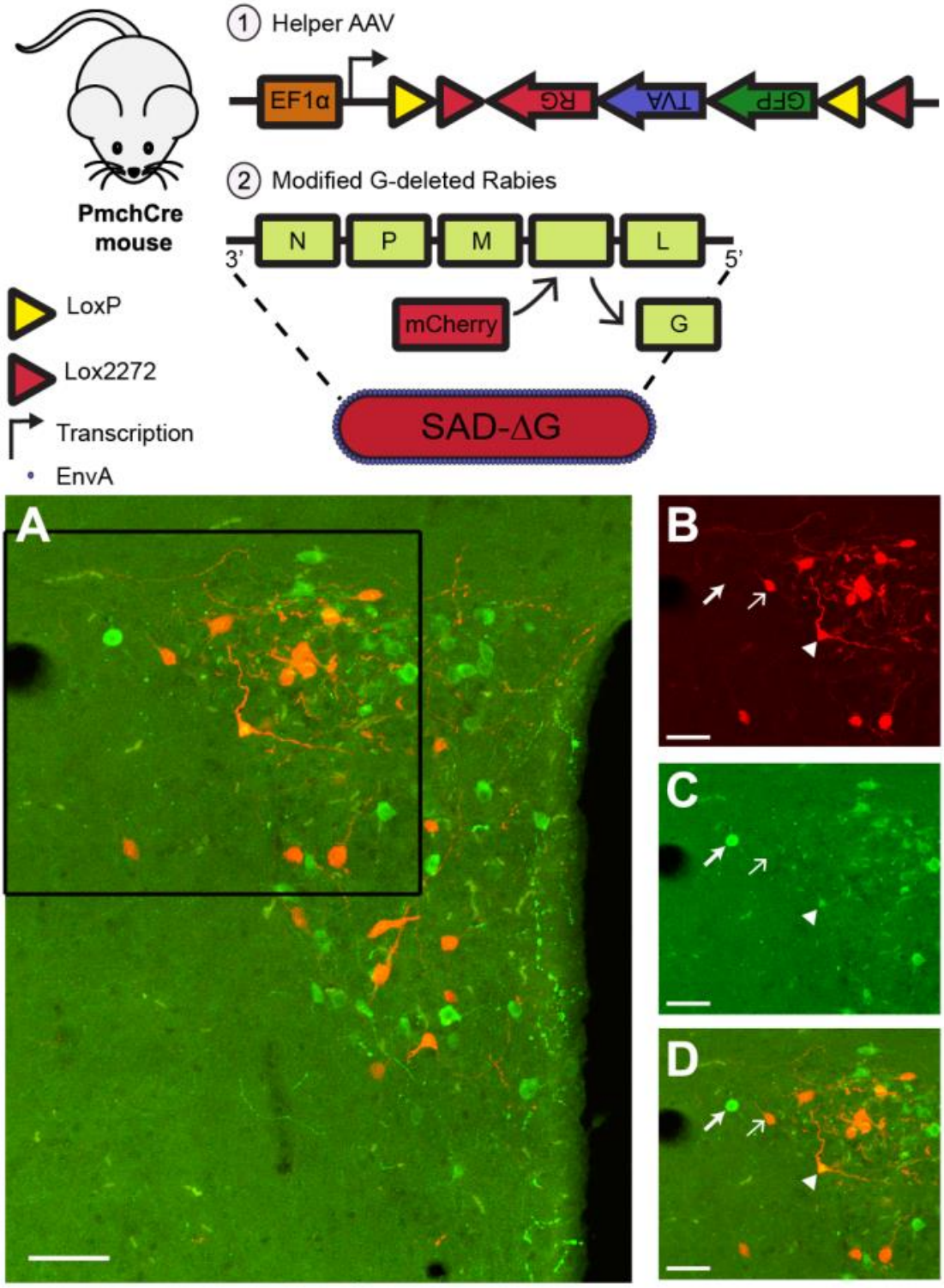

1 Helper AAV
EF1α
RG
TVA
GFP
2 Modified G-deleted Rabies
N
P
M
L
3'
5'
mCherry
G
SAD-ΔG
PmchCre mouse
LoxP
Lox2272
Transcription
EnvA
A
B
C
D

Figure 3.5: Expression of OTR Venus transgene in MCH-containing neurons in OTR venus transgenic mice. Immunoreactivity of

(A) MCH (red fluorescence; open arrow) and

(B) OTR-Venus (green fluorescence; closed arrow) and

(C) colocalization (merge; closed arrowhead) in the lateral hypothalamic area of an OTR Venus transgenic male mouse. Scale bar = 50um.

