Ablation of Neurons Expressing Melanin-Concentrating Hormone (MCH) in Adult Mice Improves Glucose Tolerance Independent of MCH Signaling

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Melanin-concentrating hormone (MCH)-expressing neurons have been ascribed many roles based on studies of MCH-deficient mice. However, MCH neurons express other neurotransmitters, including GABA, nesfatin, and cocaine–amphetamine-regulated transcript. The importance of these other signaling molecules made by MCH neurons remains incompletely characterized. To determine the roles of MCH neurons *in vivo*, we targeted expression of the human diphtheria toxin receptor (DTR) to the gene for MCH (*Pmch*). Within 2 weeks of diphtheria toxin injection, heterozygous *Pmch*^{DTR/+} mice lost 98% of their MCH neurons. These mice became lean but ate normally and were hyperactive, especially during a fast. They also responded abnormally to psychostimulants. For these phenotypes, ablation of MCH neurons recapitulated knock-out of MCH, so MCH appears to be the critical neuromodulator released by these neurons. In contrast, MCH-neuron-ablated mice showed improved glucose tolerance when compared with MCH-deficient mutant mice and wild-type mice. We conclude that MCH neurons regulate glucose tolerance through signaling molecules other than MCH.

Introduction

The neuropeptide melanin-concentrating hormone (MCH) was first discovered in teleost fish (Kawauchi et al., 1983) but has since been found in humans, rodents, and other mammals (Nahon et al., 1989; Presse et al., 1990; Qu et al., 1996). In mice, MCH binds to a single G-protein-coupled receptor (MCHR1) (Kokkotou et al., 2001). The cell bodies of neurons that express MCH reside primarily in the lateral hypothalamus (LH) and adjacent zona incerta (Bittencourt et al., 1992). MCH neurons project extensively throughout the brain, and MCHR1 is expressed widely (Bittencourt et al., 1992; Hervieu et al., 2000). MCH inhibits target neurons (Rao et al., 2008; Wu et al., 2009), but neurons expressing MCH also express GAD67, the major biosynthetic enzyme for GABA (Elias et al., 2008), and two secreted polypeptides, nesfatin and cocaine–amphetamine-regulated transcript (CART) (Elias et al., 2001; Fort et al., 2008). MCH neurons have low intrinsic activity both *in vitro* and *in vivo* but are stimulated by glucose (van den Pol et al., 2004; Burdakov et al., 2005; Hassani et al., 2009).

To date, most investigations into the physiologic role of MCH neurons have manipulated MCH or its receptor. As might be expected from the projection pattern of MCH neurons, a wide variety of phenotypes have been described. MCH knock-out mice

are lean and hyperactive, with increased wakefulness (Shimada et al., 1998; Kokkotou et al., 2005; Willie et al., 2008). Expression of MCH is upregulated during fasting, and the hyperactivity and wakefulness of MCH knock-out mice become more pronounced during a 24 h fast (Qu et al., 1996; Willie et al., 2008). The MCHR1 is highly expressed in the nucleus accumbens shell and caudate–putamen, and MCH knock-out mice display signs of hyperdopaminergia (Pissios et al., 2008). Glucose sensing by MCH neurons is important for maintaining normal blood glucose; when MCH neurons are made congenitally insensitive to glucose, glucose tolerance is altered in adulthood (Kong et al., 2010), but the importance of MCH for this phenotype remains unclear.

The function of MCH neurons, as opposed to MCH itself, was explored by Alon and Friedman (2006). They expressed a toxic form of ataxin-3 in MCH neurons, causing progressive cell death. Those mice developed a lean phenotype with enhanced metabolism consistent with the phenotype of MCH knock-out mice. Although their results suggest that MCH is the principle neuromodulator produced by these neurons, the slow demise of MCH neurons with this strategy may have allowed compensatory mechanisms to develop that mask other functions of these neurons. For example, sudden ablation of NPY/AgRP neurons with diphtheria toxin (DT) in adult mice results in a severe starvation phenotype (Gropp et al., 2005; Luquet et al., 2005), whereas slower ablation with ataxin-3 results in a much milder phenotype (Bewick et al., 2005). Consequently, to generate mice in which MCH neurons could be rapidly ablated in adulthood, we targeted expression of the human DT receptor (DTR) to the *Pmch* locus. In so doing, we found that MCH neurons regulate glucose tolerance through signaling molecules other than MCH.

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Materials and Methods

Animals and DT treatment. All experiments were performed in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Washington. Mice expressing the *Pmch^{DTR}* allele were generated as described previously (Wu et al., 2012). All animals were extensively crossed to a C57BL/6J background (more than six generations). Unless noted otherwise, mice were group housed under a standard 12 h light/dark cycle (lights on from 7:00 A.M. to 7:00 P.M.) and fed standard chow (LabDiet 5053) *ad libitum*. Experimental mice and controls were generated by crossing heterozygous *Pmch^{DTR/+}* mice to C57BL/6J or by double heterozygous cross for experiments involving *Pmch^{DTR/DTR}* homozygotes. To ablate MCH neurons, male mice between 8 and 12 weeks of age were injected twice with DT [50 ng/g, intramuscular (i.m.); 2 d apart; List Biologicals]. DT was dissolved in saline (SAL; 0.9% NaCl).

Body weight and food intake. Mice were individually housed and placed on a standard diet formulated for food intake measurements (D12450B; Research Diets) for 7 d before body weights and food intakes were measured daily. After baseline food intake was established (5 d), DT was injected and daily measurements continued for 14 d after the initial DT injection.

Locomotion and wakefulness analysis. For recording of 24 h locomotor activity, mice were individually housed starting 3 d after DT injection. On day 10 after DT injection, home cages were placed in activity meters with infrared beams to monitor x- and z-axes (Opto-M3; Columbus Instruments). Total activity (beam breaks) was summed over 30 min intervals. For basal activity, mice were given *ad libitum* food and water, and activity was monitored for two consecutive 24 h periods. On the third day, food was removed at 5:00 P.M., whereas activity monitoring continued throughout 24 h of fasting. To analyze patterns of rest and activity, we used the method of Pack et al. (2007), binning total activity into 10 s intervals and scoring bouts of inactivity (zero beam breaks) of >40 s as a bout of rest.

