MC4R-expressing glutamatergic neurons in the paraventricular hypothalamus regulate feeding and are synaptically connected to the parabrachial nucleus

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Activation of melanocortin-4 receptors (MC4Rs) restrains feeding and prevents obesity; however, the identity, location, and axonal projections of the neurons bearing MC4Rs that control feeding remain unknown. Reexpression of MC4Rs on single-minded 1 (SIM1)$^+$ neurons in mice otherwise lacking MC4Rs is sufficient to abolish hyperphagia. Thus, MC4Rs on SIM1$^+$ neurons, possibly in the paraventricular hypothalamus (PVH) and/or amygdala, regulate food intake. It is unknown, however, whether they are also necessary, a distinction required for excluding redundant sites of action. Hence, the location and nature of obesity-preventing MC4R-expressing neurons are unknown. Here, by deleting and reexpressing MC4Rs from cre-expressing neurons, establishing both necessity and sufficiency, we demonstrate that the MC4R-expressing neurons regulating feeding are SIM1$^+$, located in the PVH, glutamatergic and not GABAergic, and do not express oxytocin, corticotropin-releasing hormone, vasopressin, or prodynorphin. Importantly, these excitatory MC4R-expressing PVH neurons are synaptically connected to neurons in the parabrachial nucleus, which relays visceral information to the forebrain. This suggests a basis for the feeding-regulating effects of MC4Rs.

Melanocortins, working through melanocortin-4 receptors (MC4Rs), regulate energy balance (1–3). Proopiomelanocortin (POMC)-expressing neurons release the MC4R agonist α-melanocyte-stimulating hormone (α-MSH) and promote negative energy balance, whereas agouti-related protein (AgRP)-expressing neurons, releasing the antagonist AgRP, do the opposite. Consistent with these roles, optogenetic (4) and chemogenetic (5) stimulation of POMC neurons causes hypophagia and weight loss. Conversely, optogenetic (4) or chemogenetic (6) stimulation of AgRP neurons produces hyperphagia and weight gain, with prolonged effects being mediated by AgRP through its action on MC4Rs (7). Importantly, the α-MSH/MC4R pathway also operates in humans, as evidenced by massive obesity in individuals lacking either α-MSH or MC4Rs (8, 9).

Despite the established importance of MC4Rs (3), the neural mechanisms by which they regulate energy balance, and in particular feeding, are still unknown. In a prior study, we investigated the role of MC4Rs in the paraventricular hypothalamus (PVH), specifically on SIM1 (single-minded 1)-expressing neurons (2). The impetus for focusing on the PVH included the following: (*i*) MC4R expression is dense in the PVH (10–12), (*ii*) it receives strong input from AgRP and POMC neurons (13, 14), (*iii*) injection of MC3/4R ligands into the PVH affects feeding (14–16), (*iv*) PVH-directed lesions cause obesity (17, 18), and most importantly, (*v*) haploinsufficiency of SIM1, a transcription factor required for PVH development, results in obesity (19, 20). Accordingly, using transgenic approaches, we restored *Mc4r* expression selectively in SIM1 neurons in mice that otherwise lack MC4Rs (2). Of note, restoration in SIM1 neurons rescued ∼60% of the obesity and 100% of the hyperphagia of MC4R-null mice, indicating that MC4Rs on SIM1 neurons regulate feeding. More recently it was found that the obesity-reducing effect of MC4R reexpression is absent when SIM1 neurons lack the ability to release glutamate, suggesting that glutamate release by SIM1 neurons, either by a MC4R-expressing subset or a downstream non–MC4R-expressing subset, is required for the reexpression rescue response (21). For reasons that are unclear, however, in the setting of unmanipulated MC4R expression, loss of glutamate release by SIM1 neurons minimally affects energy balance (21).

There are aspects of *Sim1-Cre*-mediated reactivation of MC4R expression that are worthy of further consideration. First, *Sim1-Cre* mice also express cre activity in sites outside the PVH where

Significance

Both in rodents and humans, melanocortin-4 receptors (MC4Rs) suppress appetite and prevent obesity. Unfortunately, the underlying neural mechanisms by which MC4Rs regulate food intake are poorly understood. Unraveling these mechanisms may open up avenues for treating obesity. In the present study we have established that MC4Rs on neurons in the paraventricular nucleus of the hypothalamus are both necessary and sufficient for MC4R control of feeding and that these neurons are glutamatergic and not GABAergic and do not express the neuropeptides oxytocin, corticotropin-releasing hormone, prodynorphin, or vasopressin. In addition, we identify downstream projections from these glutamatergic neurons to the lateral parabrachial nucleus, which could mediate the appetite suppressing effects.