Figure 3.5

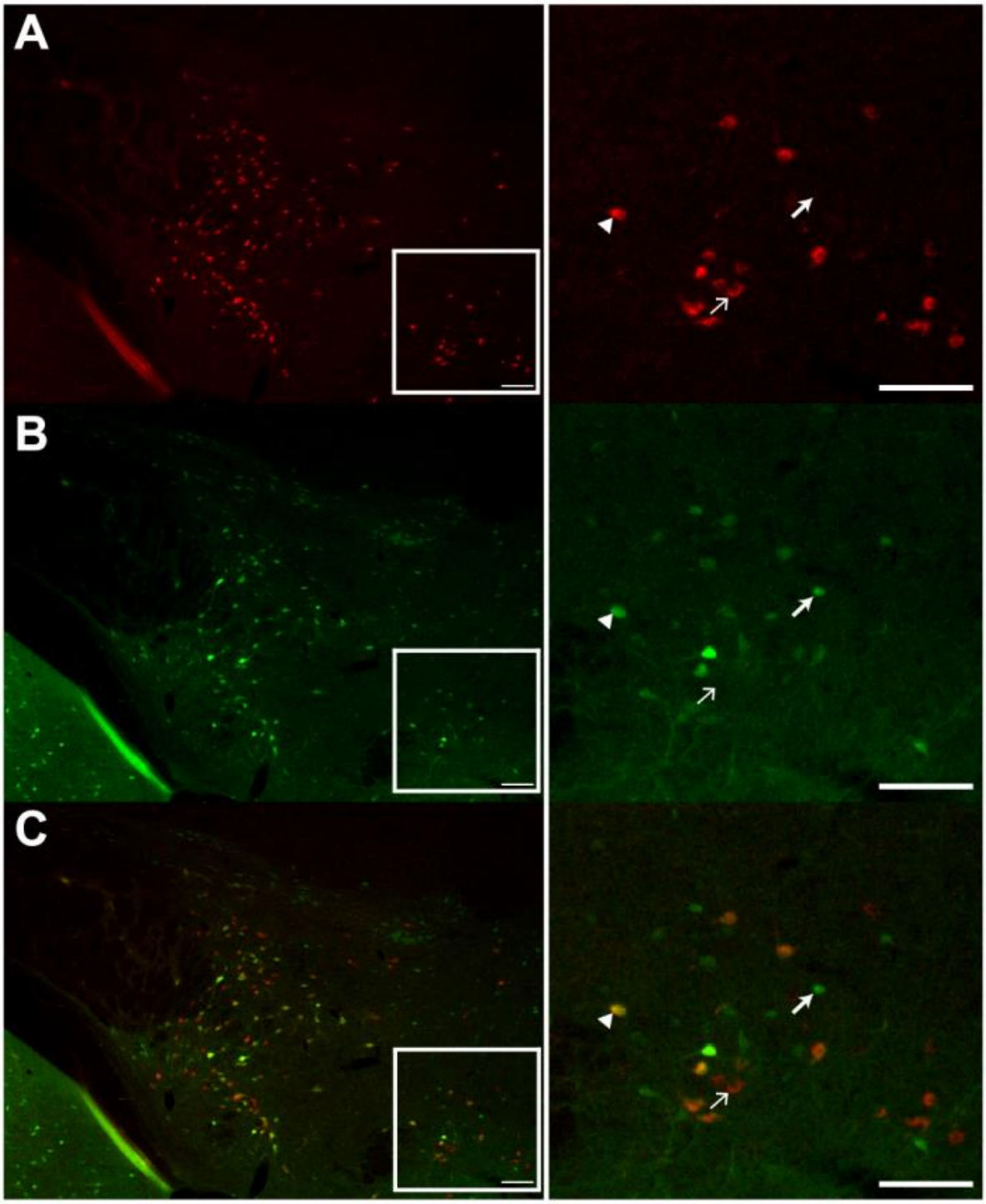


Figure 3.6: Animals received i.p. injection of vehicle or 3mg/kg GW803430, an MCH1R antagonist, and i.c.v. administration of PBS, MCH, OT, or MCH+OT and were tested for (A) Marble-burying behavior [one-way ANOVA $F(7, 121) = 7.229$, $^*P < 0.0001$, n = 13-18/group; Bonferroni post-hoc test revealed a significant difference $P < 0.05$ between OT vs. GW + OT, MCH vs. GW + MCH, MCH + OT vs. GW + MCH + OT, but not vehicle and GW], (B) Distance Traveled [one-way ANOVA; $F(7, 56) = 0.1606$, $P = 0.99$, n = 7-11/group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups]