Psychomotor activation. For experiments using psychostimulants, group-housed mice were injected with DT 10 d before the first day of testing. At 12:00 P.M., mice were placed individually into activity meters to acclimate for 3 h before injection with vehicle [water, 10 μ l/g, intraperitoneal (i.p.)]. On the next day, after acclimatization, mice were injected with GBR 12909 (1-[2-[bis(4-fluorophenyl)-methoxy]ethyl]-4-[3-phenylpropyl]piperazine) (20 mg/kg, i.p.; Sigma). Water was used as vehicle, because GBR 12909 is insoluble in SAL. Total activity was monitored in 5 min intervals for 3 h after injection. For cocaine sensitization, a separate cohort of DT-injected mice was injected on 6 consecutive days after a 3 h acclimatization, and total activity was summed over the next 1 h. On the first 2 d, mice received SAL (10 μ l/g, i.p.). On each of the next four days, mice received cocaine (20 mg/kg, i.p.; Sigma).

Glucose tolerance. For glucose tolerance testing, mice were selected from each genotype, which did not differ in body weights, and were individually housed for 7 d on ALPHA-dri bedding (Shepherd Specialty Papers) before DT was injected. Ten days after DT injection, mice were fasted overnight (~17 h). The next morning (between 10:00 A.M. and 11:00 A.M.) fasting blood glucose was measured from a nick in the tail

Figure 1. Immunohistochemical characterization of *Pmch^{DTR}* allele expression in the LH. **A**, As revealed by GFP expression (green), the *Pmch^{DTR}* allele was expressed in most neurons that expressed MCH (red) of heterozygous *Pmch^{DTR/+}* mice injected with SAL (top). After injection with DT (bottom), almost all GFP-expressing neurons were killed after 10 d, and very few MCH neurons remained. **B**, In homozygous *Pmch^{DTR/DTR}* mice injected with SAL (top), MCH was absent, but GFP expression matched the wild-type pattern of MCH expression. After injection with DT (bottom), GFP-expressing neurons were almost never seen. Scale bar, 250 μ m.

using a FreeStyle Lite glucometer (Abbott Laboratories). Blood glucose was again measured 15, 30, 45, 60, and 120 min after i.p. injection of D-glucose (250 mg/ml; Hospira). The volume of D-glucose injected was adjusted for a dose of either 1 or 2 g/kg body weight.

Immunohistochemistry. At the end of each experiment, mice were perfused transcardially with 4% paraformaldehyde, and brains were collected to evaluate the extent of MCH-neuron ablation. Frozen sections (25 μ m thick) containing the LH were immunostained with antibodies against MCH (rabbit anti-MCH; 1:1000; Phoenix Pharmaceuticals; or goat anti-MCH; 1:1000; catalog #sc-14509; C-20; Santa Cruz Biotechnology), hypocretin/orexin (goat anti-orexin-A; 1:1000; catalog #sc-8070; C-19; Santa Cruz Biotechnology), or green fluorescent protein (GFP) (rabbit anti-GFP; 1:1000; Invitrogen), followed by Cy2- or Cy3-conjugated secondary antibodies (1:300; Jackson ImmunoResearch). The number of positive cells per section (five sections per mouse) was compared between DT- and SAL-injected mice and between DT-injected *Pmch^{DTR/+}* mice and wild-type littermates.

Figure 2. Specificity of *Pmch*^{DTR} allele expression. **A, C.** As revealed by GFP expression (green), the *Pmch*^{DTR} allele was not expressed in neighboring neurons that express hypocretin (Hcrt, red), nor is the pattern of hypocretin staining affected by injection of DT (**B, D**). Scale bar, 250 μ m.

Quantifying neuronal loss. Ten days after receiving either DT or SAL, *Pmch*^{DTR/+} mice were killed for either quantitative RT-PCR or immunohistochemistry. For RT-PCR, mice were cervically dislocated and their hypothalami rapidly dissected and flash frozen. Total RNA was extracted with an RNeasy Mini kit (Qiagen). PCR amplification of *Pmch* was quantified using Brilliant II SYBR Green QRT-PCR 1-step master mix kit on an MX3000P real-time PCR system (Stratagene). The expression of *Pmch* was normalized to *Actb*. For immunohistochemistry, brains were harvested as described in the previous section, and 12 coronal sections from each mouse covering the extent of the LH were costained for MCH and GFP and examined on a Leica SL confocal microscope at the W.M. Keck Center for Advanced Studies in Neural Signaling (Seattle, WA). MCH- and GFP-positive cells were identified visually.

Statistics. All analyses and graphical representations were done using Microsoft Excel and GraphPad Prism. All Student's *t* tests were two tailed and unpaired. Significant isolated comparisons were done using Bonferroni's *post hoc* analyses when applicable. All statistical results are presented in the figures and legends. All error bars indicate SEM.

Results

Characterization of the *Pmch*^{DTR} allele

Unlike humans, mice are normally resistant to DT, because the murine DTR has a very low affinity for DT (Palmiter, 2001). Targeted recombination was used to replace the endogenous coding sequence of MCH with that of the human DTR (*Pmch*^{DTR}; Wu et al., 2012). An internal ribosome entry site allows expression of enhanced GFP as a marker.

