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MCH Neuron Activity Is Sufficient for Reward and Reinforces Feeding

Pelin Dilsiz[a] Iltan Aklan[a, c] Nilufer Sayar Atasoy[a, c] Yavuz Yavuz[b] Gizem Filiz[a] Fulya Koksalar[a] Tayfun Ates[a] Merve Oncul[a] Ilknur Coban[a] Edanur Ates Oz[a] Utku Cebecioglu[a] Muhammed Ikbal Alp[a] Bayram Yilmaz[b] Deniz Atasoy[a, c]

[a]Department of Physiology, School of Medicine, Regenerative and Restorative Medical Research Center (REMER), Istanbul Medipol University, Istanbul, Turkey; [b]Department of Physiology, School of Medicine, Yeditepe University, Istanbul, Turkey; [c]Department of Pharmacology, Iowa Neuroscience Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA, USA

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Abstract
Background: Melanin-concentrating hormone (MCH)-expressing neurons have been implicated in regulation of energy homeostasis and reward, yet the role of their electrical activity in short-term appetite and reward modulation has not been fully understood. ***Objectives:*** We investigated short-term behavioral and physiological effects of MCH neuron activity manipulations. ***Methods:*** We used optogenetic and chemogenetic approaches in *Pmch-cre* transgenic mice to acutely stimulate/inhibit MCH neuronal activity while probing feeding, locomotor activity, anxiety-like behaviors, glucose homeostasis, and reward. ***Results:*** MCH neuron activity is neither required nor sufficient for short-term appetite unless stimulation is temporally paired with consumption. MCH neuronal activation does not affect short-term locomotor activity, but inhibition improves glucose tolerance and is mildly anxiolytic. Finally, using two different operant tasks, we showed that activation of MCH neurons alone is sufficient to induce reward. ***Conclusions:*** Our results confirm diverse behavioral/physiological functions of MCH neurons and suggest a direct role in reward function.

Introduction

Lateral hypothalamic (LH) melanin-concentrating hormone (MCH)-expressing neurons have long been implicated in energy homeostasis, reward, and sleep-wake regulation. Intracerebroventricular (i.c.v.) administration of MCH causes an acute increase in food intake and its long-term infusion promotes weight gain, while MCH receptor antagonists have an anti-obesity effect [1–3]. Overexpression of the *Pmch* gene causes mild weight gain, whereas ablation of the MCH peptide or its receptor

Pelin Dilsiz, Iltan Aklan, and Nilufer Sayar Atasoy contributed equally to this work.

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E-Mail karger@karger.com
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Deniz Atasoy
Department of Pharmacology, Roy J. and Lucille A. Carver College of Medicine, University of Iowa
51 Newton Rd.
Iowa City, IA 52242 (USA)
E-Mail deniz-atasoy@uiowa.edu

causes leanness and resistance to high-fat-diet-induced obesity [4–7]. Similarly, mice with ablation of MCH neurons display an improved glucose tolerance and late onset leanness [8, 9].

Although the mechanism through which MCH neurons may influence appetite is not clear, a role in connecting homeostatic needs with reward function has been suggested [10–14]. Consistently, ex vivo MCH neuronal activity is increased by glucose [15, 16], and this activation is suggested to be involved in reward function as well as peripheral glucose homeostasis [10, 16, 17]. Despite this, it is not clear whether MCH neuronal activity can acutely change short-term appetite since much of the manipulations involve congenital ablations of MCH neurons, their expressed genes, or pharmacological administration of MCH receptor agonists and antagonists. Few studies have addressed the role of electrical activity changes of MCH neurons, yielding variable results depending on the organism and time of stimulation [10, 17, 18], and no acute effect of inhibition has been reported. Using both optogenetics and chemogenetics, we investigated behavioral and physiological effects of acute MCH neuronal activity manipulations. We showed that changes of MCH neuronal activity are neither required nor sufficient to alter short-term appetite (measured here by food intake) unless the stimulation is time locked with consumption. Furthermore, we discovered that MCH neuronal activation alone is rewarding independently of any oral or post-ingestive cues.

Materials and Methods

Mice

Mice were housed at 22–24 °C on a 12-h light (06:00) and dark (18:00) cycle with ad libitum access to water and standard mouse chow unless otherwise noted. Cre recombinase-expressing line *Tg(Pmch-cre)1Lowl/J* (Jackson Labs Stock 014099) [16] were backcrossed with C57BL/6 (Jackson Labs Stock 000664) for maintenance. Behavioral experiments were conducted with 8- to 10-week-old male and female mice.

Recombinant Adeno-Associated Viral Vectors and Virus Production

Recombinant adeno-associated virus production was performed as previously described [19]. The Cre-dependent recombinant adeno-associated viral vectors (rAAV) plasmids used in this study were purchased from Addgene (http://www.addgene.org/) and are as follows: *rAAV2/1-CAG-FLEX-tdTomato* (10^{13} genomic copies/mL), *rAAV2/1-EF1a-DIO-hM3D(Gq)-mCherry* (6×10^{12} genomic copies/mL), *rAAV2/8-EF1a-DIO-hM4D(Gi)-mCherry* (10^{11} genomic copies/mL), and *rAAV2/1-EF1a-FLEX-hChR2(H134R)-eYFP-WPRE-HGHpA* (1.87×10^{14} genomic copies/mL).

Stereotaxic Viral Injections and Optical Fiber Implantation

Intracranial injections were performed as described previously [20]. Briefly, mice (P30 to P40) were anaesthetized with isoflurane in the stereotaxic instrument (David Kopf Instruments, Tujunga, CA, USA). Before drilling the skull for injection, the scalp was incised carefully and a total of 600 nL of intracranial injection per side was performed with a pulled glass pipette with a tip diameter of 20–40 μm (Wiretrol; Drummond Scientific, Broomall, PA, USA). rAAV was injected at coordinates around the LH (bregma: –1.30 mm, midline: ±1.00 mm, and dorsal surface [relative to the brain surface]: –4.90 and 4.70 mm, 300 nL at each Z-position/side, total 1.2 μL/mouse). For chemogenetic stimulation assays, the scalp was stitched together and the mice were allowed at least 2 weeks for recovery and transgene expression.

For in vivo photostimulation assays, ferrule capped optical fiber (200-μm core diameter, NA = 0.50, ThorLabs) was implanted over the LH and fixed with dental cement over the skull. The fiber tip coordinates are: *x*: ±1.00, *y*: –1.300, and *z*: –4.500 (online suppl. Fig. 1E; for all online suppl. material, see www.karger.com/doi/10.1159/000501234). The mice were allowed 3 weeks for postoperative recovery. For post hoc histological evaluation of optogenetic activation, the animals were photostimulated for 30 min immediately after which they were sacrificed and transcardially perfused with 4% PFA.

Electrophysiology

P50-P60 mice were deeply anaesthetized with isoflurane and decapitated. The brains were obtained and placed in an ice-cold cutting solution including: 234 mM sucrose, 28 mM $NaHCO_3$, 7 mM dextrose, 2.5 mM KCl, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM sodium ascorbate, 3 mM sodium pyruvate, and 1.25 mM NaH_2PO_4, aerated with 95% O_2/5% CO_2. Coronal slices (300 μm thick) containing the LH area were sectioned using a vibratome (Leica VT1000S) and placed in artificial cerebrospinal fluid containing: 119 mM NaCl, 25 mM $NaHCO_3$, 11 mM D-glucose, 2.5 mM KCl, 1.25 mM $MgCl_2$, 2 mM $CaCl_2$, and 1.25 mM NaH_2PO_4, aerated with 95% O_2/5% CO_2 for 30 min at room temperature. MCH neurons were identified with an mCherry fluorescent tag and loose seal patched using electrodes with 4- to 5-MΩ tip resistances using artificial cerebrospinal fluid as the intracellular solution and 5 μM clozapine added during recording.