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MC4Rs are found, most notably the medial amygdala (2) (Allen Brain Institute, Transgenic Characterization, Sim1-Cre;Ai14). It has recently been proposed that MC4Rs in the medial amygdala affect feeding (22). Because of this, uncertainty exists regarding the role of MC4Rs on PVH neurons vs. other neurons in regulating feeding behavior. Second, because MC4R deficiency causes obesity and hyperleptinemia, which is expected to increase melanocortinergic tone, neurons bearing reexpressed MC4Rs in an otherwise MC4R-null background are likely to be "hyper-stimulated," possibly exaggerating their role in regulating feeding. Finally, reexpression studies are silent on the existence of functionally important redundant sites of actions—an important possibility that has been suggested by others (23). For these and other reasons, the location and nature of hyperphagia-preventing MC4R-expressing neurons is uncertain. In the present study we address these issues by using the following approaches. First, by deleting and reexpressing MC4Rs from cre-expressing neurons, we identify neurons on which MC4Rs are both necessary and sufficient for regulating feeding. This allows for a robust assessment of function. Second, by stereotaxically injecting adeno-associated virus expressing cre (AAV-Cre) into different anatomical locations, we examine the role of MC4Rs in the PVH vs. other sites. Third, by using an extensive panel of neuron-specific cre-expressing mice, we more specifically define the neurotransmitter/neuropeptide nature of the food intake-regulating MC4R-expressing neurons. Finally, we use channelrhodopsin (ChR2)-assisted circuit mapping (24, 25) to probe downstream sites through which melanocortin effects are mediated.

Results

Generation and Validation of *Mc4r$^{lox/lox}$* Mice. *Mc4r$^{lox/lox}$* mice were generated using homologous recombination in embryonic stem cells (*SI Materials and Methods*). To confirm that a functionally null *Mc4r* allele results from cre-mediated excision, *Mc4r$^{lox/lox}$* mice were mated to germ-line cre-deleter mice [*EIIa*-Cre (26)], and the resulting cre-modified *Mc4r* alleles were then bred to homozygosity (*Mc4r$^{\Delta/\Delta}$* mice). As expected, *Mc4r$^{\Delta/\Delta}$* mice are markedly overweight (Fig. S1*A*), have increased fat mass (Fig. S1*B*), are hyperphagic (Fig. S1*C*), and have increased linear length (Fig. S1*D*). This phenotype is essentially identical to that seen in two other lines of *Mc4r* null mice (2, 3), indicating that the cre-deleted *Mc4r$^{\Delta/\Delta}$* allele is a null allele.

Deletion of MC4Rs from SIM1 Neurons. We previously established that MC4Rs on SIM1 neurons are sufficient for normal regulation of feeding (2). To test whether they are also necessary, we generated mice that lack MC4Rs on SIM1 neurons (*Sim1-Cre*; *Mc4r$^{lox/lox}$* mice). We found that *Sim1-Cre;Mc4r$^{lox/lox}$* mice displayed increased body weight, increased fat mass, hyperphagia, and increased linear growth compared with their controls (*Mc4r$^{lox/lox}$* mice) (Fig. 1). The intermediate effect on body weight is consistent with our prior *Sim1-Cre* reactivation study and the role of MC4R-expressing SIM1 neurons in regulating feeding but not energy expenditure (2). Thus, in addition to being sufficient (2), MC4Rs on SIM1 neurons are also necessary for feeding regulation. These results demonstrate the importance of MC4Rs on SIM1 neurons in restraining feeding.

Manipulation of MC4Rs on Glutamatergic and GABAergic Neurons. Because the PVH contains many glutamatergic and few GABAergic neurons (27), we hypothesized that MC4R-expressing neurons important for feeding would be glutamatergic. To address this, we generated mice lacking MC4Rs on glutamatergic neurons [*Vglut2 (Slc17a6)-ires-Cre;Mc4r$^{lox/lox}$* mice]. As predicted, *Vglut2-ires-Cre;Mc4r$^{lox/lox}$* mice have markedly increased body weight, increased fat mass, are hyperphagic, and have increased linear growth (Fig. 1). The phenotype observed is comparable to that