(C) Open Field center:periphery ratio [one-way ANOVA; $F(7, 80) = 0.5999$, $P = 0.75$, n = 7-11/group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups]

(D) Time spent in light compartment of the Light dark box [one-way ANOVA $F(7, 49) = 1.688$, $P = 0.13$, n = 6-8/group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups]

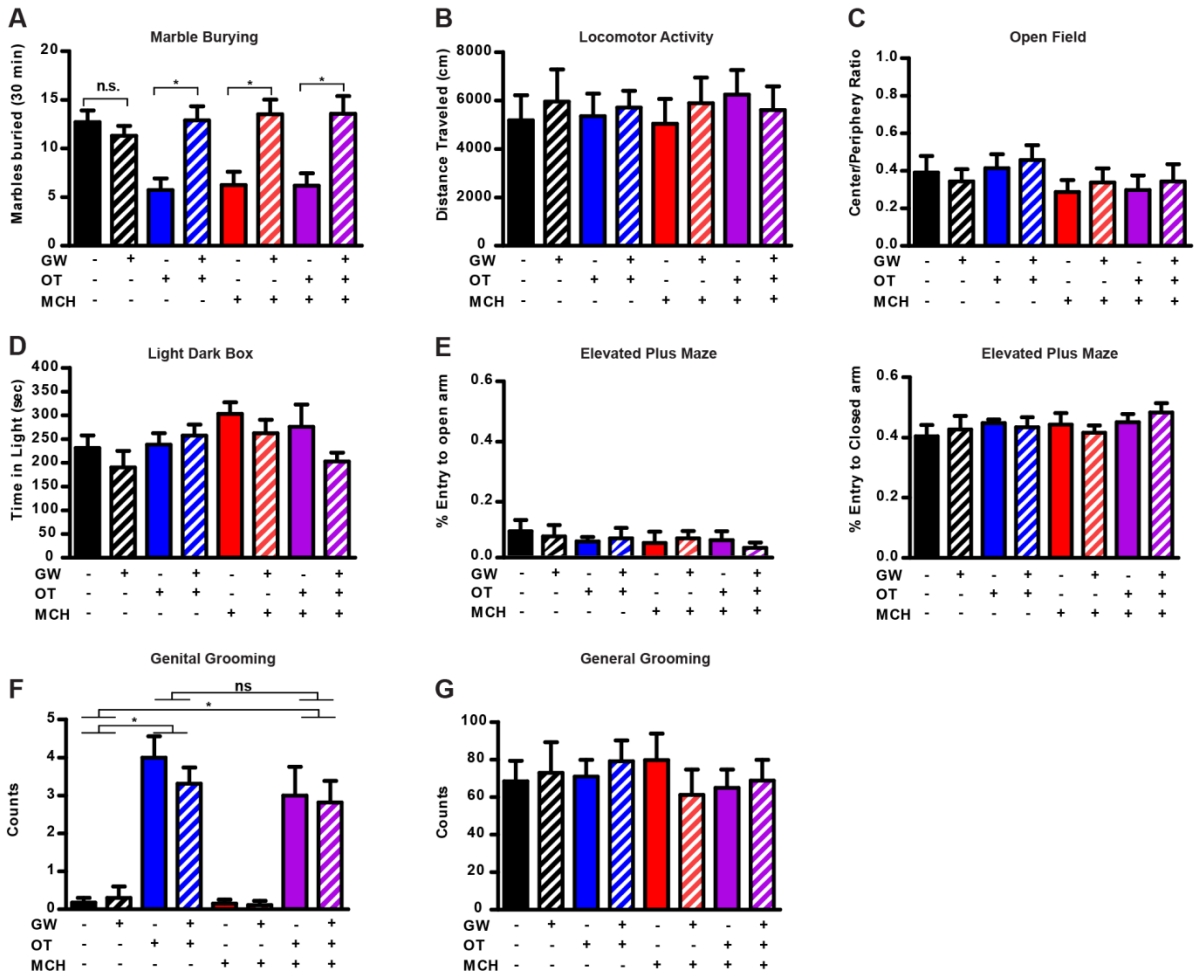
(E) EPM % entries to open arm [one-way ANOVA; $F(7, 44) = 0.3180$, $P = 0.94$, n = 6-8/group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups] and closed arm [one-way ANOVA; $F(7, 44) = 0.6085$, $P = 0.75$, n = 6-8/group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups]

