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## Melanin concentrating hormone modulates oxytocin-mediated marble burying

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### 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. The oxytocin system has been linked to the regulation of repetitive behavior in both animal models and humans, but many of its downstream targets have still to be found. We report that the melanin-concentrating hormone (MCH) system is a target of the oxytocin system in regulating one repetitive behavior, marble burying. First we report that nearly 60% of MCH neurons express oxytocin receptors, and demonstrate using rabies mediated tract tracing that MCH neurons receive direct pre-synaptic input from oxytocin neurons. Then we show that MCH receptor knockout (MCHR1KO) mice and MCH ablated animals display increased marble burying response while central MCH infusion decreases it. Finally, we demonstrate the downstream role of the MCH system on oxytocin mediated marble burying by showing that central infusions of MCH and oxytocin alone or together reduce it while antagonizing the MCH system blocks oxytocin-mediated reduction of this behavior. Our findings reveal a novel role for the MCH system as a mediator of the role of oxytocin in regulating marble-burying behavior in mice.

### Keywords

Rabies circuit mapping; MCHR1; Orbitofrontal cortex; Perseverative behavior

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## 1. Introduction

Marble-burying is a repetitive digging behavior that is perseverative and resistant to changes based on familiarity. It has been suggested as a screening test for anxiolytic response, but more recently has been proposed as modeling repetitive behaviors characteristic of mental disorders such as obsessive-compulsive disorder (OCD) or autism spectrum disorder (ASD) (Thomas et al., 2009; Greene-Schloesser et al., 2011; Angoa-Perez et al., 2013; Aguilar-Valles et al., 2015; Mitra et al., 2016a, 2016b, 2017).

The oxytocin (OT) system is a neuropeptide system that has gained increasing importance in our understanding of repetitive behaviors. 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).

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 (LH) (Hirose et al., 2016). One of the best characterized neuropeptide systems in the LH is the melanin-concentrating hormone (MCH) system.

MCH is a 19 amino acid peptide made in two specific hypothalamic nuclei, the 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, 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). 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 (Bodfish et al., 2000; de Bildt et al., 2004; Kim and Lord, 2010; Mandy et al., 2011).

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 role of the MCH system in repetitive behavior and to determine whether there is an interaction between the MCH and OT system in regulating repetitive behavior.

## 2. Materials and methods

### 2.1. Animals

All animal experiments were carried out in accordance with the University of California, Irvine's Animal Institutional Animal Care and Use Committee (IACUC #2002–2343).

**2.1.1. Diphtheria Toxin (DT) mediated ablation of MCH neurons—***mchCre/+; 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). We crossed the *iDTR* strain to *PmchCre* strain rendering Cre expressing MCH neurons sensitive to DT. Adult male animals were group housed under controlled conditions (temperature  $21\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ ; 12 h light–dark cycle, lights on at 7:00 a.m.) with free access to water and food. All operant procedures were performed during the light phase (between 10 a.m. and 4 p.m.).

**2.1.2. MCHCre mouse line to demonstrate monosynaptic inputs to MCH neurons in vivo—**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.

**2.1.3. Wild-type mice used for ICV administration of peptides—**Male CD1 mice (Charles Rivers, Wilmington, MA, USA) 7–11 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 h light/dark cycle, with food and water ad lib.

#### 2.1.4. OTR venus transgenic mice used to demonstrate MCH colocalization—

Immunoreactivity of OXTR-Venus knock-in male mice brain tissue was acquired from the lab of Dr. Katsuhiko Nishimori from Tohoku University, Miyagi, Japan.

### 2.2. Surgery

All stereotaxic surgery was conducted in Dr. Xiangmin Xu's lab using a stereotaxic machine (MyNeuroLab two arm stereotaxic with Angle Two software). Under isoflurane anesthesia, 6 week aged *Pmch-cre* mice were injected with 0.2  $\mu$ l 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,  $-1.82$  mm; mediolateral,  $+0.91$  mm; and dorsoventral,  $-5.25$  (Paxinos and Franklin, 2001)). Following surgery, mice were allowed to recover for three weeks 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.

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 g of a mixture of ketamine and xylazine (Ketamine 100 mg/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 0.5 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.