Food Intake Studies

The animals were single housed following postoperative recovery and handled for 3 days. The mice were fed ad libitum. By the beginning of the light cycle, food consumption was monitored for 2 h for baseline. For DREADD activation experiments 0.01 mg/kg clozapine or saline was administered i.p., and the food intake was measured by weighing for 2 more hours. For a subset of experiments (Fig. 2; online suppl. Fig. 1) 3 mg/kg CNO was used. For light cycle manipulations, the premanipulation 2-h food consumption was taken as baseline. For dark onset experiments, acute chemogenetic activation and inhibition were performed at the beginning of the dark cycle with ad libitum-fed *Pmch-cre* mice. Consumption during the same circadian period of the previous day was used for baseline comparison, also with saline injections. Similar timelines were used for optogenetic experiments except that the CLZ/CNO injection was replaced by photostimulation at 20 Hz applied over the LH through a 473-nm diode laser (Doric Lenses Inc., QC, Canada). The pulse protocol used throughout this study

was 20-Hz, 10-ms pulses for 1 s, repeated every 4 s; photostimulation took 2 h. For all studies, the food intake was measured manually and the spilled amount, which accumulated below the meshwired floor of the cage floor, was subtracted.

Glucose and Insulin Tolerance Tests

The blood glucose concentrations of 16-h food-deprived MCH^{hM3Dq} and MCH^{hM4Di} mice were recorded by an automatic glucose monitor (t = 0 min; Accu-Check Performa Nano; Roche Diagnostics, Germany). The blood was obtained from the tail vein. Thirty minutes after the first measurement, CNO (3 mg/kg) or saline was administered i.p.. At the 60-min time point, 20% glucose (10 mL/kg) [21] was injected i.p. and blood glucose concentrations were measured at 90, 120, 150 min.

For the insulin tolerance test, blood glucose levels of ad libitum-fed MCH^{hM3Dq} and MCH^{hM4Di} mice were measured and, 30 min later, i.p. injection of CNO (3 mg/kg) or saline was performed. Insulin (0.75 IU/kg; Humulin R; Eli Lilly, Indianapolis, IN, USA) was administrated i.p. at the 60-min time point. Blood glucose concentrations were measured at 90, 120, and 150 min. The first blood glucose recordings were obtained at 09:00 for both ITT and GTT.

Self-Stimulation, Open Field, and Elevated Plus Maze

Nose Poke Self-Stimulation Assay

Two nose poke ports were located on opposite sides of Coulbourn Habitest test cages (Coulbourn Instruments, Allentown, PA, USA). By the beginning of the dark cycle, MCH^{ChR2} and MCH^{tdTom} mice had been acclimatized in test cages for 3 consecutive days, 1 h each. The number of times the mice poked each port was recorded. The less preferred operandum was paired with a 20-Hz photostimulation of 10 s per entrance (10-ms pulses of 15 mW, 473 nm) on the test day. During the test period, the animals were allowed 1 h for 2 days with active and inactive nose poke ports. Nose poke counts were monitored by Graphic State software and recorded by a CCD camera.

Lever Press Self-Stimulation Assay

Habitest test cages were supplied with 2 levers placed on opposite sides. MCH^{ChR2} and MCH^{tdTom} mice were acclimatized in test cages for 1 h for 3 consecutive days. During the acclimatization period, the mice were monitored to count the number of times they pressed each lever. The less preferred operandum was paired with a 20-Hz photostimulation of 10 s per entrance (10-ms pulses of 15 mW, 473 nm) on the test day. The mice were allowed 1 h on test days for 2 days with active and inactive levers. Lever press counts were displayed by Graphic State software and recorded by a CCD camera.

Food Consumption-Paired MCH Stimulation Assay

MCH^{ChR2} and MCH^{tdTom} mice were placed in Coulbourn Habitest cages (Coulbourn Instruments) with one automated feeder installed. The mice were acclimatized for automated pellet delivery for 3 days and consumption of 20-mg pellets (Bio-Serv, Flemington, NJ, USA) was recorded. The testing session took 1 day (24 h), in which 20-Hz photostimulation was applied for 15 s each time the mouse removed a pellet from the feeder for consumption.

Open Field Analysis

For open field (OF) analysis, MCH^{ChR2} and MCH^{tdTom} mice were acclimatized to the testing room before testing. For acclimatization, following the postoperative recovery period, the mice were transferred to the testing room; each mouse had an individual cage. For 3 days, the mice were acclimatized to the testing room and to the cages without handling. The operator handled the mice for another 3 days in order to reduce any stress. The animals were placed in an OF test chamber (40 × 40 × 40 cm) and stimulated with the pulse protocol or CLZ/CNO during the test, which lasted 30 min [22, 23]. The assay was traced with a CCD camera and ANY-maze software.

Elevated Plus Maze

An elevated plus maze (EPM) with 2 open arms (5 × 35 cm) and 2 closed arms (5 × 35 × 20 cm) was elevated for 35 cm. MCH^{ChR2} and MCH^{tdTom} mice were habituated in the test room for an hour. The mice were placed in the center of the maze, facing forward toward the open arm, and were photostimulated (or chemogenetically activated by CLZ/CNO just prior to the test) and monitored for 5 min [24–26]. The tests were recorded with a CCD camera and analyzed with ANY-maze software.

Immunohistochemistry and Imaging

The animals were photostimulated for 30 min and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer fixative (pH 7.4). The brains were removed and post-fixed with the same solution for 4 h. Brain sections (75 μm) were sliced with a vibratome and washed in 0.1 M phosphate-buffered saline with Triton X-100 (0.1% PBST). Brain sections were blocked in 5% normal goat serum/PBST for 1 h at room temperature and incubated overnight at +4 °C in blocking solution containing the primary antibody (anti-cFos, 1:5,000; Cell Signaling). The slices were then rinsed with PBST 3 times, incubated with the secondary antibody (Alexa Flour 488, 1:500; Invitrogen) for 1 h in room temperature, and washed with PBST 3 more times. The brain sections were transferred to microscope slides and mounted with Fluoromount (Sigma F4680) for imaging. Brain images were collected by confocal microscopy (Carl Zeiss, Thornwood, NY, USA).

Results

MCH Neurons Are neither Sufficient nor Required for Acute Appetite for Chow Food

Given the extensive literature on MCH neurons and long-term body weight regulation, we evaluated whether acute manipulation of MCH neuronal activity has any influence on short-term appetite regulation. To achieve this, we used DREADDs chemogenetic activity manipulation tools. We transduced MCH neurons in *pmch-cre* reporter mice with bilateral LH injections of *cre*-dependent chemogenetic activator rAAV2-DIO-hM3Dq-mCherry or inhibitor rAAV2-DIO-hM4Di-mCherry viruses. We then i.p. delivered the clozapine ligand to acutely activate or inhibit MCH neurons (Fig. 1a–f). Neither activation nor inhibition of MCH neurons had any significant impact on the short-term food intake in the day time and at dark onset (Fig. 1g, h; online suppl. Fig. 1a–d).