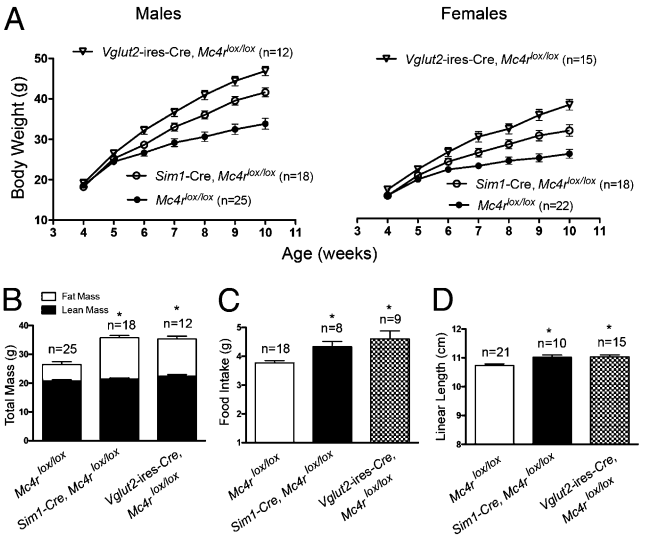

Fig. 1. Effects of deleting MC4Rs from SIM1$^+$ and VGLUT2$^+$ neurons on energy balance. (*A*) Body weight of *Vglut2*-ires-Cre;*Mc4r$^{lox/lox}$*, *Sim1*-Cre; *Mc4r$^{lox/lox}$*, and *Mc4r$^{lox/lox}$* mice (males and females). Body weights are significantly different (*$P < 0.05$) compared with *Mc4r$^{lox/lox}$* mice starting at 6 wk for *Vglut2*-ires-Cre;*Mc4r$^{lox/lox}$* male mice, 5 wk for *Vglut2*-ires-Cre; *Mc4r$^{lox/lox}$* female mice, and 7 wk for *Sim1*-Cre;*Mc4r$^{lox/lox}$* male and female mice. (*B–D*) Fat mass and lean mass (*B*), daily food intake (ad libitum) (*C*), and linear length (*D*) of *Mc4r$^{lox/lox}$*, *Vglut2*-ires-Cre;*Mc4r$^{lox/lox}$*, and *Sim1*-Cre; *Mc4r$^{lox/lox}$* male mice at 12–15 wk. Data are presented as mean ± SEM. *$P <$ 0.05; one-way ANOVA compared with *Mc4r$^{lox/lox}$* mice.

seen in *Mc4r* null mice. Thus, MC4Rs on glutamatergic neurons are necessary for preventing hyperphagia and obesity.

To determine whether MC4Rs on glutamatergic neurons are also sufficient, we selectively reexpressed MC4Rs on glutamatergic neurons in an otherwise MC4R-null background (*Vglut2-ires-Cre;Mc4r$^{loxTB/loxTB}$* mice). Notably, reexpression of MC4Rs on glutamatergic neurons essentially completely rescued increased weight gain, fat stores, hyperphagia, and depressed respiratory exchange ratio (Fig. 2 *A–D*) and normalized increased linear length, hyperglycemia, and hyperinsulinemia (Table S1). It is important to note that the apparent near-complete rescue of the *Mc4r* obesity phenotype in *Vglut2-ires-Cre; Mc4r$^{loxTB/loxTB}$* mice is far greater than the partial rescue observed in *Sim1-Cre;Mc4r$^{loxTB/loxTB}$* mice (2) and is presumably due to reactivation of MC4R expression in a larger fraction of the body weight-regulating neurons. We also assessed O_2 consumption in these mice (Table S1), but the marked differences in body composition prevent meaningful interpretation of the results (28, 29). Collectively, these results demonstrate that MC4Rs on glutamatergic neurons are both necessary and sufficient for prevention of hyperphagia and obesity.

In contrast to MC4Rs on glutamatergic neurons, MC4Rs on GABAergic neurons play little or no role in regulating energy balance: no reduction in obesity was observed in mice with reactivation of the MC4R allele in GABAergic neruons [*Vgat-ires (Slc32a1)-Cre;Mc4r$^{loxTB/loxTB}$* mice] (Fig. S2).

AAV-Cre Injections to Determine the Role of MC4Rs on PVH Neurons. The above studies demonstrate that the MC4R-expressing neurons controlling feeding are both SIM1$^+$ and glutamatergic. Whether these neurons exclusively reside in the PVH is uncertain. To resolve this, we stereotaxically injected AAV-Cre into the PVH of mice bearing cre-sensitive *Mc4r* alleles. We also injected AAV-Cre into the medial amygdala because, like the PVH, it has neurons that are SIM1$^+$, glutamatergic, and *Mc4r*-expressing (2, 10, 11, 27). Of relevance, the medial amygdala is heavily

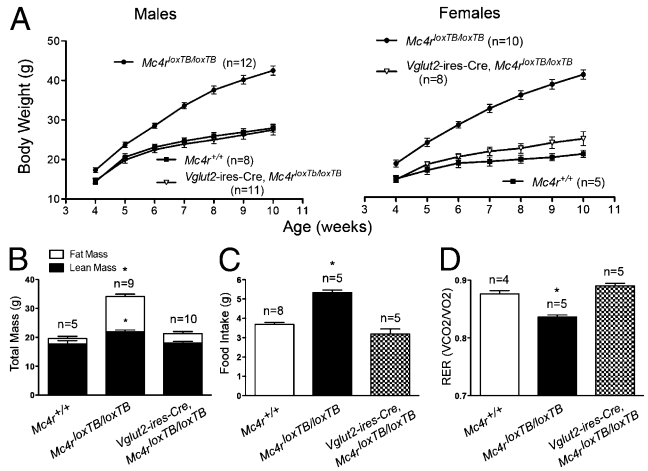

Fig. 2. Effect of reactivating MC4Rs on VGLUT2$^+$ neurons on energy balance. (*A*) Body weight of *Mc4r*$^{+/+}$, *Mc4r*$^{loxTB/loxTB}$, and *Vglut2*-ires-Cre; *Mc4r*$^{loxTB/loxTB}$ mice (males and females). Body weights of all groups are significantly different (*$P < 0.05$) compared with *Mc4r*$^{loxTB/loxTB}$ mice at all data points analyzed. (*B–D*) Fat mass and lean mass (*B*), daily food intake (ad libitum) (*C*), and respiratory exchange rate (*D*) of *Mc4r*$^{+/+}$, *Mc4r*$^{loxTB/loxTB}$, *and Vglut2*-ires-Cre;*Mc4r*$^{loxTB/loxTB}$ male mice at 12–14 wk. Data are presented as mean ± SEM. *$P < 0.05$; one-way ANOVA, all groups compared with *Mc4r*$^{loxTB/loxTB}$ mice.