### 2.3. Drugs

**2.3.1. Diphtheria toxin (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 (16 ng/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.

**2.3.2. 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; Toshinai et al., 2010) and OT (Arletti et al., 1985; Argiolas et al., 1986; Amico et al., 2004). The injection cannula was connected via PE50 tubing to a 50  $\mu$ l Hamilton microsyringe fitted to a microinjection pump (KDS 200, KD Scientific). Infusions were administered in a volume of 2  $\mu$ l over 2 min, and an additional 2 min was allowed for diffusion before the infusion cannulas were removed. 3 mg/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 3 mg/kg dose (Gehlert et al., 2009).

## 2.4. Behavioral testing

**2.4.1. Locomotion, stereotypic activity and open field**—Locomotion was monitored in an open field test chamber (40 × 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 travelled was measured by infrared beam arrays and recorded, analyzed and calculated by Activity Monitor 5 software (Med Associates, Inc.). The horizontal, vertical, and stereotypic activities were also recorded and analyzed. 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 × 33.75) vs the peripheral area defined as the 6.25 cm strip surrounding the center area.

## 2.5. 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.

**2.5.1. 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.

**2.5.2. 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). 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.

**2.5.3. 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.

**2.5.4. 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 s 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.

## 2.6. 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. 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<sup>+</sup>PmchCre<sup>+</sup> mice and iDTR<sup>+</sup>PmchCre<sup>-</sup> littermates. For c-Fos immunohistochemistry, mice were anesthetized 90 min after marble-burying and perfused transcardially. Brain sections were incubated with rabbit anti-c-Fos antibody (1:5000; 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. 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).

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 μm 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 h 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.

**2.6.1. Histology**—Lateral ventricle guide cannula placements in CD1 mice were assessed after the behavioral experiments. The injection sites were verified by using a bright-field microscope to visualize the thionin staining.

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

### 3. Results

#### 3.1. The anatomical interactions between the OT and MCH systems

Oxytocin has been shown to depolarize MCH cells but no other GABAergic neurons in the LH, suggesting a selective innervation of this system (Yao et al., 2012). To determine whether OT neurons 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 (Fig. 1A–D, S1). Only a subpopulation of cells presynaptic to MCH neurons from the PVN express the neuropeptide oxytocin (0.7%  $\pm$  0.4%).

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%  $\pm$  1.6% overlap with MCH (Fig. 2) whereas 4% of OT neurons express MCHR1 as reported by Chee et al. (2013).

#### 3.2. The MCH system regulates marble burying

Acute administration of OT has been shown to decrease repetitive stereotypical behaviors in inbred mouse models of ASD (Teng et al., 2013). To first 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.

The ablation efficiency in DT MCH-ablated mice 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  $\pm$  64.71 vs. 5.2  $\pm$  2.185; Fig. 3). MCHR1 KO and diphtheria toxin (DT) treated  $iDTR^{+}PmchCre^{+}$  (MCH-ablated) mice showed a significant increase in marble-burying behavior and stereotypy measurement compared to their respective controls (Fig. 4A–B, S2 A-B). Consistent with these results, icv infusion of MCH in wildtype mice decreased marble-burying in comparison to vehicle infusion (Fig. 4C) but did not affect stereotypy (Figure S2 C). 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 S2 D-F). Increased locomotor activity was detected in MCHR1KO as previously reported



(Figure S2 G (Marsh et al., 2002; Smith et al., 2005, 2008; Zhou et al., 2005)), but no changes in locomotor activity were detected in adult DT treated  $iDTR^{+}PmchCre^{+}$  vs. DT treated  $iDTR^{+}PmchCre^{-}$  control mice (Figure S2 H), and wild type mice infused centrally with MCH vs. vehicle (Figure S2 I).

MCH-ablated and MCH-intact mice show differential c-fos activation following the 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  $iDTR^{+}PmchCre^{-}$  (Fig. 5A). No significant differences were found between groups in the medial or ventral orbital cortex (Fig. 5B–D).