The cell bodies of MCH neurons reside throughout the LH and zona incerta. Because robust antibodies against DTR are lacking, GFP expression was used as a surrogate for DTR expression. In the LH of adult heterozygous *Pmch*^{DTR/+} mice, double-label immunohistochemistry with antibodies against MCH and GFP revealed that $89 \pm 1\%$ of MCH-positive cells appeared to express GFP ($n = 4$; Fig. 1A). Among MCH-positive cells, some expressed GFP strongly, whereas others did so weakly, and some appeared not to express GFP. Expression of GFP was specific to MCH neurons; it was not expressed in neighboring hypocretin neurons, and DT did not alter expression of hypocretin (Fig. 2). Ten days after adult heterozygous *Pmch*^{DTR/+} mice received injection of DT (50 ng/g, i.m.), the LH contained only $2 \pm 1\%$ of the

number of MCH neurons counted in mice injected with SAL (Fig. 1A). Most sections from the LH of DT-treated *Pmch*^{DTR/+} mice lacked any MCH-positive cells, whereas similar sections from mice treated with SAL showed >30 MCH-positive cells. Similarly, quantitative RT-PCR on total RNA isolated from hypothalami revealed that injection of DT reduced *Pmch* transcript levels to $1.2 \pm 0.1\%$ that of mice injected with SAL ($n = 4$ per group), in agreement with previous results (Wu et al., 2012). Thus, two independent methods for quantifying the extent of ablation revealed that 98–99% of MCH neurons in *Pmch*^{DTR/+} mice were killed by DT treatment.

Homozygous *Pmch*^{DTR/DTR} mice do not express MCH but show abundant GFP expression in the pattern expected for MCH (Fig. 1B). We refer to these mice as MCH-deficient mutant mice. After injection of DT to adult homozygous *Pmch*^{DTR/DTR} mice, all GFP-expressing neurons in the LH were killed within 10 d (Fig. 1B).

Adult ablation of MCH neurons produces leanness, hyperactivity

Studies of MCH knock-out mice suggest that MCH is haploinsufficient (Shimada et al., 1998). Indeed, heterozygous *Pmch*^{DTR/+} mice had body weights similar to wild-type littermates for at least the first 2 months of life. After DT injection, heterozygous *Pmch*^{DTR/+} mice developed mild leanness over the next 2 weeks (Fig. 3; two-way ANOVA; genotype $\times$ time; $F_{(13,182)} = 5.83$, $p < 0.0001$; $n = 8$ per group). The degree of leanness was similar to that reported for MCH knock-out mice (Jeon et al., 2006). However, food intake was not significantly altered by ablating MCH neurons. During the week before DT injection, heterozygous *Pmch*^{DTR/+} and wild-type mice ate 13.9 ± 0.4 and 14.2 ± 0.4 kcal/d, respectively (mean $\pm$ SEM). During the 2 weeks after DT injection, neuron-ablated mice tended to eat less than controls (14.2 ± 0.4 and 15.1 ± 0.3 kcal/d), but the difference was not significant (two-way ANOVA; genotype $\times$ time, $F_{(1,14)} = 2.34$, $p = 0.148$; genotype, $F_{(1,14)} = 1.54$, $p = 0.235$). Although food intake was initially reported to decrease in MCH knock-out mice on a mixed genetic background (Shimada et al., 1998), these mice on other backgrounds eat normally (Kokkotou et al., 2005).

MCH knock-out mice are more active than wild-type littermates during both *ad libitum* feeding and 24 h fasting (Kokkotou et al., 2005; Willie et al., 2008). Ten days after injection of DT, adult heterozygous *Pmch*^{DTR/+} mice and wild-type littermates showed increased locomotion in their home cages during both fed and fasted conditions (Fig. 4A,B). In both cases, this difference only occurred during the dark period; during the light period, the genotypes were similarly active (Fig. 4C,D; two-way ANOVA; genotype $\times$ period of day; fed, $F_{(1,13)} = 13.82$, $p = 0.0026$; fasted, $F_{(1,13)} = 10.11$, $p = 0.0073$; Bonferroni's *post hoc* analysis; $n = 7$ –8 per group).

MCH knock-out mice spend less time asleep than wild-type littermates (Willie et al., 2008). Several groups have validated a rapid, non-invasive method to characterize temporal patterns of sleep by analyzing locomotor activity (Pack et al., 2007; Kudo et

al., 2011; Fisher et al., 2012). Adopting a similar approach to screen MCH-neuron-ablated mice for changes in their patterns of rest and activity, we recorded locomotion in 10 s bins and scored a bout of zero beam breaks lasting longer than 40 s as an episode of rest. Adult mice lacking MCH neurons spent less time at rest than wild-type littermates, similar to MCH knock-out mice (Fig. 5A; two-way ANOVA; genotype $\times$ period of day; $F_{(1,13)} = 5.48$, $p = 0.036$; Bonferroni's *post hoc* analysis; $n = 7$ –8 per group). The difference occurred entirely during the dark period; during the light period, both genotypes spent similar amounts of time at rest. The decrease in time spent at rest during the dark period was likely attributable to a decreased number of rest bouts, although average bout length also decreased slightly and neither reached statistical significance (Fig. 5C,E). During a 24 h fast, the difference between genotypes in time spent at rest became more pronounced (Fig. 5B; two-way ANOVA; genotype $\times$ period of day; $F_{(1,13)} = 4.94$, $p = 0.045$; Bonferroni's *post hoc* analysis). The decreased time that MCH-neuron-ablated mice spent at rest was attributed to a decreased number of rest bouts; average bout length did not differ between genotypes (Fig. 5D,F; two-way ANOVA; genotype $\times$ period of day; number of bouts, $F_{(1,13)} = 5.93$, $p = 0.030$; Bonferroni's *post hoc* analysis).

Because adult MCH-neuron-ablated mice spent less time at rest, their increased locomotor activity might have been explained merely by increased wakefulness. However, after adjustment for time spent at rest, MCH-neuron-ablated mice still showed an increased rate of activity during the dark period. Rate of activity was calculated by dividing total activity (beam breaks/12 h) by the time spent active during that period (12 h minus time spent at rest). During the dark period, MCH-neuron-ablated mice moved at a rate of 38 ± 7 beam breaks/min active, whereas wild-type mice moved at 22 ± 3 beam breaks/min active (two-way ANOVA; genotype; fed, $F_{(1,26)} = 6.77$, $p = 0.015$; fasted, $F_{(1,26)} = 6.35$, $p = 0.018$; Bonferroni's *post hoc* analysis, $*p < 0.05$ for dark period).