connected to the hypothalamus, has previously been associated with obesity (30, 31), and MC4Rs in this site have been proposed to affect feeding (22). Finally, we also tested two SIM1$^-$ sites, the nucleus of the solitary tract (NTS) and the lateral parabrachial nucleus (L-PBN), because they have glutamatergic and *Mc4r*-expressing neurons, and importantly, injections of MC3/4 receptor agonists into these sites affect energy balance (10, 11, 23, 27).

AAV-Cre–mediated deletion of MC4Rs in the PVH of *Mc4r*$^{lox/lox}$ mice increased body weight gain and fat mass and caused hyperphagia (Fig. 3 *A–D*). Conversely, reexpression of MC4Rs in the PVH of *Mc4r*$^{loxTB/loxTB}$ mice markedly reduced body weight gain and fat mass and greatly reduced hyperphagia (Fig. 3 *E–H*). Thus, MC4Rs on neurons in the PVH are both necessary and sufficient for prevention of hyperphagia. This demonstrates that the PVH is the major anatomic site through which MC4Rs restrain feeding. In contrast, injection of AAV-Cre into the anterior or posterior medial amygdala or the L-PBN of *Mc4r*$^{loxTB/loxTB}$ mice had no effect on the body weight gain (Fig. S3 *A–F*). This suggests that MC4Rs in the medial amygdala and L-PBN do not play a major role in regulating energy balance. Of interest, AAV-Cre injection into NTS [and also the nearby dorsal motor nucleus of the vagus nerve (DMV)] of *Mc4r*$^{loxTB/loxTB}$ mice modestly reduced body weight gain and fat mass (Fig. 3 *I–K*). This occurred in the absence of detectable changes in food intake (Fig. 3*L*), raising the possibility that energy expenditure was increased (Fig. 3 *K* and *L*). In total, the above results demonstrate that MC4Rs in the PVH, and not the medial amygdala, L-PBN, or NTS, regulate feeding.

The Role of MC4Rs on PVH Neuropeptide-Expressing Neurons. Both oxytocin (OXT)- and corticotropin-releasing hormone (CRH)-expressing PVH neurons have been proposed to mediate the satiety effects of MC4Rs (32–35). To directly test the role of MC4Rs on these neurons, as well as two other neuropeptide-expressing neurons in the PVH, we used *Oxt-ires-Cre* (36), *Crh-ires-Cre* (37), arginine *vasopressin (Avp)-ires-Cre* (37, 38), and *prodynorphin (Pdyn)-ires-Cre* (37) knockin mice. To our surprise, for all four "neuropeptide"*-ires-Cre;Mc4r*$^{loxTB/loxTB}$ mice, there was no diminution of the obesity seen in MC4R-deficient obese *Mc4r*$^{loxTB/loxTB}$ control mice (Fig. 4 *A–D* and Fig. S4). Of note, OXT and CRH neurons are distinct populations of PVH neurons (Fig. 4*E*). Last, obesity was also not reduced in "double cre" *Crh-ires-Cre, Oxt-ires-Cre, Mc4r*$^{loxTB/loxTB}$ mice (Fig. 4*F*). Thus, the PVH MC4R-expressing neurons that regulate feeding are SIM1$^+$ and glutamatergic but do not express OXT, CRH, arginine vasopressin or prodynorphin.

The L-PBN Is Downstream of MC4R-Expressing Glutamatergic PVH Neurons. Having established that food intake-regulating MC4R-expressing neurons are glutamatergic and are in the PVH, we used ChR2-assisted circuit mapping (CRACM) to identify neurons