### 3.3. MCH mediates OT-induced reduction of marble burying

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 3 mg/kg dose of GW803430, significantly reversed the MCH, OT, and MCH+OT mediated reduction in marble burying (Fig. 6A). Baseline marble-burying behavior was not significantly different from vehicle at the 3 mg/kg i.p dose of GW803430 (Fig. 6A). 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 (Fig. 6B). 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 (Fig. 6C). We also tested exploratory and anxiety behaviors in these animals. No significant difference between groups was detected in open-field assay, light-dark box, elevated plus maze, or locomotor activity (Fig. 6D–F). i.c.v. infusion of OT have been shown to increase genital grooming in mice (Amico et al., 2004).

## 4. 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 marble-

burying behavior – a type of repetitive behavior. Our data also show that this role is mediated at least in part by interactions between the MCH and oxytocin systems.

First we identify an anatomical link between MCH and OT neurons. 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.

Then, our data show that disruption of the MCH system increases marble burying. Both MCHR1 knock out animals and MCH ablated mice display significant increases in marble-burying while, MCH icv infusions decrease marble-burying behavior. Acute blockade via MCHR1 antagonist or stimulation via central MCH infusion, does not induce stereotypy, although a more chronic disruption of the MCH system, as modeled through MCHR1 KO and DT mediated MCH ablation, leads to the development of a weak stereotypy.

MCH ablated mice show a significant decrease in neuronal activation measured by c-Fos immunoreactivity in the lateral orbital cortex, an area important in regulating repetitive behavior. 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 marble-burying and stereotypical behavior in mice.

To determine the interactions 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 marble-burying behavior in comparison to control. Antagonizing MCHR1 using GW803430 blocked the reduction in marble-burying behavior not only in MCH infused animals but also OT and MCH + OT groups demonstrating that OT acts through the MCH system to reduce marble-burying behavior. Our data reveal that MCH mediates OT regulation of marble-burying behavior. The data also 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 marble-burying does not reflect an anxiety response (Thomas et al., 2009; Greene-Schloesser et al., 2011; Angoa-Perez et al., 2013; Aguilar-Valles et al., 2015; Mitra et al., 2016a, 2016b, 2017). As previously reported, we also found that OT infusions increased genital grooming but MCH infusions did not (Amico et al., 2004). MCH antagonist administration 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.

This illustrates the selective interaction between the MCH system in modulating OT response to a specific type of compulsive behavior, marble-burying, without affecting anxiety or locomotion. In conclusion, we identify a novel role for the MCH system and identify a previously undefined neurocircuit by which OT regulates marble-burying 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 marble-burying behavior. The behavioral and anatomical findings in our study warrant further investigation of the role of this circuit in disorders symptomatic of compulsive behaviors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.neuropharm.2017.09.008>.