(F) Bouts of genital grooming [one-way ANOVA; $F(7, 79) = 15.53$, $^*P < 0.0001$, n = 9-13/group; Bonferroni post-hoc test revealed a significant difference $P < 0.05$ between vehicle vs. OT, vehicle vs. GW + OT, vehicle vs. MCH + OT, vehicle vs. GW + MCH + OT, GW vs. OT, GW vs. GW + OT, GW vs. MCH + OT, GW vs. GW + MCH + OT, OT vs. MCH, OT vs. GW

+ MCH, GW + OT vs. MCH, GW + OT vs. GW + MCH, MCH vs. MCH + OT, MCH vs. GW + MCH + OT, GW + MCH vs. MCH + OT, GW + MCH vs. GW + MCH + OT

(G) Bouts of general grooming [one-way ANOVA; $F(7, 78) = 0.2828$, $P = 0.96$, n = 9-13/group]. Data are presented as the means ± SEM.

Figure 3.6


A
Marble Burying
Marbles buried (30 min)
n.s.
B
Locomotor Activity
Distance Traveled (cm)
C
Open Field
Center/Periphery Ratio
D
Light Dark Box
Time in Light (sec)
E
Elevated Plus Maze
% Entry to open arm
Elevated Plus Maze
% Entry to Closed arm
Genital Grooming
ns
Counts
F
General Grooming
Counts
G
GW
OT
MCH