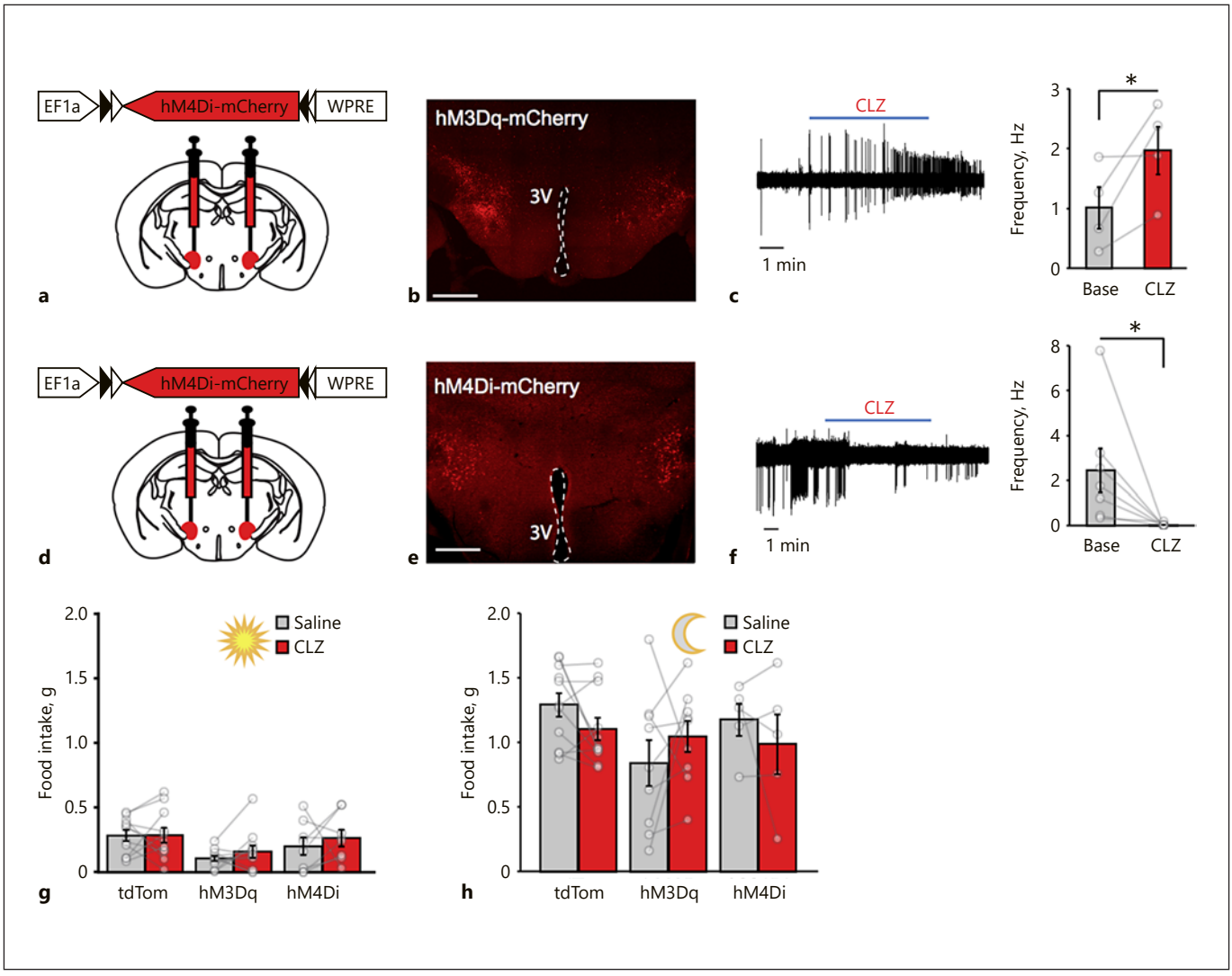


Fig. 1. MCH neuron activity is neither necessary nor sufficient for chow food appetite. **a** Schematic diagram of hM3Dq-mCherry expression in MCH neurons in *Pmch-cre* mice. **b** Representative image of transduced MCH neurons in the LH. Scale bar, 500 μm. **c** Left: representative loose seal recordings from an $MCH^{hM3Dq\text{-}mCherry}$ neuron showing increased spontaneous action currents upon bath perfusion of clozapine (CLZ, 5 μM). Right: Quantification of change in the action current firing rate before and after clozapine perfusion ($n = 4$ neurons, mean ± SEM: base = 1.05 ± 0.35 Hz, CLZ = 1.97 ± 0.40 Hz; $p = 0.0462$, one-tailed t test). **d** Schematic diagram of hM4Di-mCherry expression in MCH neurons in *Pmch-cre* mice. **e** Representative image of transduced MCH neurons in the LH. Scale bar, 500 μm. **f** Left: representative loose seal recordings from an $MCH^{hM4Di\text{-}mCherry}$ neuron showing suppression of spontaneous action currents upon bath perfusion of CLZ (5 μM). Right: quantification of change in the action current firing rate before and after CLZ perfusion ($n = 6$ neurons, mean ± SEM: base = 2.45 ± 0.97 Hz, CLZ = 0.03 ± 0.03 Hz; $p = 0.024$, one-tailed t test; GABAA blocker picrotoxin, 50 μM, was added to the bath to increase the baseline spontaneous firing rate). Summary graph of daytime (**g**, $n = 11$ tdTom mice, $n = 11$ hM3Dq-mCherry mice, $n = 8$ hM4Di-mCherry mice; mean ± SEM for each bar in g: tdTom saline = 0.28 ± 0.04, tdTom CLZ = 0.29 ± 0.06, hM3Dq saline = 0.11 ± 0.02, hM3Dq CLZ = 0.16 ± 0.05, hM4Di saline = 0.20 ± 0.07, and hM4Di CLZ = 0.26 ± 0.06; two-way ANOVA, interaction $F[2, 27] = 0.2597$, $p = 0.7731$; effect of treatment $F[1, 27] = 1.051$, $p = 0.3143$; effect of virus injection $F[2, 27] = 4.496$, $p = 0.0206$; saline vs. CLZ Sidak's adjusted p values: $p > 0.9999$ [tdTom], $p = 0.7883$ [hM3Dq], and $p = 0.7785$ [hM4Di]) and dark onset (**h**, $n = 11$ tdTom mice, $n = 9$ hM3Dq-mCherry mice, $n = 5$ hM4Di-mCherry mice; mean ± SEM for each bar in g: tdTom saline = 1.28 ± 0.09, tdTom CLZ = 1.10 ± 0.09, hM3Dq saline = 0.84 ± 0.18, hM3Dq CLZ = 1.04 ± 0.12, hM4Di saline = 1.17 ± 0.12, hM4Di CLZ = 0.98 ± 0.23; two-way ANOVA, interaction $F[2, 22] = 1.877$, $p = 0.1768$; effect of treatment $F[1, 22] = 0.3212$, $p = 0.5767$; effect of virus injection $F[2, 22] = 1.809$, $p = 0.1874$; saline versus CLZ Sidak's adjusted p values: $p = 0.5124$ [tdTom], $p = 0.5291$ [hM3Dq], and $p = 0.7739$ [hM4D]) food intake in response to saline or CLZ (0.01 mg/kg) administration in ad libitum-fed *Pmch-cre* mice expressing tdTomato, hM3Dq-mCherry, or hM4Di-mCherry in MCH neurons. Each bar represents 2 h of food intake. * $p < 0.05$.