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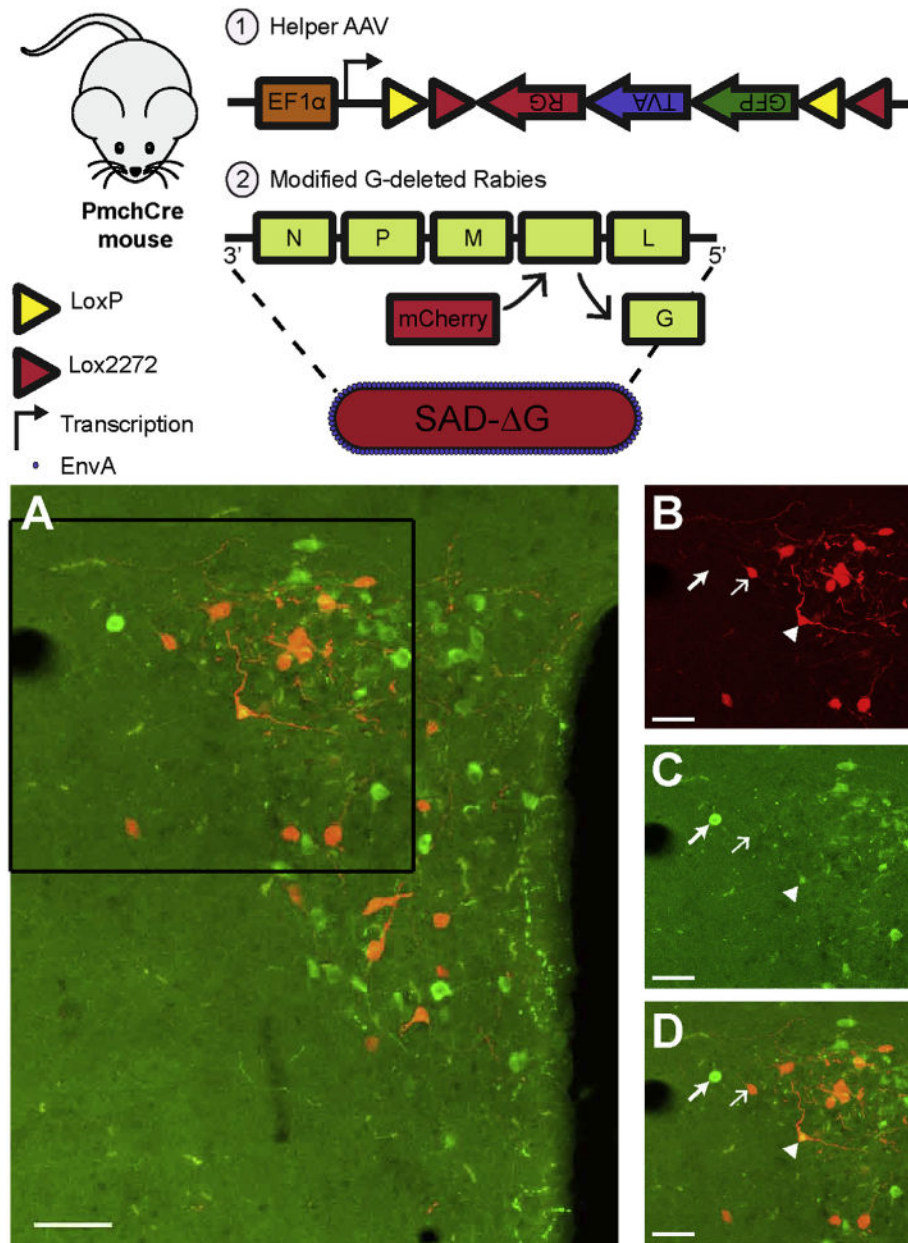
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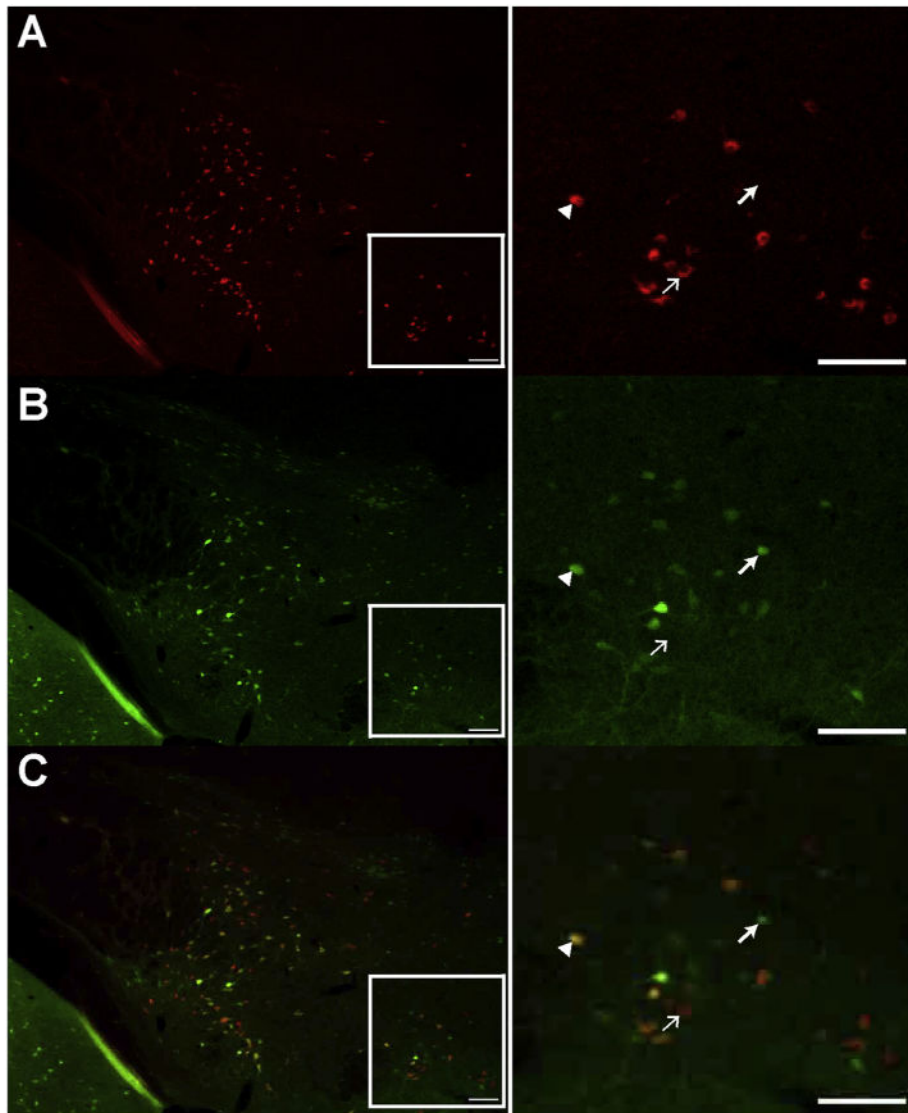
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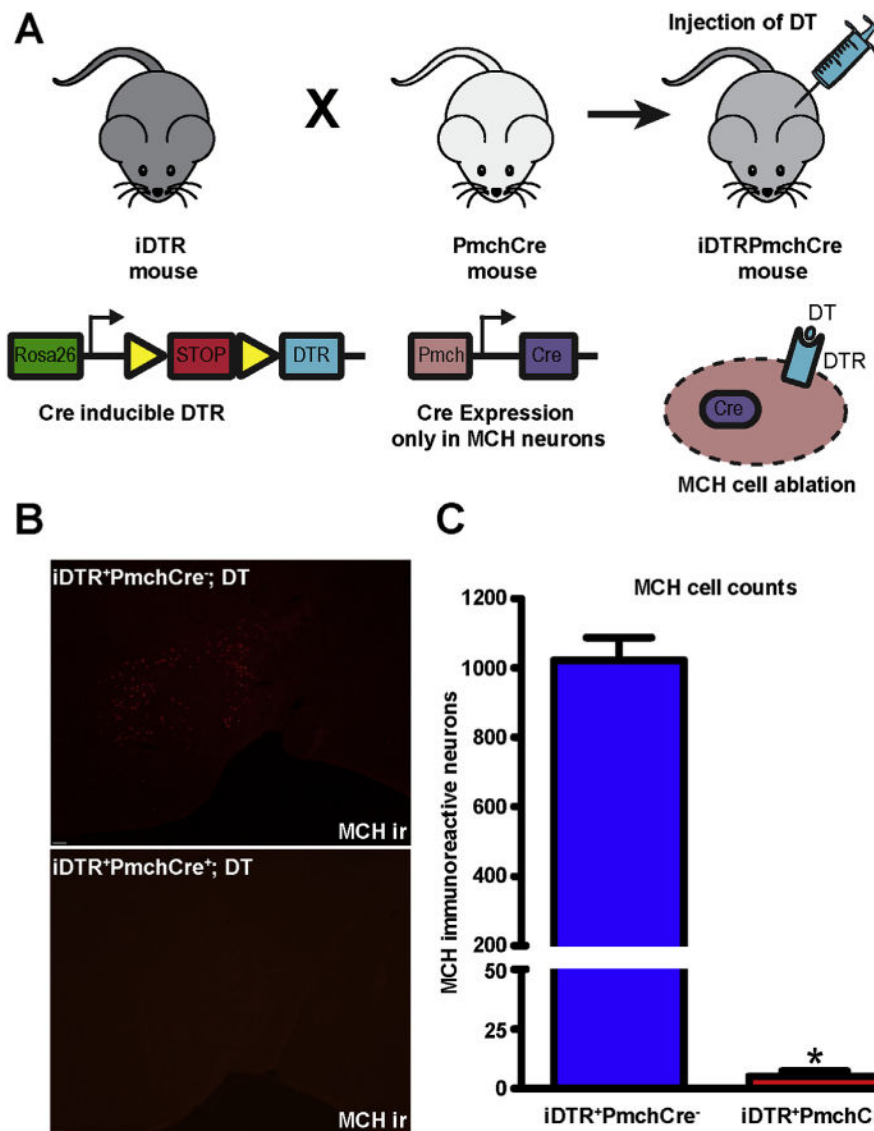
**Fig. 1.** SADDg-mCherry(EnvA) retrogradely labeled cells in PVN expressing oxytocin. A. Schematic representation of MCHCre mouse line and tracing strategy of monosynaptic inputs to MCH neurons in vivo. We used MCHCre transgenic mice in which only MCH cells express Cre-recombinase. First, we injected 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 SAD GmCherry 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