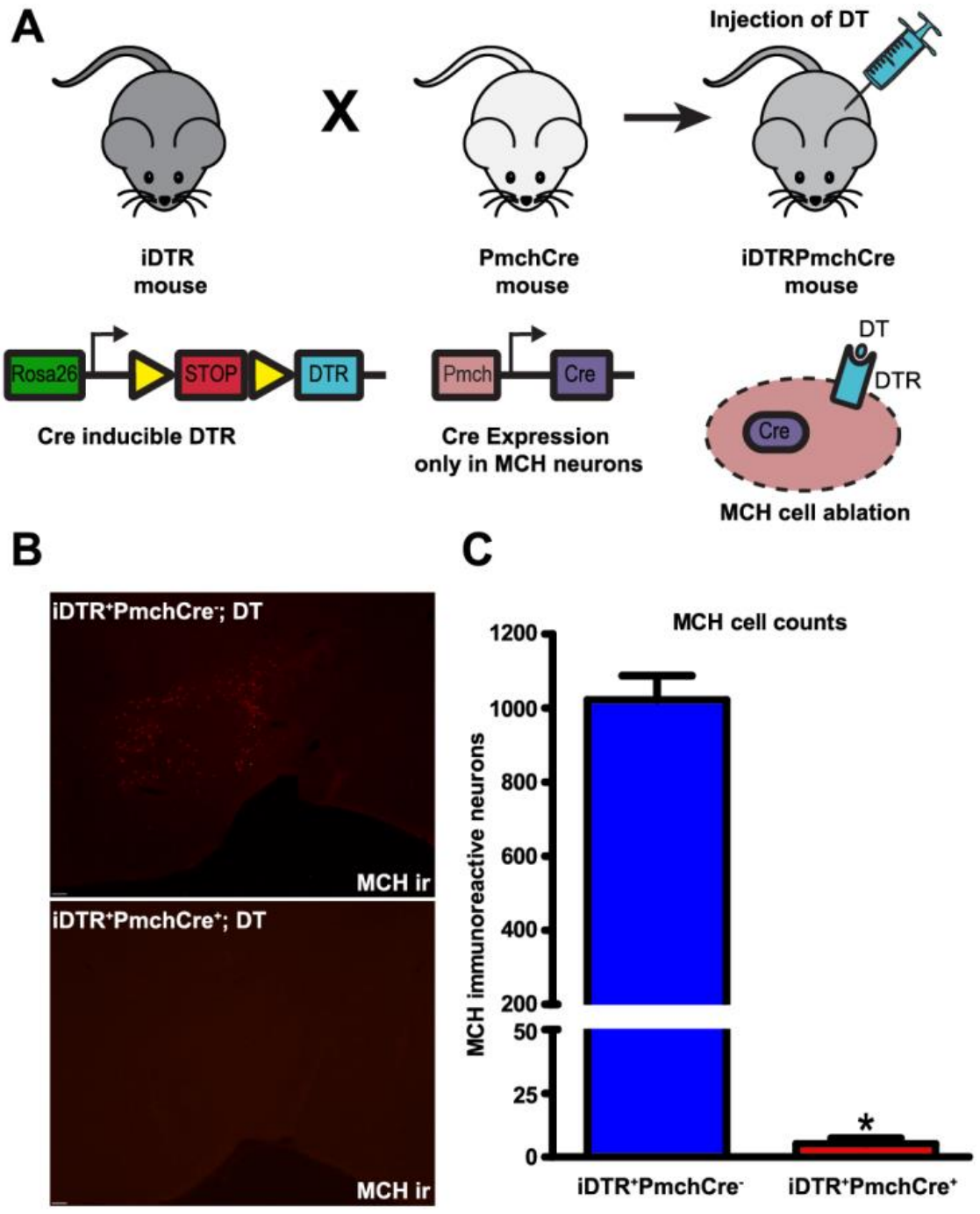
Supplementary Figure 3.1: Diphtheria Toxin (DT) mediated ablation of MCH neurons

A) Schematic demonstrating how MCH cells are ablated using the DTR system. We crossed the iDTR strain to PmchCre strain rendering Cre expressing MCH neurons sensitive to DT.

B) MCH immunoreactivity (red fluorescence) in the lateral hypothalamus and zona incerta of $iDTR^{+}PmchCre^{-}$ and $iDTR^{+}PmchCre^{+}$ mice following DT (Scale bar 100um).

C) Cell counts of MCH immunoreactivity in DT treated $iDTR^{+}PmchCre^{-}$ and $iDTR^{+}PmchCre^{+}$ mice. Values represent mean ± SEM and were analyzed by unpaired t-test; $t(19)=14.93$, $^{*}P < 0.0001$, n = 10-11.

Supplementary Figure 3.1



Supplementary Figure 3.2: cFos immunoreactivity in DT treated iDTR^{+}PmchCre^{-} and iDTR^{+}PmchCre^{+} 90 min. following Marble Burying in the

A) Lateral septal nucleus, intermediate part (LSI) [unpaired t-test; t(6) = 3.292, *P = 0.02]

B) Retrosplenial granular cortex (RSG) [unpaired t-test; t(6) = 2.492, *P = 0.048]

C) Retrosplenial agranular cortex (RSA) [unpaired t-test; t(6) = 3.303, *P = 0.02].