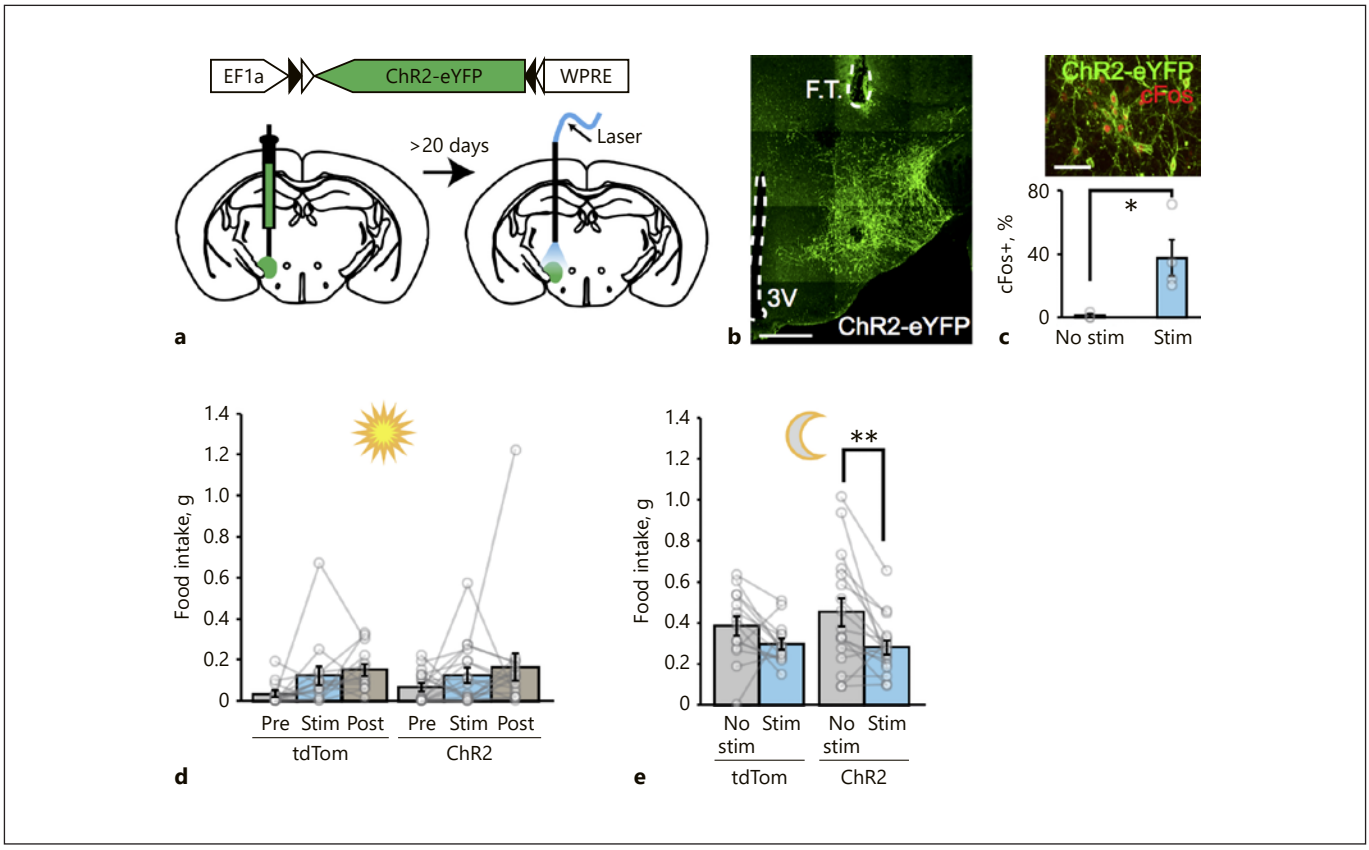


Fig. 2. Optogenetic stimulation of MCH neurons does not increase acute food intake. **a** Schematic representation of ChR2-eYFP expression in MCH neurons in *Pmch-cre* mice and their photostimulation. **b** Representative photomicrographs showing ChR2-eYFP-expressing MCH neurons in the LH. Scale bar, 250 µm. FT, fiber track. **c** Top: representative image of ChR2-eYFP-transduced MCH neurons with cFos expression (red) in response to 20 Hz photostimulation. Scale bar, 100 µm. Bottom: quantification of the percentage of cFos-expressing $MCH^{ChR2\text{-}eYFP}$ neurons in stimulated (Stim) vs. unstimulatied (No stim) mice. $p = 0.0222$ (one-tailed t test), mean ± SEM in %: No stim = 1.03 ± 1.03, Stim = 37.5 ± 11,53. Summary graph of daytime (**d**, $n = 14$ tdTom mice, $n = 17$ ChR2-eYFP mice, mean ± SEM in g: tdTom pre-Stim = 0.034 ± 0.01, Stim = 0.12 ± 0.05, post-Stim = 0.15 ± 0.03; ChR2 pre-Stim = 0.07 ± 0.02, Stim = 0.12 ± 0.04, post-Stim = 0.17 ± 0.07; Sidak's adjusted p values for pre-Stim vs. Stim: $p = 0.5390$ [tdTom], $p = 0,8408$ [ChR2], two-way ANOVA: interaction, $F[2, 58] = 0.08368$, $p = 0.9198$; effect of stimulation, $F[2, 58] = 4.204$, $p = 0.0197$; and effect of viral injections $F[1, 29] = 0.1525$, $p = 0.6990$) food intake before, during, and after photostimulation, and dark cycle (**e**, $n = 14$ tdTomato mice, $n = 17$ ChR2-eYFP mice, mean ± SEM in g: tdTom No stim = 0.39 ± 0.05, Stim = 0.30 ± 0.03; ChR2 No stim = 0.46 ± 0.07, Stim = 0.28 ± 0.03; Sidak's adjusted p values for No stim vs. Stim: $p = 0.2485$ [tdTom], $p = 0,0040$ [ChR2]; 2-way ANOVA: interaction, $F[1, 29] = 1.299$, $p = 0.2637$; effect of stimulation, $F[1, 29] = 11.76$, $p = 0.0018$; and effect of viral injections $F[1, 29] = 0.1864$, $p = 0.6692$) food intake with or without photostimulation of MCH neurons in ad libitum-fed *Pmch-cre* mice. Each bar represents 2 h of food intake. * $p < 0.05$, ** $p < 0.01$.

It is estimated that activated MCH neurons may fire at rates of up to 10–40 Hz in vivo [27, 28]. To ensure that chemogenetic activation did not underestimate a possible role of MCH neuron activity on feeding, we also used optogenetic stimulation as an alternative approach. For this, we transduced MCH neurons with rAAV2-DIO-ChR2-eYFP virus for *cre*-dependent *Channelrhodopsin-2* expression and placed an optical fiber over the LH (Fig. 2a–c; online suppl. Fig. 1e). Similar to the chemogenetic activation, optogenetic stimulation of MCH neurons at 20 Hz did not cause any significant increase in food intake (Fig. 2d, e; online suppl. Fig. 1f). On the contrary, the dark onset food consumption was slightly decreased in MCH neuron-stimulated mice. These results suggest that, under these conditions, MCH neuronal activity is not sufficient to drive the consumption of chow food and is dispensable for acute regulation of appetite.