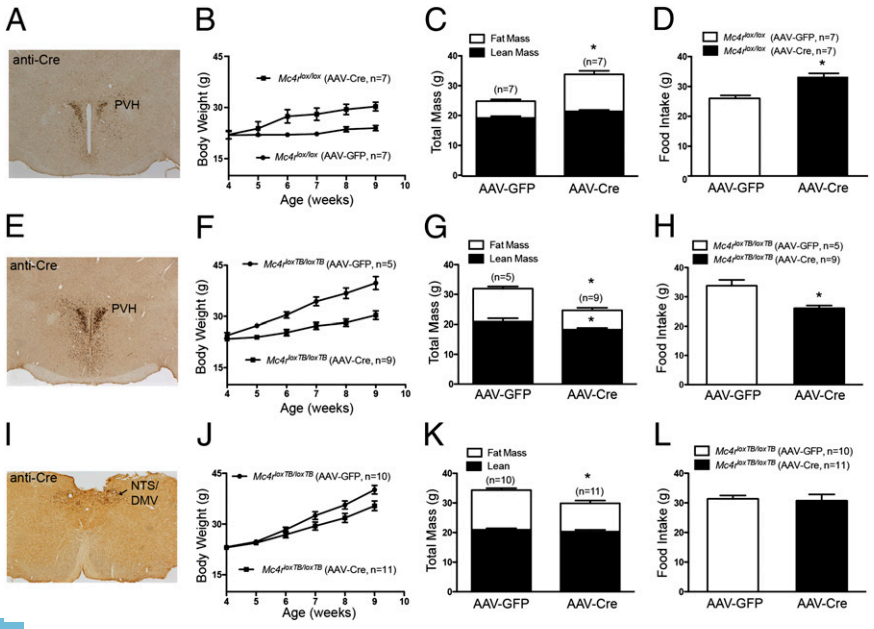

Fig. 3. Site-specific deletion or reactivation of MC4Rs using stereotaxic AAV-Cre injections. (*A–D*) AAV-Cre injected bilaterally into the PVH of *Mc4r*$^{lox/lox}$ mice. (*A*) Representative image showing immunohistochemistry for Cre. Body weight (*B*), fat and lean mass (*C*), and food intake (*D*) of AAV-Cre vs. AAV-GFP injected *Mc4r*$^{lox/lox}$ male mice. Body weights are significantly different (*$P < 0.05$) compared with AAV-GFP injected controls starting at 6 wk of age. (*E–H*) AAV-Cre injected bilaterally into the PVH of *Mc4r*$^{loxTB/loxTB}$ mice. (*E*) Representative image showing immunohistochemistry for Cre. Body weight (*F*), fat and lean mass (*G*), and food intake (*H*) of AAV-Cre vs. AAV-GFP injected *Mc4r*$^{loxTB/loxTB}$ male mice. Body weights are significantly different (*$P < 0.05$) compared with AAV-GFP injected controls starting at 5 wk of age. (*I–L*) AAV-Cre injected bilaterally into the NTS/DMV of *Mc4r*$^{loxTB/loxTB}$ mice. (*I*) Representative image showing immunohistochemistry for Cre. Body weight (*J*), fat and lean mass (*K*), and food intake (*L*) of AAV-Cre vs. AAV-GFP injected *Mc4r*$^{loxTB/loxTB}$ male mice. Body weights are significantly different (*$P < 0.05$) compared with AAV-GFP injected controls starting at 8 wk of age. In all studies, bilateral AAV-Cre injections were done in 4-wk-old mice. Mice lacking bilateral hits were not included in the study. Fat and lean mass were measured at 10–11 wk of age. Data are presented as mean ± SEM. *$P < 0.05$; unpaired *t* test compared with AAV-GFP injected controls.

that are synaptically and functionally downstream. Cre-dependent AAV expressing ChR2-mCherry was stereotaxically injected into the PVH of *Vglut2-ires-Cre* mice that also bear the cre-dependent *R26-loxSTOPlox-L10-GFP* reporter allele (37). The mCherry tag on ChR2 permits identification of all PVH glutamatergic projections, whereas the *R26-loxSTOPlox-L10-GFP* reporter, which targets GFP to ribosomes, allows for visualization of vesicular glutamate transporter 2 (VGLUT2$^+$) glutamatergic cell bodies (Fig. 5*A*). Abundant mCherry-expressing fibers were observed in the NTS and the nearby DMV (Fig. 5*B*). These projections are of interest because an influential model posits that MC4R-expressing PVH neurons promote satiety by projecting to and activating neurons in the NTS, increasing their sensitivity to gut-derived, afferent vagal satiety signals (32, 39). With this in mind, we used CRACM to determine whether NTS neurons are indeed downstream of PVH glutamatergic neurons. Recordings were performed in the caudal NTS (medial and commissural subdivisions of NTS) (40), an area enriched in mCherry-expressing fibers, and which is known to receive afferent vagal information from the gut. Unexpectedly, we failed to detect light-evoked excitatory postsynaptic currents (EPSCs) in 93% (37 of 40) of NTS neurons tested (Fig. 5*C*). In contrast, we readily detected light-evoked EPSCs in 100% (15 of 15) of DMV neurons tested (Fig. 5*D*). These were blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), an α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor antagonist, confirming their glutamatergic nature (Fig. 5*D*). Thus, remarkably, the PVH glutamatergic neurons projecting to the dorsal vagal complex are largely synapsing on preganglionic vagal motor neurons in the DMV, and importantly, seem to provide little input to the NTS neurons that receive satiety information from the gut. With this in mind, we then checked for connectivity at an upstream node in the linear, ascending pathway that relays satiety information from the gut to the forebrain, namely the L-PBN (41–46). Recent studies have strongly implicated the L-PBN in regulating feeding (47, 48). PVH glutamatergic neurons send dense ChR2-mCherry–expressing projections to the central subdivision of the L-PBN (Fig. 5 *E* and *F*), and importantly, we detected light-evoked EPSCs in 55% (6 of 11) of VGLUT2$^+$ neurons (identified by GFP-expression from the L10-GFP reporter allele) in this location of L-PBN (Fig. 5*G* and Fig. S5). Again, EPSCs were blocked by CNQX. In contrast, we failed to detect light-evoked EPSCs in 100% (10 of 10) of the nonglutamatergic L-PBN neurons (i.e., neurons lacking GFP) (Fig. 5*H*). Thus, PVH glutamatergic neurons are synaptically connected to a major subset of glutamatergic neurons in the L-PBN.