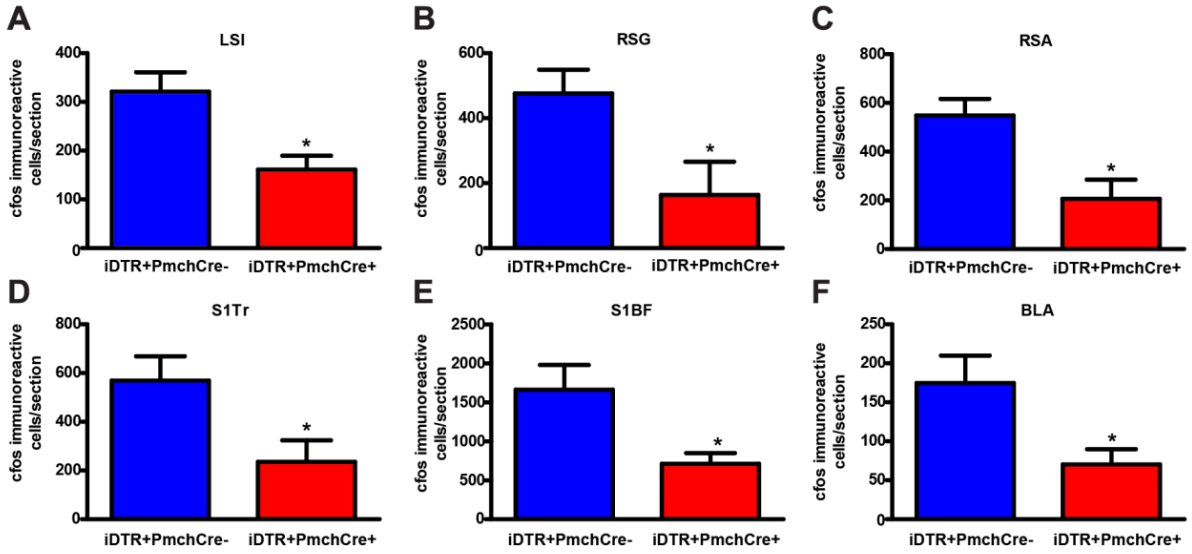
D) Primary somatosensory cortex, trunk region (S1Tr) [unpaired t-test; t(6) = 2.506, *P = 0.046].

E) Primary somatosensory cortex, barrel field (S1BF) [unpaired t-test; t(6) = 2.748, *P = 0.03].

F) Basolateral amygdaloid nucleus, anterior part (BLA) [unpaired t-test; t(6) = 2.594, *P = 0.04].

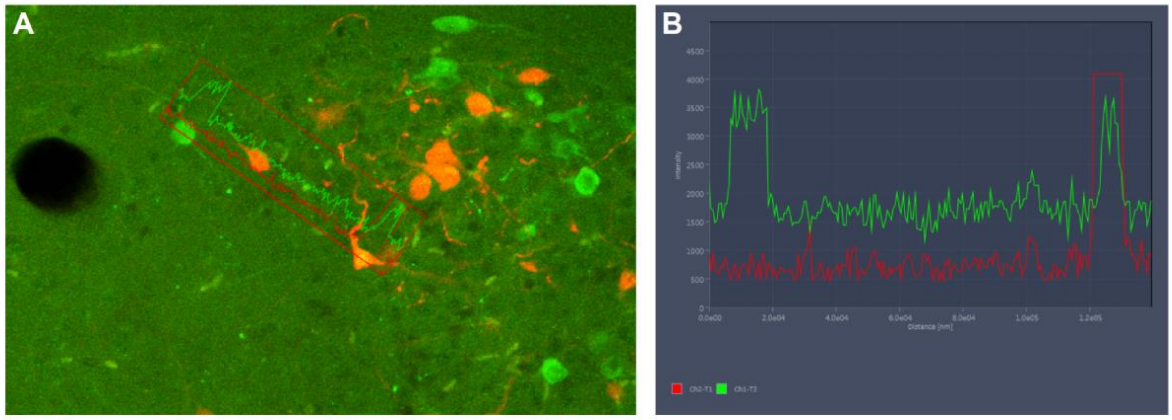
Values represent mean ± SEM (n = 4/group).

Supplementary Figure 3.2



Supplementary Figure 3.3: A-B) Spectral analysis of confocal image of colocalization of an oxytocin neuron and presynaptically labeled neuron.