Adult ablation of MCH neurons increases locomotor responses to psychostimulants

The MCH receptor is highly expressed in the shell of the nucleus accumbens, and MCH knock-out mice display signs of hyperdopaminergia, including hypersensitivity to the selective dopamine reuptake blocker GBR 12909 (Pissios et al., 2008). To test psychomotor response to GBR 12909, we first acclimatized mice to locomotor chambers for 3 h before injecting vehicle (water). Locomotion remained consistently low in both MCH-neuron-ablated mice and wild-type littermates with no significant difference between genotypes (Fig. 6A). The next day, after acclimatization, the same mice received GBR 12909 (20 mg/kg, i.p.). This dose increased locomotion in both genotypes compared with the previous injection of vehicle. However, the response of MCH-neuron-ablated mice to GBR 12909 was significantly greater than that of wild-type mice (Fig. 6A; two-way ANOVA,

Figure 3. Body weights after MCH-neuron ablation in adult mice. In the 2 weeks after injection of DT, heterozygous *Pmch^{DTR/+}* mice gained body weight more slowly than wild-type littermates that also received DT ($n = 8$ per group).

Figure 4. Locomotor activity of MCH-neuron-ablated mice when fed and fasted. **A**, Ten days after injection of DT, total activity over 30 min intervals was recorded in the home cage with *ad libitum* access to chow. Data were averaged over 2 consecutive days. **B**, Shortly before the dark period (shaded), chow was removed while activity monitoring continued over the next 24 h of fasting. **C, D**, Activity was totaled for the light and dark periods during *ad libitum* feeding and fasting, respectively. The increased activity of heterozygous *Pmch^{DTR/+}* mice became more pronounced during a 24 h fast but remained confined to the dark period; activity during the light period was similar between genotypes ($***p < 0.001$, $n = 7$ –8 per group).

genotype $\times$ time, GBR 12909, $F_{(36,288)} = 2.87$, $p < 0.0001$, $n = 5$ per group).

MCH-neuron-ablated mice were also tested for locomotor sensitization to repeated cocaine administration. Because this protocol has not been reported for MCH knock-out mice, we simultaneously tested homozygous *Pmch^{DTR/DTR}* littermates. When naive to cocaine, heterozygous *Pmch^{DTR/+}* mice injected with DT 10 d before testing and homozygous *Pmch^{DTR/DTR}* mice injected with SAL were both hypersensitive to the locomotor-increasing effects of cocaine (20 mg/kg, i.p.) compared with wild-type littermates (Fig. 6B; two-way ANOVA; genotype; $F_{(2,11)} = 4.27$, $p = 0.043$; Bonferroni's *post hoc* analysis; $n = 4$ –5 per group). After repeated, daily injection of cocaine, wild-type mice showed a significant increase in locomotion on days 3 and 4, reaching a level equivalent to MCH-deficient mice; neither MCH-neuron-ablated mice nor MCH-deficient mutant mice sensitized to cocaine (Fig. 6C; one-way ANOVA, $F_{(3,9)} = 13.1$, $p = 0.0013$; Bonferroni's *post hoc* analysis, $**p < 0.01$ for comparison with day 1). In their responses to cocaine, MCH-neuron-ablated mice appeared indistinguishable from MCH-deficient mutant mice.

Figure 5. Analysis of time spent at rest in MCH-neuron-ablated mice when fed and fasted. **A**, During the dark period heterozygous *Pmch*^{DTR/+} mice fed *ad libitum* spent less time at rest than wild-type littermates, but during the light period resting times were similar (** $p < 0.01$; $n = 7$ –8 per group). **B**, During a 24 h fast, this difference in activity during the dark period became more pronounced (*** $p < 0.001$). **C**, **E**, During *ad libitum* feeding, this difference in time spent at rest arose from a slight but not significant decrease in both number and length of resting bouts. **D**, **F**, During a 24 h fast, the difference in time spent at rest came from a decreased number of resting bouts, with average bout length unchanged (** $p < 0.01$).

Adult ablation of MCH neurons improves glucose tolerance, even in the absence of MCH signaling

MCH neurons increase their firing rate *in situ* when extracellular glucose increases (Burdakov et al., 2005). Ten days after DT injection, MCH-neuron-ablated mice showed reduced area under the curve (AUC) during a glucose tolerance test (Fig. 7A; Student's *t* test; $p = 0.006$; $n = 8$ per group). In contrast, MCH-deficient mice had normal glucose tolerance at this age (Fig. 7B; Jeon et al., 2006). Hypothesizing that this phenotype of MCH-neuron ablation resulted from the loss of a non-MCH neurotransmitter in MCH neurons, we measured glucose tolerance in homozygous *Pmch*^{DTR/DTR} mice injected with DT. Like heterozygotes, these mice showed improved AUC compared with homozygous littermates injected with SAL (Fig. 7B; one-way ANOVA; $F_{(3,25)} = 6.85$, $p = 0.0016$; Bonferroni's *post hoc* analysis; $n = 6$ –8 per group). Heterozygous *Pmch*^{DTR/+} mice and homozygous *Pmch*^{DTR/DTR} mice had similar AUCs after injection with DT. As a control, wild-type mice injected with DT showed glucose tolerance similar to those injected with SAL (Fig. 7C; Student's *t* test; $p = 0.45$; $n = 7$ per group).