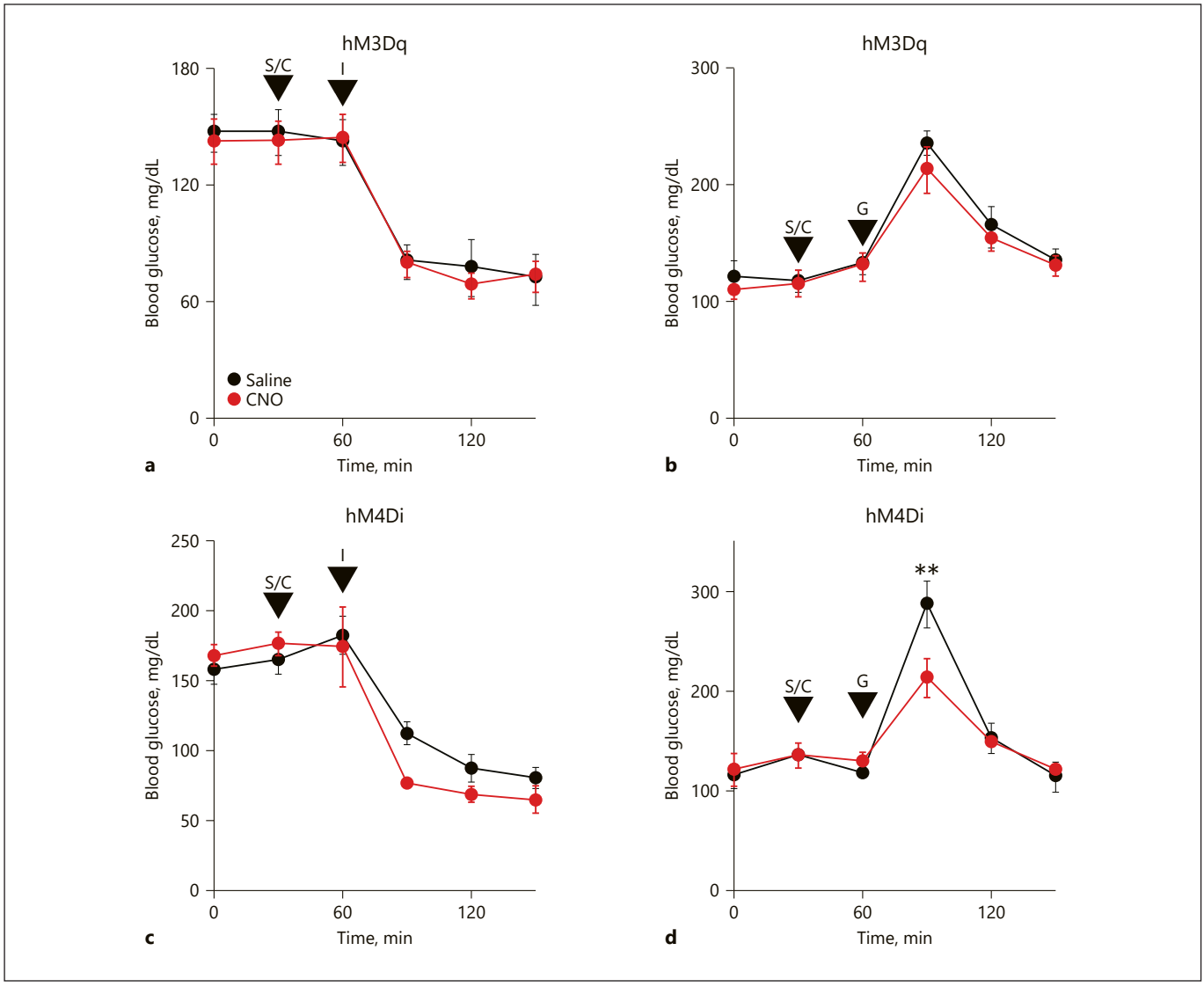


Fig. 3. Effect of MCH neuron activity manipulations on glucose homeostasis. **a** Insulin tolerance plot for MCH-specific hM3Dq-mCherry-expressing mice (n = 9 hM3Dq mice, two-way ANOVA, interaction F[5, 80] = 0.2218, p = 0.9522; effect of time, F[5, 80] = 61.89, p < 0.0001; effect of CNO treatment F[1, 16] = 0.04625, p = 0.8324). **b** glucose tolerance plot for hM3Dq-mCherry mice (n = 6 hM3Dq mice, two-way ANOVA, interaction F[5, 50] = 0.3099, p = 0.9047; effect of time, F[5, 50] = 53.34, p < 0.0001; effect of CNO treatment F[1, 10] = 0.217, p = 0.6513). **c** insulin tolerance plot for MCH-specific hM4Di-mCherry-expressing mice (n = 6 hM4Di mice, two-way ANOVA, interaction F[5, 50] = 2.113, p = 0.0792; effect of time, F[5, 50] = 65.98, p < 0.0001; effect of CNO treatment F[1, 10] = 0.5847, p = 0.4621). **d** Glucose tolerance test in MCH-specific hM4Di-mCherry-expressing mice (n = 6 hM4Di mice, two-way ANOVA, interaction F[5, 50] = 4.052, p = 0.0036; effect of time, F[5, 50] = 40.75, p < 0.0001; effect of CNO treatment F[1, 10] = 0.5603, p = 0.4714; Sidak's adjusted p = 0.0014 at 90 min, saline vs. CNO) in response to i.p. saline or CNO administration. S, saline; C, CNO; I, insulin; G, glucose.** p < 0.01.

Effect of Acute MCH Neuronal Activity Manipulations on Glucose Homeostasis, Short-Term Locomotor Activity, and Anxiety

Ex vivo recordings suggest that the activity of MCH neurons is sensitive to the extracellular glucose concentration, and genetic manipulations of MCH neurons can profoundly influence glucose homeostasis [15–17]. To investigate whether acute changes in MCH neuron activity have a role in glucose regulation, we stimulated and inhibited MCH neurons chemogenetically as described above while monitoring glucose tolerance and insulin sensitivity. In agreement with a recent report, acute che-

mogenetic stimulation of MCH neurons did not cause significant changes in glucose tolerance or insulin sensitivity (Fig. 3; online suppl. Fig. 2a–d) [17]. Conversely, acute chemogenetic inhibition of MCH neurons improved both glucose tolerance and insulin sensitivity (Fig. 3; online suppl. Fig. 2e–h). Consistent with previous reports that used genetic manipulations [16, 17], these results suggest that inhibition of MCH neuronal activity improves whole-body glucose metabolism.

Alterations in energy expenditure, in the form of locomotor activity, have been suggested to contribute to body weight changes seen in genetic MCH pathway manipulations. While MCH receptor-1 knockout mice or MCH neuron-ablated mice are hyperactive [8, 29], infusion of MCH or MCHR1 agonists had no effect on locomotion [2, 30], suggesting a discrepancy between genetic and pharmacologic approaches. Therefore, we evaluated the effect of short-term manipulations of MCH neuronal activity on locomotor activity. Neither chemogenetic silencing nor optogenetic activation had any significant effect on locomotion (Fig. 4c–e, h–j), which is different from the results of a recent study examining acute chemogenetic activation of MCH neurons [17]. However, it must be noted that we focused on the short-term effects of unilateral activation, whereas the former study analyzed a period of 8 h in which the entire MCH neuron population was activated.

Finally, given the contradictory reports about the effects of pharmacologic and genetic MCH pathway manipulations on anxiety-like behavior [31–34], we probed the influence of acute MCH neuron activity changes on this behavioral domain. We evaluated the effect of bilateral chemogenetic activation/inhibition and unilateral optogenetic activation of MCH neurons in OF and EPM tasks. We did not observe any significant difference in the OF test (Fig. 4a–j; online suppl. Fig. 3a, b) for either MCH inhibition or activation. On the other hand, optogenetic activation reduced the average speed in the open arm of the EPM (Fig. 4k–n; online suppl. Fig. 3c, d), whereas chemogenetic inhibition significantly increased the number of entries into the open arm, suggesting that suppression of the MCH neuron output may have an anxiolytic effect (Fig. 4o–r). Collectively, these experiments reveal that MCH neuron electrical activity manipulations can rapidly modulate glucose homeostasis and anxiety-like behavior without a significant effect on the short-term locomotor activity.

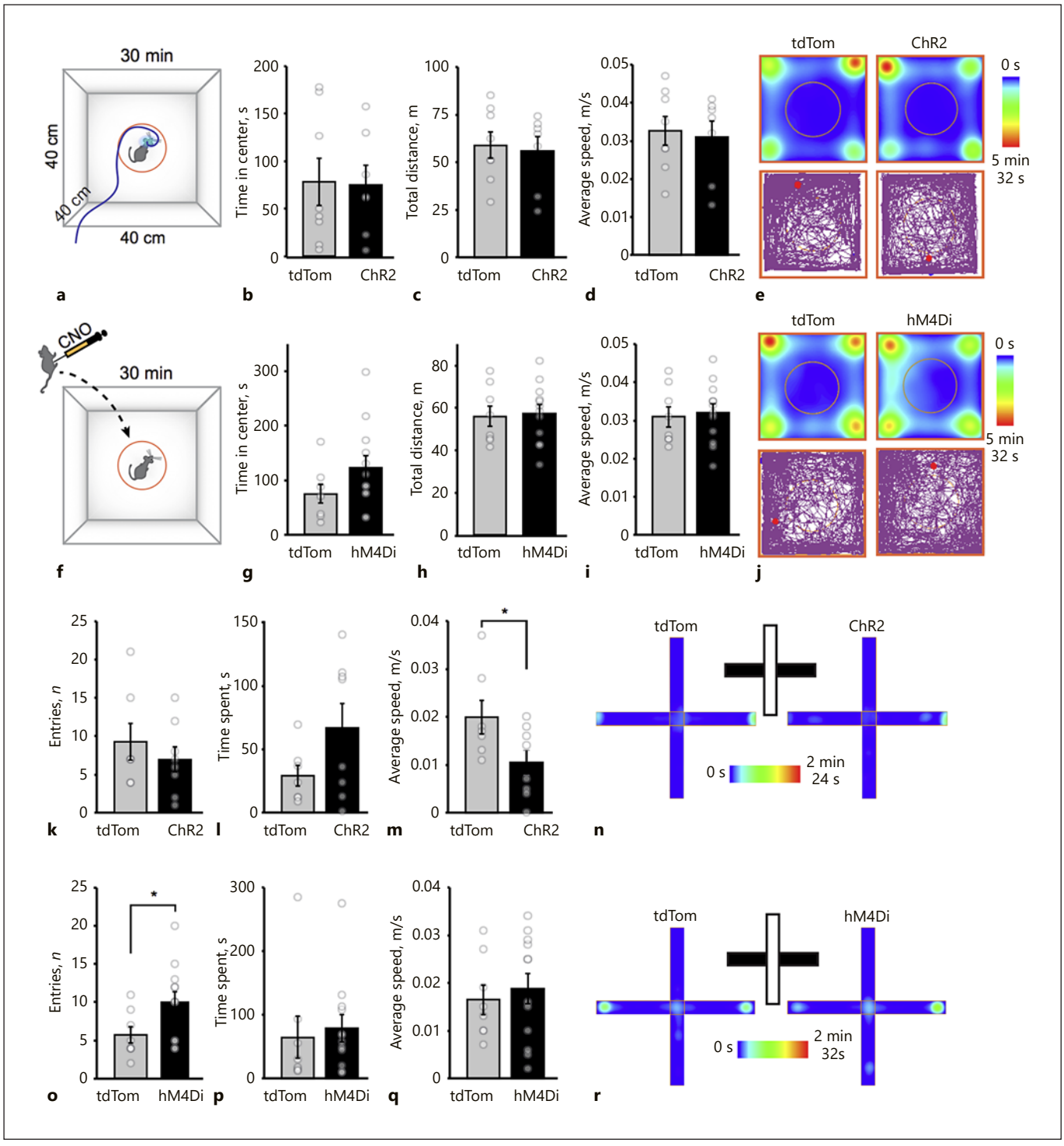
MCH Neuron Activation during Feeding Increases Chow Food Consumption