Supplementary Figure 3.3



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Chapter 4

Conclusion

Summary of Findings

In the present studies we defined the brain nuclei and subpopulations of neurons that provide direct innervation to MCH neurons. We also report a novel role for the MCH system in repetitive behavior. In Chapter 2 we first explored the regulation of the MCH system by first identifying the specific monosynaptic inputs to LHA MCH neurons using modified rabies-mediated circuit mapping technique in a Cre dependent transgenic mouse line, pMCHCre. We analyzed the distribution of presynaptic neurons from each brain area and found that MCH neurons received the strongest presynaptic inputs from the paraventricular nucleus, lateral hypothalamus, supramammillary nuclei, and mammillary bodies. Neurochemical analysis of these presynaptic inputs revealed that the LHA MCH neurons receive direct presynaptic input from oxytocin, vasopressin, MCH, orexin, histamine, and OFQ/N synthesizing neurons identifying these neuropeptides as having direct regulation of LHA MCH neurons. OFQ/N and orexin represented the strongest presynaptic inputs to the MCH system and previous work in our lab confirms that the receptors of both of these neuropeptides show high levels of colocalization in MCH neurons (Parks et al., 2014b). Taken together, these studies suggest that OFQ/N and orexin may exert more robust control of the perifornical LHA MCH neurons. This study also identified direct synaptic input from oxytocin neurons which were previously identified as having a stimulatory action on MCH cells (Yao et al., 2012). Our neuroanatomical work in

Chapter 2 confirmed a direct neuroanatomical link between these two peptides and laid the groundwork for further functional studies to investigate the OT-MCH neural circuit.

The findings reported in Chapter 3 show that disruption of the MCH system increases repetitive behavior while its activation decreases it. Melanin concentrating receptor knockout (MCHR1KO) mice and MCH ablated animals show increased repetitive behavior while central MCH infusion decreases it. The regulation of repetitive behavior in both animal models and human trials has also been linked to the OT system. Based on our findings in Chapter 2, which demonstrate that MCH neurons receive direct presynaptic input from oxytocin neurons we investigated whether the neuropeptide OT acted through the MCH system to influence repetitive behavior. Immunohistochemical analysis revealed that nearly 60% of MCH neurons express oxytocin receptors. Pharmacological studies further demonstrated the existence of an interaction between the MCH and oxytocin systems. We discovered that central infusions of MCH and oxytocin alone or together reduce repetitive behavior while antagonizing the MCH system blocks oxytocin-mediated reduction of repetitive behavior. This finding suggests that MCH neurons are downstream of oxytocin cells regulating repetitive behavior. Using a combination of genetic, neuroanatomical, and pharmacological approaches we report a novel role for the MCH system in regulating oxytocin mediated repetitive behavior.

Furthermore, disruption of the MCH system by ablating MCH neurons in adulthood leads to a significant decrease in MCH neuronal activation measured by cFos immunoreactivity in the lateral orbital cortex, an area important in regulating repetitive behavior. Both our behavioral and anatomical observations complement the finding that optogenetic activation of the lateral orbital cortex in an animal model that displays pathological repetitive behavior, the Sapap3

mutant mice, alleviates perseverative behavior (Burguiere et al., 2013). The lateral orbital cortex is part of the orbitofrontal cortex; this subsection of the prefrontal cortex is shown to be important in OCD like behaviors (Ahmari et al., 2013; Burguiere et al., 2013), and also expresses MCHR1 in high levels (Hervieu et al., 2000). Thus, long term disruption of the MCH system leads to decreased activation of the lateral orbital cortex contributing to an increase in repetitive behavior. The behavioral and anatomical findings from our studies warrant further investigation of the role of the MCH system in disorders symptomatic of perseverative behaviors.

In conclusion, the work described in this dissertation has identified several neuropeptide systems that can directly regulate MCH neurons to mediate some of the diverse functions that this system regulates. Our work has characterized a novel interaction between MCH and oxytocin neurons which may be involved in regulating repetitive behavior, and has identified a novel phenotype in MCHR1 knockout mice and MCH ablated animals that strongly suggests that the MCH system may regulate perseverative behaviors. This work contributes to our overall understanding of the neuroanatomical and neurochemical regulation of MCH neurons and serves as a framework for future studies investigating the functions of the novel MCH neural circuits defined in these studies.

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