Discussion

Although many laboratories have studied mice lacking MCH or its receptor, we hypothesized that developmental compensation for the absence of MCH in these mice might have attenuated their phenotypes. Therefore, we targeted DTR expression to MCH neurons to produce sudden loss of MCH in adult mice. Our method for killing MCH neurons removed almost all of these

Figure 6. Locomotor responses to psychostimulants. **A**, Ten days after DT injection, mice were injected intraperitoneally with vehicle and monitored for locomotor activity for 3 h. The next day the same mice were injected with the dopamine reuptake inhibitor GBR 12909 (20 mg/kg, i.p.). Data are represented as beam breaks per 5 min interval for 3 h after injection. MCH-neuron-ablated mice responded to GBR 12909 with significantly increased locomotion compared with wild-type mice; during vehicle injection the genotypes responded similarly ($n = 5$ per group). **B**, For 6 consecutive days, a separate cohort of DT-injected mice was injected with SAL (days 1 and 2) and then cocaine (days 3–6, 20 mg/kg, i.p.). The graph presents total locomotor activity (beam breaks) during the hour after injection on each day. After SAL locomotion did not differ between genotypes, but on the first 2 d of cocaine exposure, locomotion was significantly elevated in both MCH-neuron-ablated and MCH knock-out mice (*Pmch*^{DTR/DTR}) compared with wild-type littermates (* $p < 0.05$ for comparison with wild type; $n = 4$ –5 per group). MCH-neuron-ablated mice were not statistically different from MCH knock-out mice. **C**, Relative to their locomotor activity on the first day of cocaine, wild-type mice sensitized to repeated injections (** $p < 0.01$ for comparison to day 1). Neither MCH-neuron-ablated nor MCH knock-out mice sensitized to cocaine.

cells from the LH within 2 weeks. This reduction is greater than one would have expected, because 11% of MCH neurons appeared to lack GFP (and, therefore, lack DTR). However, we know that only a few molecules of DT are needed to kill a cell, so MCH neurons that express just a few DT receptors might not appear to express GFP but could still be susceptible to DT. Also, because GFP is expressed from an internal ribosome entry site following the coding sequence for the DTR, GFP expression might be lower than DTR expression. Regardless, DT treatment

produced nearly complete loss of MCH neurons within 2 weeks. The phenotypes of MCH-neuron-ablated mice did not support our original hypothesis that developmental compensation attenuates the phenotypes of MCH knock-out mice.

Although MCH is often cited as orexi-genic, the role of MCH neurons in modulating food intake remains equivocal. Our study found no effect of MCH-neuron ablation in adult mice on food intake within 2 weeks of DT injection, at a time when MCH neurons are absent histochemically and other phenotypes of MCH-deficiency are clear. Acute food intake increases robustly when MCH is injected intracerebroventricularly (Qu et al., 1996), and the first description of MCH knock-out mice reported hypophagia (Shimada et al., 1998). When these MCH knock-out mice, which were on a mixed 129/Sv × C57BL/6 background, were bred onto pure 129/Sv or C57BL/6 backgrounds, they ate normally (Kokkotou et al., 2005). The mice used in our study were on a C57BL/6 background. Central injection of nesfatin-1 or CART, which are both expressed in MCH neurons, decreases food intake (Lambert et al., 1998; Yang et al., 2005; Oh-I et al., 2006; Stengel et al., 2012). Although these peptides are also expressed in non-MCH neurons throughout the brain, the orexi-genic effect of MCH release from MCH neurons may be countered by anorexi-genic effects from nesfatin-1 and CART. This possibility is consistent with our observation that ablating MCH neurons in adult mice leaves food intake acutely normal.

In times of caloric scarcity, animals must balance wakefulness and activity (e.g., food seeking) against the need to conserve energy. Our results indicate that MCH neurons downregulate activity during a fast primarily by releasing a product of the *Pmch* gene. Previous studies of mice deficient in MCH signaling revealed that knock-out of MCH differs from knock-out of MCHR1; more specifically, MCH knock-out mice sleep less, but MCHR1 knock-out mice sleep more (Adamantidis et al., 2008; Willie et al., 2008). Willie et al. (2008) speculated that the phenotype of MCH knock-out mice reflects loss of neuropeptide-glycine-glutamate and neuropeptide-glutamate-isoleucine (NGE and NEI) in addition to MCH. Ablation of MCH neurons yielded a phenotype similar to knock-out of MCH. Because we targeted the DTR to the promoter for *Pmch*, DT injection ablated neurons that express MCH and NGE and NEI. Therefore, our results confirm the phenotype of MCH knock-out mice, while enhancing the idea that NGE and NEI regulate locomotion and patterns of rest and activity. Furthermore, because ablating MCH neurons altered patterns of locomotor activity to a degree similar to knock-out of MCH, the products of *Pmch* appear to be the key neuromodulators released by MCH neurons to regulate these behaviors.

Figure 7. Glucose tolerance tests. **A**, Ten days after DT injection, heterozygous *Pmch*^{DTR/+} mice showed improved AUC during glucose tolerance test compared with wild-type littermates injected with DT (0-glucose, 2 g/kg, i.p.; ***p* < 0.01; *n* = 8 per group). These data have been replicated in two other cohorts. **B**, A similar effect was seen comparing DT-injected with SAL-injected littermates either heterozygous or homozygous for the *Pmch*^{DTR} allele (0-glucose, 1 g/kg, i.p.; **p* < 0.05, ***p* < 0.01, ^{n.s.}*p* > 0.05; *n* = 6–8 per group). The graph shows data combined from two replicate experiments using cohorts of *Pmch*^{DTR/+}, *Pmch*^{DTR/DTR} littermates. **C**, Wild-type mice injected with DT display glucose tolerance similar to those injected with SAL (*n* = 7 per group).