To determine whether the glutamatergic PVH neurons that project to the L-PBN express MC4Rs, we injected retrogradely transported fluorescent red retrobeads into the L-PBN of *Vglut2-ires-Cre*;*R26-loxSTOPlox-L10-GFP* mice and then tested for effects of an MC4R-selective agonist on membrane potential and firing rate. We assessed effects on the L-PBN–projecting, glutamatergic (beads$^+$,GFP$^+$) PVH neurons as well as the PVH glutamatergic neurons that project elsewhere (beads$^-$,GFP$^+$) (Fig. 5*I*). Of interest, the MC4R agonist depolarized and increased the firing rate of 65% (11 of 17) of the L-PBN–projecting glutamatergic neurons (Fig. 5*J*). In contrast, the MC4R agonist activated only 10% (1 of 10) of the non–L-PBN-projecting neurons (Fig. 5*K*). Thus, functional MC4Rs are present on the majority of glutamatergic neurons that project to the L-PBN. In total, these findings suggest a circuit (MC4R-expressing, glutamatergic PVH neuron→glutamatergic L-PBN neuron) by which MC4R action could decrease feeding.

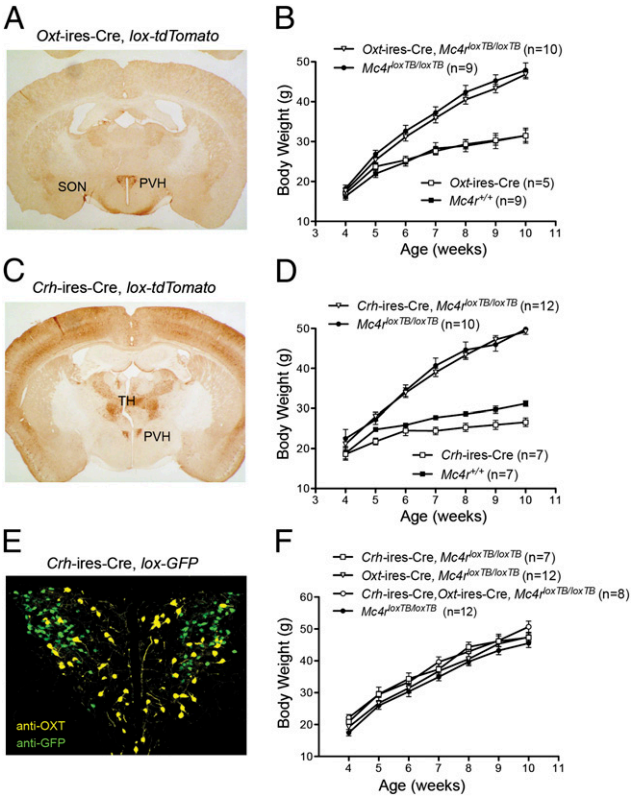

Fig. 4. MC4Rs on OXT$^+$ and CRH$^+$ neurons do not regulate body weight. (*A* and *C*) Immunohistochemistry for tdTomato expression in *Oxt*-ires-Cre; *lox-tdTomato* mice (*A*) and *Crh*-ires-Cre;*lox-tdTomato* mice (*C*). (*B*, *D*, and *F*) Body weight curves for *Oxt*-ires-Cre;*Mc4r*$^{loxTB/loxTB}$ (*B*), *Crh*-ires-Cre;*Mc4r*$^{loxTB/loxTB}$ (*D*), and *Oxt*-ires-Cre;*Crh*-ires-Cre; *Mc4r*$^{loxTB/loxTB}$ (*F*) male mice. (*E*) Double immunohistochemistry for OXT (yellow) and GFP (green) in the PVH of *Crh*-ires-Cre; *lox-GFP* mice. Data are presented as mean ± SEM. n indicates number of mice per group.