Our results suggest that acute stimulation of MCH neurons is not sufficient to drive food intake. This is in striking contrast to the behavior observed upon activation of other orexigenic populations such as arcuate nu-

Fig. 4. Effect of MCH neuron activity manipulations on anxiety-like behaviors and short-term locomotor activity. **a–d** OF test for mice with ChR2-eYFP-expressing MCH neurons. **a** Schematic diagram describing the OF assay during 20 Hz photostimulation of LH. The time spent (**b**, mean ± SEM in s: tdTom = 78.29 ± 24.8, ChR2 = 75.2 ± 20.6, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.9265$), the total distance (**c**, mean ± SEM in m: tdTom = 58.92 ± 6.89, ChR2 = 55.89 ± 7.45, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.7698$), and the average speed (**d**, mean ± SEM in m/s: tdTom = 0.033 ± 0.004, ChR2 = 0.031 ± 0.004, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.7771$) were similar between tdTomato and ChR2 mice ($n = 8$ tdTomato, $n = 7$ ChR2-eYFP mice). **e** Mean heat maps and representative traces for each group are shown. Blue dots mark the beginning and red dots mark the end points of the mice's positions. **f–i** OF test for mice with hM4Di-mCherry-expressing MCH neurons. **f** Schematic diagram describing the OF assay followed by i.p. CNO administration. The time spent (**g**, mean ± SEM in s: tdTom = 75.05 ± 16.80, hM4Di = 122.86 ± 22.42, tdTom vs. hM4Di unpaired two-tailed *t* test, $p = 0.1385$), the total distance (**h**, mean ± SEM in m: tdTom = 56.03 ± 4.83, hM4Di = 57.50 ± 4.06, tdTom vs. hM4Di unpaired two-tailed *t* test, $p = 0.8193$), and the average speed (**i**, mean ± SEM in m/s: tdTom = 0.031 ± 0.0027, hM4Di = 0.032 ± 0.0023, tdTom vs. hM4Di unpaired two-tailed *t* test, $p = 0.7829$) were similar between tdTomato and hM4Di-mCherry mice ($n = 8$ tdTomato, $n = 12$ hM4Di mice). **j** Mean heat maps and representative traces for each group are shown. Blue dots mark the beginning and red dots mark the end points of the mice's positions. **k–m** EPM test during MCH neuron stimulation. The number of entries to the open arms (**k**, mean ± SEM: tdTom = 9.29 ± 2.40, ChR2 = 7.00 ± 1.67, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.4388$) and the time spent in the open arms (**l**, mean ± SEM in s: tdTom = 29.00 ± 8.22, ChR2 = 66.86 ± 19.06, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.1068$) were comparable between the ChR2 and tdTomato groups, whereas the average speed in the open arms (**m**, mean ± SEM in m/s: tdTom = 0.020 ± 0.0035, ChR2 = 0.011 ± 0.0026, tdTom vs. ChR2 unpaired two-tailed *t* test, $p = 0.04557$) was significantly decreased in the ChR2 group ($n = 7$ tdTomato, $n = 8$ ChR2-eYFP mice). **n** Mean heat maps for each group. **o–q** EPM test in MCH-specific hM4Di- or tdTomato-expressing mice. The number of entries to the open arms was significantly increased in the hM4Di group (**o**, mean ± SEM: tdTom = 5.75 ± 1.06, hM4Di = 10.00 ± 1.41, tdTom vs. hM4Di unpaired two-tailed *t* test, $p = 0.04252$), while the time spent in the open arms (**p**, mean ± SEM in s: tdTom = 65.00 ± 32.93, hM4Di = 79.54 ± 21.15, tdTom vs. hM4Di unpaired two tailed *t* test, $p = 0.7007$) and the average speed in the open arms (**q**, mean ± SEM in m/s: tdTom = 0.017 ± 0.0030, hM4Di = 0.019 ± 0.0032, tdTom vs. hM4Di unpaired two-tailed *t* test, $p = 0.6246$) were similar in the 2 groups ($n = 8$ tdTomato, $n = 12$ hM4D mice). **r** Mean heat maps for each group.

(For figure see next page.)

cleus AgRP neurons and LH^{vGAT} neurons [20, 35]. However, if MCH neurons have a reinforcing role during feeding rather than simply increasing the level of hunger, they would be expected to become active during or shortly after consumption. Given their implication in food reward, we reasoned that pairing the activation of MCH neurons with chow food consumption might increase the reward value of the consumed pellet, leading to overeating. To test this possibility, we paired the optogenetic stimulation of MCH neurons with chow pellet removal



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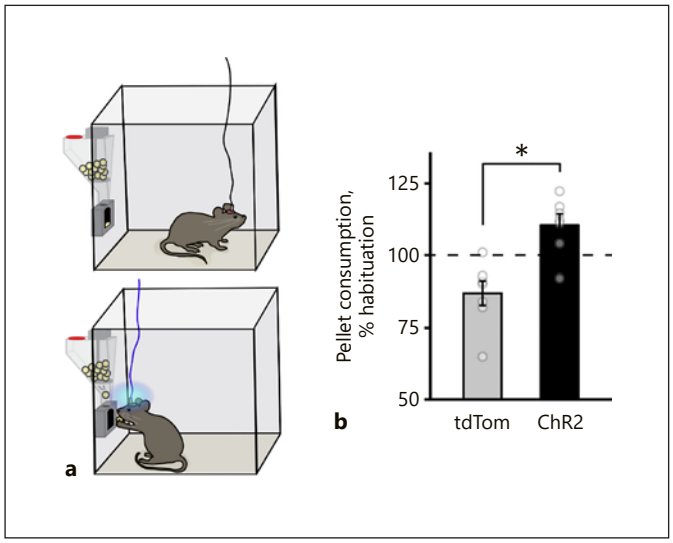

Fig. 5. MCH neuron photoactivation during feeding increases chow food consumption. **a** Schematic representation of the food-coupled MCH-photostimulation assay performed with *Pmch-cre* mice expressing tdTomato or ChR2-eYFP in MCH neurons. **b** Summary graph of the change in pellet consumption relative to a nonstimulated habituation day upon 20 Hz self-stimulation (15 s/pellet) of LH (n = 7 tdTomato mice, n = 7 ChR2-eYFP mice, mean ± SEM: % of habituation: tdTom = 86.87 ± 4.30, ChR2 + 110.67 ± 3.72, tdTomato vs. ChR2-eYFP: unpaired two-tailed t test, * p = 0.0013).

from the feeder to ensure that MCH activation occurs during consumption. It takes approximately 15 s to consume a 20-mg chow pellet; therefore, we adjusted the MCH stimulation to the 15-s time period following the pellet removal (Fig. 5a). Unlike continuous stimulation, pellet consumption-paired stimulation of MCH neurons significantly increased the total number of chow pellets consumed over a 24-h period (Fig. 5b; online suppl. Fig. 4). Taken together, these results suggest that MCH neuron activation alone may not be involved in food seeking or elevation of the hunger state but, when paired with consummatory activity, they may have role in reinforcing feeding.