Because the MCH receptor is highly expressed on medium spiny neurons of the nucleus accumbens shell (Georgescu et al., 2005), many investigations have probed how MCH signaling modulates the mesolimbic dopamine system (Smith et al., 2005, 2008; Tyhon et al., 2006, 2008; Pissios et al., 2008; Chung et al., 2009). Most have used mice lacking MCHR1, but a few have used mice lacking MCH. Our study used mice that lost MCH neurons as adults to examine phenotypes described for MCH knock-out mice. Our results suggest that MCH neurons modulate the mesolimbic dopamine system primarily by releasing products of the *Pmch* gene; other neuromodulators released by MCH neurons contribute little. The highly selective dopamine reuptake blocker GBR 12909 has been reported to induce greater locomotion in MCH knock-out mice compared with wild-type controls (Pissios et al., 2008). We found an effect of similar magnitude in MCH-neuron-ablated mice. Locomotor sensitization from repeated cocaine administration has not been reported for MCH knock-out

mice. We found that both MCH-deficient mutant mice and MCH-neuron-ablated mice were initially hypersensitive to cocaine but did not increase their locomotion during repeated injections, whereas wild-type mice did. It is not clear whether this pattern of response should be interpreted as an increase or a decrease in the functioning of the mesolimbic dopamine system; results from other models of MCH deficiency and other tests of the mesolimbic dopamine system have varied. On amphetamine, MCH knock-out mice sensitize to a dose that leaves wild-type mice unaffected (Pissios et al., 2008). Using MCHR1 knock-out mice, different groups have reported increased sensitivity to amphetamine (Smith et al., 2008) or no difference in sensitivity or conditioned place preference for amphetamine (Tyhon et al., 2008). When given cocaine, MCHR1 knock-out mice have been reported both to have no difference in sensitivity or conditioned place preference (Tyhon et al., 2008) and to have decreased sensitivity and conditioned place preference (Chung et al., 2009). Each of these studies used an independently derived MCHR1 knock-out mouse. Some were maintained on a mixed background of 129Sv × C57BL/6, whereas others were backcrossed to C57BL/6. So, the differing results might be attributed to variances in gene targeting and/or genetic background. Despite these varied results regarding MCH neurons and the mesolimbic dopamine system, the critical observation from our study is that ablation of MCH neurons in adult mice produced an effect indistinguishable from congenital deletion of MCH (and replacement with the DTR). In this regard, the key neuromodulator released by MCH neurons that affects responses to psychostimulants appears to be MCH. However, because the MCH-deficient mutant mice that we used in this study express the DTR from the *Pmch* promoter, we cannot rule out that their phenotypes arose in some part from expression of DTR in addition to deficiency of MCH.

In contrast, we found that MCH neurons regulate blood glucose by releasing neuromodulators not expressed from the *Pmch* gene. Ablation of MCH-deficient MCH neurons (i.e., injection of DT into *Pmch^{DTR/DTR}* mice) produced a result indistinguishable from ablation of MCH-replete MCH neurons. Possible mediators of this effect known to be expressed in MCH neurons are GABA, nesfatin-1, and CART (Elias et al., 2001, 2008; Fort et al., 2008). Although MCH neurons have been ablated previously using a different strategy (Alon and Friedman, 2006), our results are the first to demonstrate a role for these other neurotransmitters in MCH neurons. Glucose tolerance was not tested in the previous study, and neuronal loss with our approach was more rapid and complete.

Ablating MCH neurons in adult mice altered glucose tolerance differently than making MCH neurons congenitally insensitive to glucose. Mice whose MCH neurons express mutated glucose-sensing machinery display worsened glucose tolerance as young adults (Kong et al., 2010). The results of Kong et al. (2010) were surprising, because MCH knock-out mice have normal glucose tolerance at a similar age, and central injection of MCH promotes insulin resistance (Pereira-da-Silva et al., 2005; Jeon et al., 2006). Importantly, Kong et al. (2010) manipulated the entire MCH neuron rather than a single neurotransmitter, and their results suggested that a non-MCH neurotransmitter may be important for MCH-neuron regulation of blood glucose. Our results suggest a similar role for non-MCH neurotransmitters but with a different impact on blood glucose homeostasis. The differences between our two studies may reflect the importance of glucose sensing in MCH neurons during development. Our method for ablating MCH neurons allowed temporal control; in the current study, MCH neurons were ablated in adulthood, and

glucose tolerance was tested within 2 weeks. In the embryo, MCH neurons differentiate early (Risold et al., 2009), and recent evidence suggests that some phenotypes of congenital MCH deficiency arise from alterations during development (Mul et al., 2010; Croizier et al., 2011). In the study by Kong et al., MCH neurons were insensitive to glucose during this time, and glucose tolerance was tested months later, after chronic absence of glucose sensitivity in MCH neurons. Because of the increasing prevalence of childhood obesity and an upsurge in early-onset type 2 diabetes in the United States, the possibility that MCH neurons act during youth to impact blood glucose regulation later in life will be important to address in future studies.

The effect on glucose tolerance that we observed would be considered an improvement from the perspective of diabetes management. The burden of this disease is projected to double or triple over the next several decades (Boyle et al., 2010). Although researchers of glucose homeostasis have studied the interplay of peripheral organs for many years, neuronal populations in the brain have emerged recently as exciting new players in this field (Thorens, 2010). The results of this current study identify an important new player in the neuronal regulation of blood glucose. Understanding how MCH neurons and their other neurotransmitters regulate blood glucose could offer new strategies for diabetes management.