Discussion

MC4Rs play a critical role in regulating feeding and energy expenditure and in preventing obesity. However, despite this realization many years ago (3, 49, 50), we still lack a mechanistic neurocircuit-level understanding of how MC4Rs brings about these important actions. In a prior study, we observed that reexpression of MC4Rs in SIM1$^+$ neurons, in mice that otherwise lack MC4Rs, prevents hyperphagia and reduces obesity (2). Because the transcription factor SIM1 is required for PVH development and its haploinsufficiency results in obesity (19, 20), the above result suggested that SIM1$^+$ neurons in the PVH are responsible for MC4R-dependent regulation of feeding. However, because cre activity in *Sim1-Cre* mice is not restricted to the PVH, and because the reexpression approach possibly exaggerates effects due to enhanced feedback in otherwise null mice, and importantly, because it tests sufficiency but not necessity thus not addressing functionally redundant sites of action, the location and nature of the MC4R-expressing, SIM1$^+$, food intake-regulating neurons has been uncertain. In the present study we address these issues by (*i*) deleting as well as reexpressing MC4Rs, thus testing both necessity and sufficiency, (*ii*) using AAV-Cre to address the role of MC4Rs in the PVH vs. other sites, and (*iii*) applying a panel of neurotransmitter/neuropeptide cre-expressing mice to determine the transmitter(s) released by the food intake-regulating neurons. Through such studies we determined that the MC4R-expressing neurons regulating feeding are located in the PVH, are glutamatergic, and do not express four neuropeptides found in the PVH, including OXT and CRH, which have been proposed as mediators of MC4R-induced anorexia.

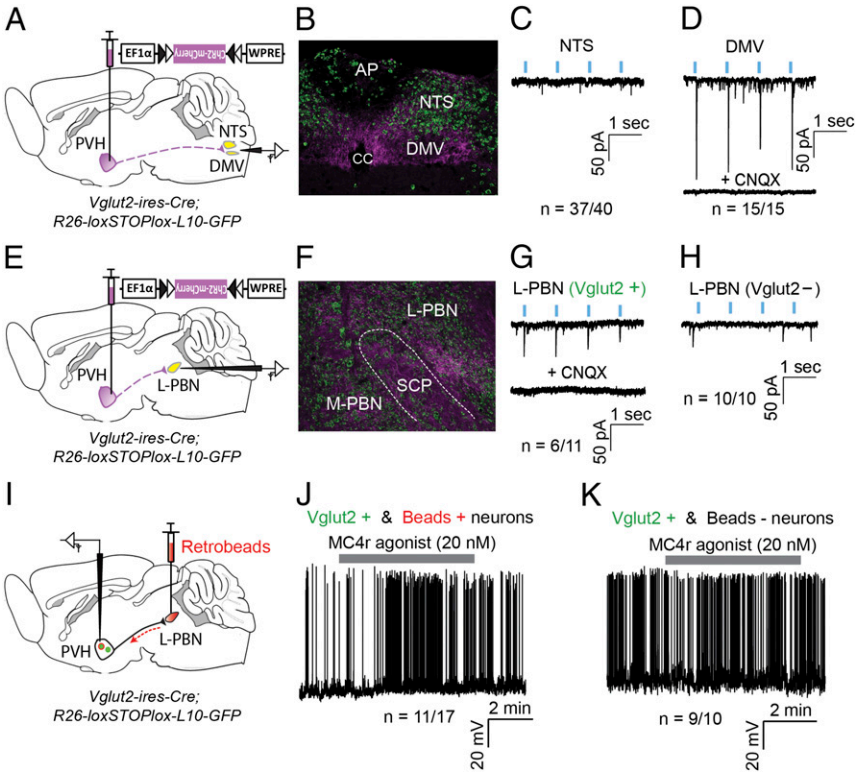

Fig. 5. Downstream neurocircuitry engaged by MC4R-expressing glutamatergic PVH neurons. (*A–D*) CRACM to probe VGLUT2 (PVH) → NTS/DMV connections. (*A*) Schematic of stereotaxic injection of AAV8-DIO-ChR2-mCherry into the PVH of *Vglut2*-ires-Cre;*R26-loxSTOPlox-L10-GFP* mice with patch-clamp recording from neurons in the NTS/DMV. (*B*) Double immunohistochemistry for mCherry (magenta) and GFP (green) in NTS/DMV. Example trace of light-evoked EPSCs recorded from neurons in NTS (*C*) and DMV (latency between onset of light and EPSCs = 5.1 + 0.2 ms) (*D*). CNQX is an AMPA receptor blocker. (*E–H*) CRACM to probe VGLUT2 (PVH) → L-PBN connections. (*E*) Schematic of stereotaxic injection of AAV8-DIO-ChR2-mCherry into the PVH of *Vglut2*-ires-Cre, *R26-loxSTOPlox-L10-GFP* mice with patch-clamp recording from neurons in the L-PBN. (*F*) Double immunohistochemistry for mCherry (magenta) and GFP (green) in L-PBN. Example trace of light-evoked EPSCs recorded from VGLUT2$^+$ (latency = 4.6 + 0.3 ms) (*G*) and VGLUT2$^-$ (*H*) neurons in L-PBN. CNQX is an AMPA receptor blocker. (*I*) Schematic showing retrobead injection into the L-PBN of *Vglut2*-ires-Cre, *R26-loxSTOPlox-L10-GFP* mice with patch-clamp recording from neurons in the PVH. Example trace from beads$^+$ (*J*) and beads$^-$ (*K*) VGLUT2$^+$ PVH neurons. n indicates number of recoded cells.