MCH Neuronal Activation Is Sufficient for Reward without Food

Our results showed that, when simultaneously paired with a consummatory event, MCH neuron activation could increase chow consumption. It has been suggested that MCH neuronal activation can add reward value only when oral sweet cues are present [10]. However, our results suggest that, even with nonsweet chow, consumption can also be increased by MCH activation. To test whether oral/postoral cues are required at all, we further investigated the reward value of direct MCH stimulation using non-food-related tasks of nose poke and lever press self-stimulation. For this, we coupled brief optogenetic activation of ChR2-expressing MCH neurons (10 s) with nose poke or lever press operant tasks. We also placed an inactive nose poke port/lever to test the specificity of activation of the laser-coupled port/lever, respectively (Fig. 6a, d). For both nose poke and lever press operant tasks, MCH activation significantly increased port entries and lever press events over the inactive instrument, suggesting that MCH activation alone is sufficient to generate a reward effect even in the absence of oral or postingestive cues (Fig. 6).

Discussion

Here we evaluated the short-term behavioral and physiological effects of MCH neuronal electrical activity manipulations. We report that acute changes in MCH neuronal activity are not sufficient to alter feeding behavior, unless the stimulation is delivered during the feeding event. In addition, inhibition of MCH neurons improves glucose tolerance and reduces anxiety-like behavior, but neither activation nor inhibition affects the short-term locomotor activity. Furthermore, using 2 different instrumental tasks, we showed that activation of MCH neurons alone is rewarding.

Diverse Physiological and Behavioral Functions of MCH Neurons

Our results on MCH neuronal activity-dependent changes in glucose tolerance and anxiety are consistent with earlier genetic and cell type-specific ablation studies [8, 16]. Rapid improvement of glucose tolerance upon MCH inhibition suggests that developmental compensation or other circuit reorganizations upon genetic manipulation do not account for the observed effect. Similarly, a weak anxiolytic effect of MCH neuronal inhibition, which was predicted from genetic ablation studies but not observed with pharmacologic administration of MCHR1 antagonists, supports a role of MCH in the stress axis.

MCH Neurons and Reward

The mechanisms that underlie MCH neuronal regulation of metabolism and reward are not completely understood. For short-term appetite regulation, our results indicate that MCH neuronal activity is neither sufficient to