References

- Adamantidis A, Salvert D, Goutagny R, Lakaye B, Gervasoni D, Grisar T, Luppi PH, Fort P (2008) Sleep architecture of the melanin-concentrating hormone receptor 1-knockout mice. *Eur J Neurosci* 27:1793–1800. [CrossRef Medline](#)
- Alon T, Friedman JM (2006) Late-onset leanness in mice with targeted ablation of melanin concentrating hormone neurons. *J Neurosci* 26:389–397. [CrossRef Medline](#)
- Bewick GA, Gardiner JV, Dhillon WS, Kent AS, White NE, Webster Z, Ghatei MA, Bloom SR (2005) Post-embryonic ablation of AgRP neurons in mice leads to a lean, hypophagic phenotype. *FASEB J* 19:1680–1682. [CrossRef Medline](#)
- Bittencourt JC, Presse F, Arias C, Peto C, Vaughan J, Nahon JL, Vale W, Sawchenko PE (1992) The melanin-concentrating hormone system of the rat brain: an immuno- and hybridization histochemical characterization. *J Comp Neurol* 319:218–245. [CrossRef Medline](#)
- Boyle JP, Thompson TJ, Gregg EW, Barker LE, Williamson DF (2010) Projection of the year 2050 burden of diabetes in the US adult population: dynamic modeling of incidence, mortality, and prediabetes prevalence. *Popul Health Metr* 8:29. [CrossRef Medline](#)
- Burdakov D, Gerasimenko O, Verkhatsky A (2005) Physiological changes in glucose differentially modulate the excitability of hypothalamic melanin-concentrating hormone and orexin neurons *in situ*. *J Neurosci* 25:2429–2433. [CrossRef Medline](#)
- Chung S, Hopf FW, Nagasaki H, Li CY, Belluzzi JD, Bonci A, Civelli O (2009) The melanin-concentrating hormone system modulates cocaine reward. *Proc Natl Acad Sci U S A* 106:6772–6777. [CrossRef Medline](#)
- Croizier S, Amiot C, Chen X, Presse F, Nahon JL, Wu JY, Fellmann D, Risold PY (2011) Development of posterior hypothalamic neurons enlightens a switch in the prosencephalic basic plan. *PLoS One* 6:e28574. [CrossRef Medline](#)
- Elias CF, Lee CE, Kelly JF, Ahima RS, Kuhar M, Saper CB, Elmquist JK (2001) Characterization of CART neurons in the rat and human hypothalamus. *J Comp Neurol* 432:1–19. [CrossRef Medline](#)
- Elias CF, Sita LV, Zamboni BK, Oliveira ER, Vasconcelos LA, Bittencourt JC (2008) Melanin-concentrating hormone projections to areas involved in somatomotor responses. *J Chem Neuroanat* 35:188–201. [CrossRef Medline](#)
- Fisher SP, Godinho SI, Potheary CA, Hankins MW, Foster RG, Peirson SN (2012) Rapid assessment of sleep-wake behavior in mice. *J Biol Rhythms* 27:48–58. [CrossRef Medline](#)
- Fort P, Salvert D, Hanriot L, Jegu S, Shimizu H, Hashimoto K, Mori M, Luppi PH (2008) The satiety molecule nesfatin-1 is coexpressed with melanin-