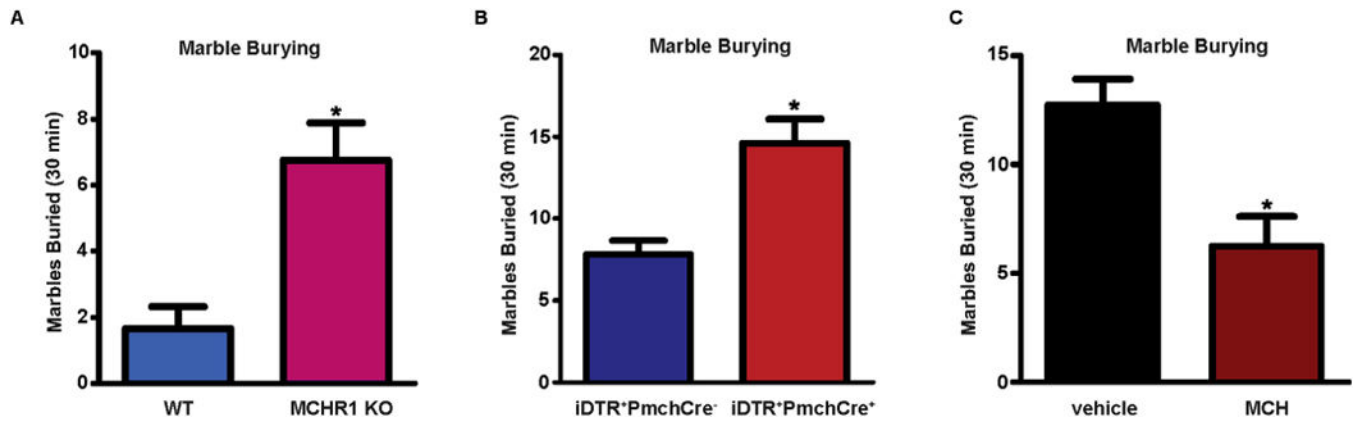
represent the viral genomic sequences encoding the wild-type nucleoprotein, phosphoprotein, matrix protein and polymerase required for rabies virus expression. The pseudotyped rabies virus can thus infect MCH cells and monosynaptically spread to cells which are directly presynaptic to MCH cells. The 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 = 50  $\mu\text{m}$ . 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 = 10  $\mu\text{m}$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** 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 = 50  $\mu$ m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