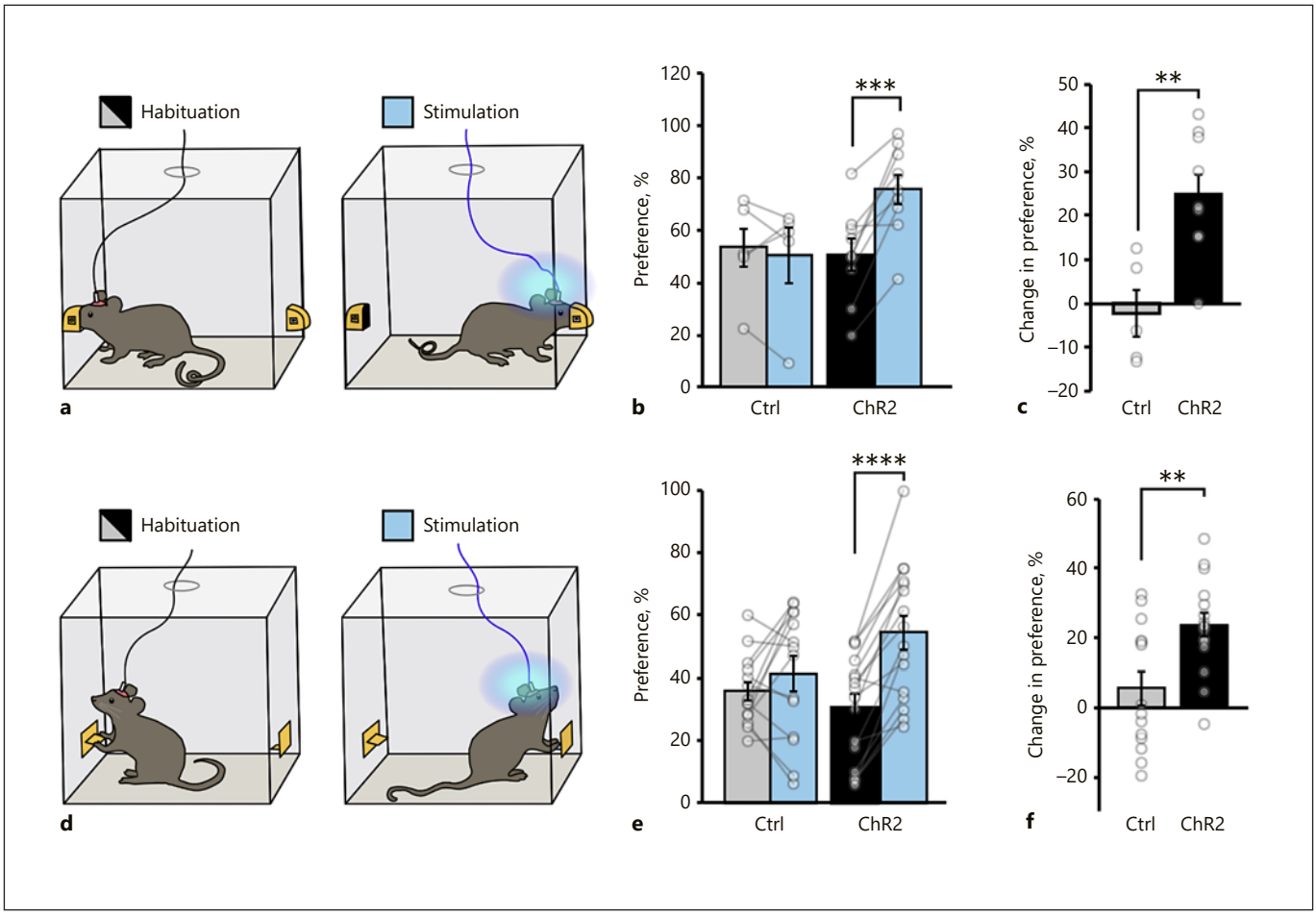

Fig. 6. Activation of MCH neurons is sufficient for a reward even in the absence of food consumption. **a–c** Schematic diagram describing the nose poke assay. **a** Mice were initially observed for nose-poke port preference. Photostimulation (20 Hz; 10 s photostimulation/port entry) was paired with the less preferred one. The preference for the laser-coupled operandum was significantly increased on stimulation days compared both to the tdTomato group and the habituation days (**b**), and the change in preference was significantly higher in the ChR2 group compared to the tdTomato animals (mean ± SEM: control habituation = 52.66 ± 8.70, control stimulation = 50.34 ± 10.44, ChR2 habituation = 50.68 ± 6.02, ChR2 stimulation = 75.56 ± 5.74, n = 5 tdTomato mice, n = 9 ChR2-eYFP mice; two-way ANOVA, interaction F[1,12] = 13.46, p = 0.0032; effect of trial F[1,12] = 9.258, p = 0.0102; effect of viral injections F[1,12] = 1.366, p = 0.2652; Sidak's adjusted p values: p = 0.9784 [tdTomato vs. ChR2-eYFP, habituation], p = 0.0508 [tdTomato vs. ChR2-eYFP, stimulation], p = 0.0002 [habituation vs. stimulation, ChR2-eYFP], and p = 0.9118 [habituation vs. stimulation, tdTomato, ** p < 0.01 *** p < 0.001]). **c** The change in preference was significantly greater in the ChR2-eYFP group compared to the tdTomato group (mean ± SEM: control = –2.32 ± 5.34, ChR2 = 24.88 ± 4.64, n = 5 tdTomato mice, n = 9 ChR2-eYFP mice; tdTomato vs. ChR2-YFP p = 0.003213, unpaired two-tailed t test, ** p < 0.01). **d–**f Lever press assay. Mice were initially observed for normal lever press frequencies, and then the less preferred lever was coupled with a laser for the stimulation day (**d**). Summary graph showing the increase in preference for the laser coupled lever on the stimulation day in ChR2-eYFP-transduced *Pmch-cre* mice only (**e**); mean ± SEM: control habituation = 35.81 ± 2.98, control stimulation = 41.41 ± 5.52, ChR2 habituation = 30.68 ± 4.19, ChR2 stimulation = 54.37 ± 5.36, n = 14 tdTomato mice, n = 16 ChR2-eYFP mice, 2-way ANOVA, interaction F(1,28) = 9.864, p = 0.0040; effect of trial F(1,28) = 25.89, p < 0.00001; effect of viral injections F(1,28) = 0.4332, p = 0.5158; Sidak's adjusted p values: p = 0.6878 (tdTomato vs. ChR2-eYFP, habituation), p = 0.1068 (tdTomato vs. ChR2-eYFP, stimulation), p = 0.3490 (habituation vs. stimulation, tdTomato, p < 0.00001; habituation vs. stimulation, ChR2-eYFP, **** p < 0.0001). The change in preference was significantly greater compared to that in the tdTomato group. **f** Mean ± SEM: control = –5.60 ± 4.80, ChR2 = 23.69 ± 3.38, (n = 14 tdTomato mice, n = 16 ChR2-eYFP mice, tdTomato vs. ChR2-YFP p = 0.003950, unpaired two-tailed t test, ** p < 0.01).

initiate nor necessary to sustain food intake, suggested by the inability to effect overall consumption by chemogenetic activation or inhibition during both day time and dark onset. Indeed, contrary to our expectations, the dark cycle food consumption was slightly reduced in mice which were subjected to continuous MCH neuron photostimulation (20 Hz). However, if activation is time locked during consumption, MCH neurons appear to increase the food intake, suggesting a reinforcing role for ongoing consumption (Fig. 5). It is unlikely that this increase is driven by a delay in satiety signals since continued stimulation does not result in an overall increase in feeding (Fig. 2; online suppl. Fig. 1f).

Earlier results using 2 bottle preference assays in which MCH activation was paired with noncaloric sugar drinking resulted in an inability to display a natural preference toward caloric sugar [10]. Use of a 2-bottle assay with water, however, did not result in a preference for the MCH-activated one, leading to the suggestion that, when paired with sweet oral cues, MCH neuron activation can ensue a reward function that is normally derived from calories. Our results from operant task self-stimulation, however, suggest that in the absence of a sweet taste or even in the absence of any oral/post oral cues MCH activation can still ensue reward. It is unlikely that the discrepancy between our results and the previous report could stem from the use of distinct transgenic *Pmch-cre* drivers, since both lines appear to label >80% of all MCH neurons. However, the longer stimulation durations used in this study (1 s per 5 licks vs. 15 s per 20-mg pellet or 10 s per operant activation) likely uncovered the sufficiency of MCH neuron activity for reward even in the absence of any other food- or taste-related stimuli.

The reward effects of LH stimulations have been previously noted in the GABAergic subpopulation, whereas glutamate-expressing neurons have been shown to be aversive [35, 36]. Our results extend these findings and show that, despite being mostly glutamatergic [11], MCH neurons can also induce self-stimulation. In agreement with the reward function, MCH axons have been localized to the VTA and the striatum, where MCHR1 is highly expressed [10, 37]. Rather than directly stimulating the motivation to eat, as seen with ARC^{AgRP} and LH^{vGAT} neurons, our results support a reinforcing role for MCH neurons. This is consistent with earlier reports showing that rats given an MCHR1 antagonist displayed reduced lever press for sucrose solution, MCH knockout rats displayed a reduced operant response to fat, and MCH neuron-ablated mice were unable distinguish caloric versus noncaloric sweets [10, 38, 39].

Surprisingly, it was the feeding-paired stimulation, but not continuous activation, that increased pellet consumption. Continuous activation even in dark onset, during which mice consume abundantly, did not yield an additional increase in the feeding response but indeed decreased it. One interpretation could be that under the continuous stimulation regime animals might be unable to discern which of their actions, i.e., feeding, nesting, or grooming, is causing the reward effect and therefore cannot reinforce that specific action. On the other hand, if MCH neuronal stimulation is time restricted to a specific action, e.g., pellet consumption, nose poke, or lever press, only then does a significant increase in preference for that action occur. This is consistent with a reinforcing role of MCH neurons rather than simply an increase in appetite.

Limitations of This Study

Our approach was based on addressing the short-term effects of acute MCH neuron activity manipulations, specifically focusing on energy homeostasis, stress, and reward. Overall, our results support a role for MCH neurons in amplification of consumption by reinforcing feeding only when activated in a specific temporal pattern. However, our study does not address whether such activation occurs during feeding, and it remains to be seen how in vivo MCH neuron activity dynamics respond to feeding of different types of diets. The reduced response to hedonically active food in MCH-compromised animal models suggests that MCH activation might take place during palatable food consumption [10, 38, 39]. However, endogenous activity dynamics of MCH neurons have only been studied during sleep-wake regulation and novelty exposure but not in the context of feeding [27, 28].

Our negative results on locomotor activity changes should be interpreted cautiously since we only evaluated the effect of daytime manipulations and we only focused on the first 30 min. It is likely that prolonged observation upon bilateral activation, as reported recently [17], may unveil a likely contribution. In addition, MCH neuron-ablated mice showed locomotor activity differences only in the dark period [8], consistent with a time-sensitive effect. Similarly, although we only used bilaterally infected MCH^{hM4Di} mice, our loss-of-function approach is limited by the penetrance of viral transduction, and therefore possible effects of inhibition on behaviors for which a small fraction of uninfected MCH neurons is sufficient, might have been underestimated. Nevertheless, our transduction rate was sufficient to observe significant impact of inhibition on glucose regulation and anxiety-like behaviors (Fig. 2, 3).

Finally, our study does not address which MCH-neuron derived signaling molecule governs reward function. Recent studies using MCH neuron-selective ablation of MCH peptide or vGlut2 expression (2 major signaling molecules secreted by MCH neurons) have arrived at different conclusions with respect to sugar reward function, likely reflecting partial overlap between these signaling pathways [11, 12].

Collectively, consistent with genetic approaches, our results support diverse behavioral and physiological functions of MCH neurons. We revealed that involvement in short-term appetite regulation might have strict temporal requirements and, while their acute inhibition promotes glucose tolerance and reduces anxiety-like behaviors, activation is sufficient to induce reward.

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Statement of Ethics

All animal generation, care, maintenance, and experimental procedures conform to the internationally accepted standards and were approved by the Istanbul Medipol University (IMU) Animal Care and Use Committee.

Disclosure Statement

The authors declare no competing financial interests.

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Author Contributions

P.D.: animal care, intracranial virus injections, behavioral experiments, imaging, and paper preparation. I.A.: behavioral experiments and paper preparation. N.S.A.: virus preparation, behavioral experiments, data analysis, figure preparation, and paper preparation. Y.Y., T.A., and M.O.: electrophysiological recordings. G.F. and F.K.: confocal imaging. I.C.: intracranial virus injections and ferule placement. E.A.O., M.I.A., U.C., and B.Y.: technical help. D.A.: study conception and design, analysis and interpretation of data, and drafting of this article.

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