- concentrating hormone in tuberal hypothalamic neurons of the rat. *Neuroscience* 155:174–181. [CrossRef Medline](#)
- Georgescu D, Sears RM, Hommel JD, Barrot M, Bolaños CA, Marsh DJ, Bednarek MA, Bibb JA, Maratos-Flier E, Nestler EJ, DiLeone RJ (2005) The hypothalamic neuropeptide melanin-concentrating hormone acts in the nucleus accumbens to modulate feeding behavior and forced-swim performance. *J Neurosci* 25:2933–2940. [CrossRef Medline](#)
- Gropp E, Shanabrough M, Borok E, Xu AW, Janoschek R, Buch T, Plum L, Balthasar N, Hampel B, Waisman A, Barsh GS, Horvath TL, Brüning JC (2005) Agouti-related peptide-expressing neurons are mandatory for feeding. *Nat Neurosci* 8:1289–1291. [CrossRef Medline](#)
- Hassani OK, Lee MG, Jones BE (2009) Melanin-concentrating hormone neurons discharge in a reciprocal manner to orexin neurons across the sleep-wake cycle. *Proc Natl Acad Sci U S A* 106:2418–2422. [CrossRef Medline](#)
- Hervieu GJ, Cluderay JE, Harrison D, Meakin J, Maycox P, Nasir S, Leslie RA (2000) The distribution of the mRNA and protein products of the melanin-concentrating hormone (MCH) receptor gene, *slc-1*, in the central nervous system of the rat. *Eur J Neurosci* 12:1194–1216. [CrossRef Medline](#)
- Jeon JY, Bradley RL, Kokkotou EG, Marino FE, Wang X, Pissios P, Maratos-Flier E (2006) MCH^{−/−} mice are resistant to aging-associated increases in body weight and insulin resistance. *Diabetes* 55:428–434. [CrossRef Medline](#)
- Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker BI (1983) Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature* 305:321–323. [CrossRef Medline](#)
- Kokkotou EG, Tritos NA, Mastaitis JW, Sliker L, Maratos-Flier E (2001) Melanin-concentrating hormone receptor is a target of leptin action in the mouse brain. *Endocrinology* 142:680–686. [CrossRef Medline](#)
- Kokkotou E, Jeon JY, Wang X, Marino FE, Carlson M, Trombly DJ, Maratos-Flier E (2005) Mice with MCH ablation resist diet-induced obesity through strain-specific mechanisms. *Am J Physiol Regul Integr Comp Physiol* 289:R117–R124. [CrossRef Medline](#)
- Kong D, Vong L, Parton LE, Ye C, Tong Q, Hu X, Choi B, Brüning JC, Lowell BB (2010) Glucose stimulation of hypothalamic MCH neurons involves K(ATP) channels, is modulated by UCP2, and regulates peripheral glucose homeostasis. *Cell Metab* 12:545–552. [CrossRef Medline](#)
- Kudo T, Schroeder A, Loh DH, Kuljis D, Jordan MC, Roos KP, Colwell CS (2011) Dysfunctions in circadian behavior and physiology in mouse models of Huntington's disease. *Exp Neurol* 228:80–90. [CrossRef Medline](#)
- Lambert PD, Couceyro PR, McGirr KM, Dall Vechia SE, Smith Y, Kuhar MJ (1998) CART peptides in the central control of feeding and interactions with neuropeptide Y. *Synapse* 29:293–298. [CrossRef Medline](#)
- Luquet S, Perez FA, Hnasko TS, Palmiter RD (2005) NPY/AgRP neurons are essential for feeding in adult mice but can be ablated in neonates. *Science* 310:683–685. [CrossRef Medline](#)
- Mul JD, Yi CX, van den Berg SA, Ruiter M, Toonen PW, van der Elst MC, Voshol PJ, Ellenbroek BA, Kalsbeek A, la Fleur SE, Cuppen E (2010) Pmch expression during early development is critical for normal energy homeostasis. *Am J Physiol Endocrinol Metab* 298:E477–E488. [CrossRef Medline](#)
- Nahon JL, Presse F, Bittencourt JC, Sawchenko PE, Vale W (1989) The rat melanin-concentrating hormone messenger ribonucleic acid encodes multiple putative neuropeptides coexpressed in the dorsolateral hypothalamus. *Endocrinology* 125:2056–2065. [CrossRef Medline](#)
- Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, Eguchi H, Yamamoto M, Imaki T, Hashimoto K, Tsuchiya T, Monden T, Horiguchi K, Yamada M, Mori M (2006) Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* 443:709–712. [CrossRef Medline](#)
- Pack AI, Galante RJ, Maislin G, Cater J, Metaxas D, Lu S, Zhang L, Von Smith R, Kay T, Lian J, Svenson K, Peters LL (2007) Novel method for high-throughput phenotyping of sleep in mice. *Physiol Genomics* 28:232–238. [CrossRef Medline](#)
- Palmiter R (2001) Interrogation by toxin. *Nat Biotechnol* 19:731–732. [CrossRef Medline](#)
- Pereira-da-Silva M, De Souza CT, Gasparetti AL, Saad MJ, Velloso LA (2005) Melanin-concentrating hormone induces insulin resistance through a mechanism independent of body weight gain. *J Endocrinol* 186:193–201. [CrossRef Medline](#)
- Pissios P, Frank L, Kennedy AR, Porter DR, Marino FE, Liu FF, Pothos EN, Maratos-Flier E (2008) Dysregulation of the mesolimbic dopamine system and reward in MCH^{−/−} mice. *Biol Psychiatry* 64:184–191. [CrossRef Medline](#)
- Presse F, Nahon JL, Fischer WH, Vale W (1990) Structure of the human melanin concentrating hormone mRNA. *Mol Endocrinol* 4:632–637. [CrossRef Medline](#)
- Qu D, Ludwig DS, Gammeltoft S, Piper M, Pellemounter MA, Cullen MJ, Mathes WF, Przypek R, Kanarek R, Maratos-Flier E (1996) A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* 380:243–247. [CrossRef Medline](#)
- Rao Y, Lu M, Ge F, Marsh DJ, Qian S, Wang AH, Picciotto MR, Gao XB (2008) Regulation of synaptic efficacy in hypocretin/orexin-containing neurons by melanin concentrating hormone in the lateral hypothalamus. *J Neurosci* 28:9101–9110. [CrossRef Medline](#)
- Risold PY, Croizier S, Legagneux K, Brischoux F, Fellmann D, Griffond B (2009) The development of the MCH system. *Peptides* 30:1969–1972. [CrossRef Medline](#)
- Shimada M, Tritos NA, Lowell BB, Flier JS, Maratos-Flier E (1998) Mice lacking melanin-concentrating hormone are hypophagic and lean. *Nature* 396:670–674. [CrossRef Medline](#)
- Smith DG, Tzavara ET, Shaw J, Luecke S, Wade M, Davis R, Salhoff C, Nomikos GG, Gehlert DR (2005) Mesolimbic dopamine super-sensitivity in melanin-concentrating hormone-1 receptor-deficient mice. *J Neurosci* 25:914–922. [CrossRef Medline](#)
- Smith DG, Qi H, Svenningsson P, Wade M, Davis RJ, Gehlert DR, Nomikos GG (2008) Behavioral and biochemical responses to d-amphetamine in MCH1 receptor knockout mice. *Synapse* 62:128–136. [CrossRef Medline](#)
- Stengel A, Goebel-Stengel M, Wang L, Kato I, Mori M, Taché Y (2012) Nesfatin-1(30–59) but not the N- and C-terminal fragments, nesfatin-1(1–29) and nesfatin-1(60–82) injected intracerebroventricularly decreases dark phase food intake by increasing inter-meal intervals in mice. *Peptides* 35:143–148. [CrossRef Medline](#)
- Thorens B (2010) Central control of glucose homeostasis: the brain-endocrine pancreas axis. *Diabetes Metab* 36 [Suppl 3]:S45–S49. [CrossRef](#)
- Tybon A, Adamantidis A, Foidart A, Grisar T, Lakaye B, Tirelli E (2006) Mice lacking the melanin-concentrating hormone receptor-1 exhibit an atypical psychomotor susceptibility to cocaine and no conditioned cocaine response. *Behav Brain Res* 173:94–103. [CrossRef Medline](#)
- Tybon A, Lakaye B, Adamantidis A, Tirelli E (2008) Amphetamine- and cocaine-induced conditioned place preference and concomitant psychomotor sensitization in mice with genetically inactivated melanin-concentrating hormone MCH(1) receptor. *Eur J Pharmacol* 599:72–80. [CrossRef Medline](#)
- van den Pol AN, Acuna-Goycolea C, Clark KR, Ghosh PK (2004) Physiological properties of hypothalamic MCH neurons identified with selective expression of reporter gene after recombinant virus infection. *Neuron* 42:635–652. [CrossRef Medline](#)
- Willie JT, Sinton CM, Maratos-Flier E, Yanagisawa M (2008) Abnormal response of melanin-concentrating hormone deficient mice to fasting: hyperactivity and rapid eye movement sleep suppression. *Neuroscience* 156:819–829. [CrossRef Medline](#)
- Wu M, Dumalska I, Morozova E, van den Pol A, Alreja M (2009) Melanin-concentrating hormone directly inhibits GnRH neurons and blocks kiss-peptin activation, linking energy balance to reproduction. *Proc Natl Acad Sci U S A* 106:17217–17222. [CrossRef Medline](#)
- Wu Q, Whiddon BB, Palmiter RD (2012) Ablation of neurons expressing agouti-related protein, but not melanin concentrating hormone, in leptin-deficient mice restores metabolic functions and fertility. *Proc Natl Acad Sci U S A* 109:3155–3160. [CrossRef Medline](#)
- Yang SC, Shieh KR, Li HY (2005) Cocaine- and amphetamine-regulated transcript in the nucleus accumbens participates in the regulation of feeding behavior in rats. *Neuroscience* 133:841–851. [CrossRef Medline](#)