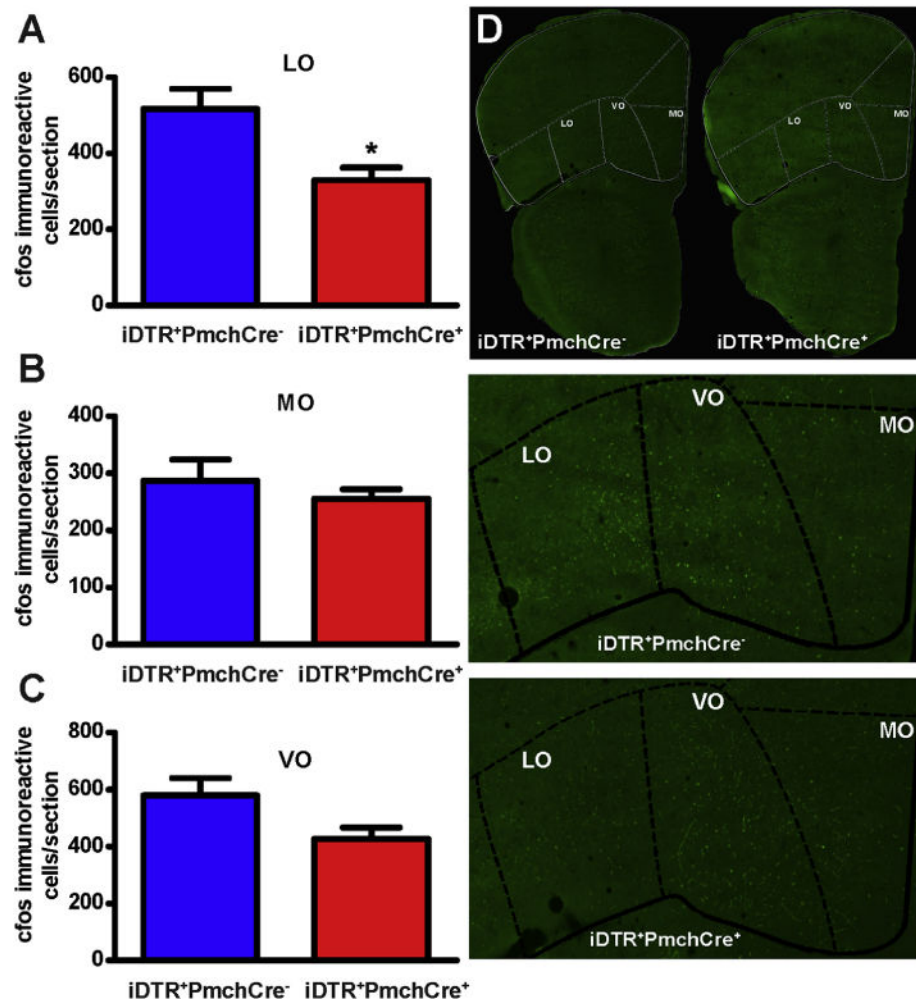


**Fig. 3.** Diphtheria Toxin (DT) mediated ablation of MCH neurons A) Schematic demonstrating how MCH cells are ablated using the DTR system. We crossed the iDTR strain to PmchCre strain rendering Cre expressing MCH neurons sensitive to DT. B) MCH immunoreactivity (red fluorescence) in the lateral hypothalamus and zona incerta of iDTR<sup>+</sup>PmchCre<sup>-</sup> and iDTR<sup>+</sup>PmchCre<sup>+</sup> mice following DT (Scale bar 100  $\mu$ m). C) Cell counts of MCH immunoreactivity in DT treated iDTR<sup>+</sup>PmchCre<sup>-</sup> and iDTR<sup>+</sup>PmchCre<sup>+</sup> mice. Values represent mean  $\pm$  SEM and were analyzed by unpaired *t*-test;  $t(19) = 14.93$ ,  $*P < 0.0001$ ,  $n = 10-11$ . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