There are several important implications of these findings. First, because the key MC4R-expressing neurons are glutamatergic, it is almost a certainty that they release glutamate and excite their downstream target neurons. Of note, this view is consistent with the recent finding that the obesity-reducing effects of MC4R reexpression in SIM1 neurons are absent when SIM1 neurons lack the ability to release glutamate (21). The key role of MC4Rs on glutamatergic neurons is in striking contrast to the situation for leptin receptors (LEPRs), for which the majority of antiobesity effects are mediated by LEPRs on GABAergic neurons (27). This dissimilarity in fast-acting transmitters highlights the different circuitries and synaptic neurobiological processes engaged by leptin vs. melanocortin action. Second, because the key MC4R-expressing neurons release glutamate, CRACM can be used to identify the downstream neurons (see below). Third, the lack of effects after manipulation of MC4Rs on OXT and CRH neurons argues strongly against the role of these neuropeptides as direct mediators of melanocortin action. This is a significant finding, especially in regard to OXT, given that other studies have suggested that PVH OXT neurons regulate feeding (32, 33, 35, 51). An important possibility that would reconcile our results with the above-mentioned studies is that MC4R-expressing glutamatergic neurons in the PVH may subsequently synapse on and activate OXT neurons, which could then suppress feeding. Alternatively, PVH OXT neurons may not regulate food intake as suggested by unperturbed feeding after inducible ablation of OXT neurons (36).

Satiety signals from the gut ascend via the afferent vagal nerve, synapsing on neurons in the NTS (41–45). This information then travels to the forebrain via direct projections from the NTS and also indirectly via a relay in the lateral parabrachial nucleus. A prevalent and attractive model proposes that PVH neurons regulating satiety do so by increasing the gain on satiety signals ascending from the gut (32, 39). A logical site of interaction would be the caudal NTS neurons, which directly receive afferent vagal signals. Indeed, a plethora of anterograde and retrograde tracing studies confirm PVH→NTS/DMV projections (52–54). Remarkably, using CRACM, we find that the PVH glutamatergic neurons projecting toward the NTS are largely targeting the preganglionic vagal motor neurons in the DMV, and not the NTS. Thus, the MC4R-expressing food intake-regulating glutamatergic neurons seem not to heavily engage the satiety signal-receiving NTS neurons. A few possibilities may reconcile the apparent discordant results. (*i*) The prior retrograde tracer injections aimed at the NTS likely also hit the nearby DMV (52). Thus, labeled neurons in the PVH are expected to include DMV-projecting neurons. (*ii*) Motor neurons in the DMV extend their dendrites to discrete regions of overlying NTS, such as the commissural and medial subnuclei, and these also receive PVH axonal projections (53). Thus, retrograde tracer could have been taken up by terminals in the NTS, which are in synaptic contact with these NTS-extending DMV dendrites. (*iii*) PVH neurons projecting to and regulating NTS neurons may not be glutamatergic. This is possible because not all PVH neurons are glutamatergic, as judged by lack of *Vglut2-ires-Cre*–dependent expression of reporter. Of note, tyrosine hydroxylase-positive PVH neurons are known to project to the NTS/DMV (52, 54). However, because the MC4Rs regulating feeding are on glutamatergic neurons, nonglutamatergic projections to the NTS, if they exist, seem not to directly mediate the effects of MC4Rs on feeding. (*iv*) Finally, we did detect connectivity to a minor population of NTS neurons (3 of 40 NTS neurons). A functionally meaningful role for this innervation cannot be excluded.

Having failed to detect significant connectivity between PVH glutamatergic neurons and NTS neurons, we then looked upstream at the L-PBN, which relays satiety information from the NTS to the forebrain (40). Of interest, more than half of the glutamatergic L-PBN neurons (mainly located in central subdivision of L-PBN) receive input from PVH glutamatergic neurons, and importantly, the majority of the L-PBN–projecting PVH neurons have MC4R activity. Given recent studies implicating

an important role for the L-PBN in regulating feeding (47, 48), this suggests a neural circuit (MC4R-expressing, glutamatergic PVH neuron→glutamatergic L-PBN neuron) by which melanocortin action in the PVH inhibits feeding. Future studies will need to critically test this important possibility.

Materials and Methods

Care of all animals and procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Mice were housed at 22–24 °C using a 12-h light/12-h dark cycle with standard pelleted mouse chow (Teklad F6 Rodent Diet 8664, 12.5% kcal from fat; Harlan Teklad) and water provided ad libitum. Body weight measurements were recorded weekly. Daily food intake was measured over 3 d and averaged to give 24-h food intake. Mice were euthanized by CO_2 narcosis. *SI Materials and Methods* provides detailed descriptions.

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