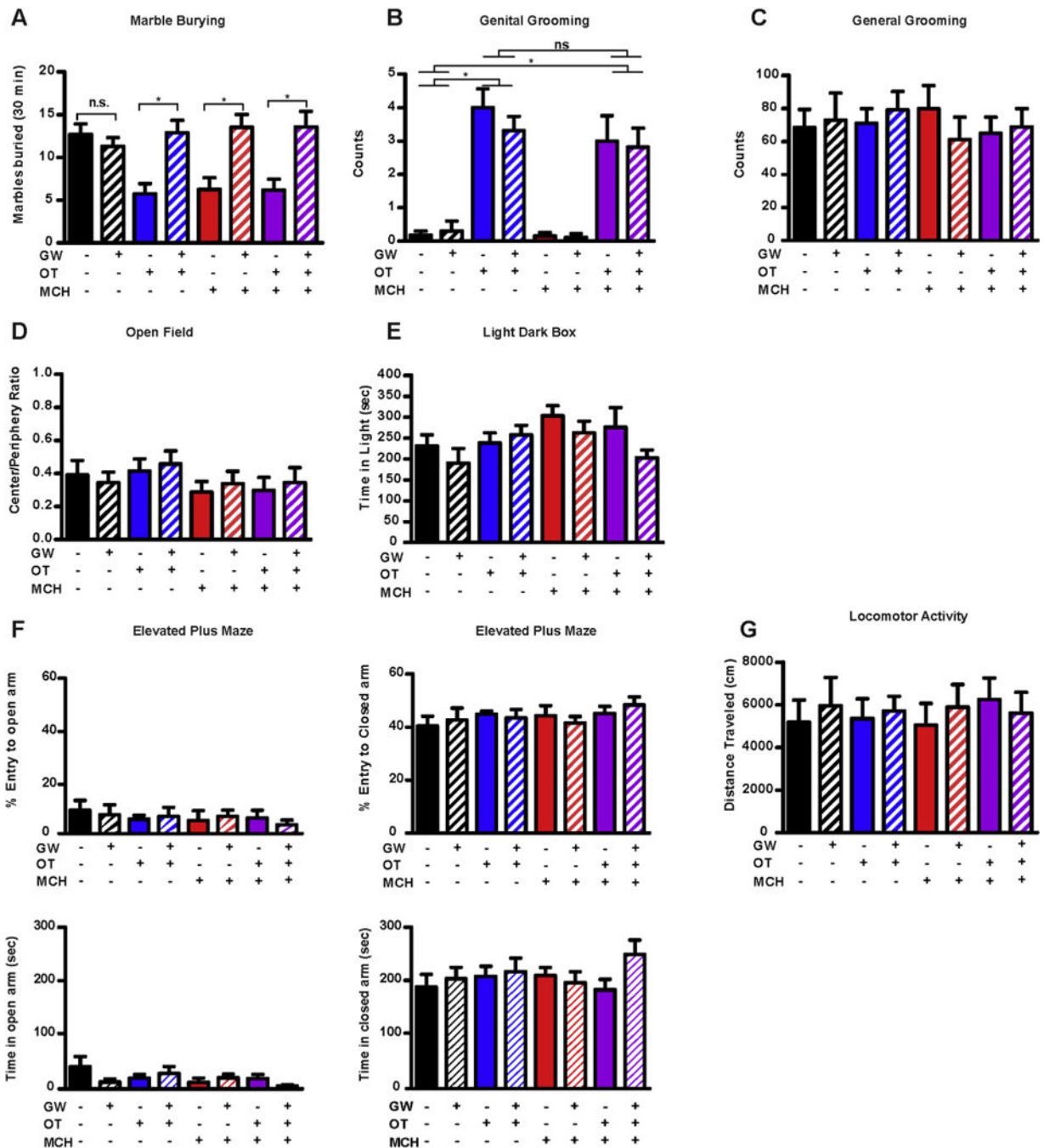


**Fig. 4.**

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<sup>+</sup>PmchCre<sup>-</sup> ( $n = 11$ ) and iDTR<sup>+</sup>PmchCre<sup>+</sup> ( $n = 10$ ), [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;  $t(32) = 3.541$ ,  $*P = 0.0012$ ,  $n = 13, 12$ ].



**Fig. 5.** cFos immunoreactivity in DT treated iDTR<sup>+</sup>PmchCre<sup>-</sup> and iDTR<sup>+</sup>PmchCre<sup>+</sup> 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<sup>+</sup>PmchCre<sup>-</sup> and iDTR<sup>+</sup>PmchCre<sup>+</sup> 90 min following Marble Burying. Values represent mean  $\pm$  SEM ( $n = 4$ /group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 6.** Animals received i.p. injection of vehicle or 3 mg/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) 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 C) 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  $\pm$  SEM D) 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] E) 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] F) 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]; Time spent in open arm [one-way ANOVA;  $F(7, 44) = 1.310$ ,  $P = 0.27$ ,  $n = 6-8$ /group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups] and time spent in closed arm [one-way ANOVA;  $F(7, 44) = 0.9306$ ,  $P = 0.49$ ,  $n = 6-8$ /group; Bonferroni's multiple comparison post-hoc test revealed no significant difference between groups] G). Distance Travelled [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].