Exploring the Roles of Neurons that Express Melanin-Concentrating Hormone (MCH)

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Abstract

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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 (CART). 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 two weeks of diphtheria toxin (DT) 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. In leptin-deficient obese mice ($Lep^{ob/ob}$), ablation of MCH neurons lowered body weight only slightly, leaving leptin-deficient mice grossly overweight, similar to the effect of knocking out MCH. For these phenotypes, ablation of MCH neurons recapitulated knockout of MCH, so MCH appears to be the critical neuromodulator. In contrast, MCH-neuron-ablated mice showed

improved glucose tolerance when compared to MCH-deficient mutant mice or wild-type mice. I conclude that the important roles of neurons expressing MCH are predominantly achieved by the release of MCH itself. Congenital absence of MCH is neither ameliorated nor exacerbated by development. The roles of non-MCH neurotransmitters released by MCH neurons are likely minor but include modulation of blood glucose homeostasis.

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INTRODUCTION

The quest to understand how the brain controls physiology and behavior began with the study of brain lesions nearly two hundred years ago. Today, although the knowledge of brain function and the tools available for its study have become incredibly complex, inactivating components of the brain in living animals remains essential to the field. Throughout the experiments presented in this dissertation, I use a modern lesioning technique to kill the population of neurons that expresses the neurotransmitter melanin-concentrating hormone (MCH). By applying this technique to living, adult mice, I show how these cells contribute to physiology and behavior.

How can we explore the functioning of the brain?

The earliest brain lesions were crude. In the 1820s, Jean Pierre Flourens, a founder of experimental neuroscience, destroyed cerebral hemispheres, the cerebellum or the brain stem of living rabbits and pigeons. In so doing, he established that distinct regions of the brain control perception, motor coordination and basic life support. Around this time, the physicians Broca and Wernicke independently discovered that their patients who had trouble speaking were missing small but specific regions of their brains. Likewise, the curious case of Phineas Gage suggested that a particular region of the brain shapes personality without affecting other aspects of behavior and physiology. These early studies of brain lesions allowed neuroscientists to ascribe functions to gross anatomical features of the brain. As techniques for visualizing the brain's cytoarchitecture became more sophisticated, neuroanatomists began distinguishing subregions of the brain, and experimentalists developed methods to make lesions smaller and more precise. In the 1950s, a region called the lateral hypothalamus was identified through electrolytic lesions as critical for the intake of food and water (Teitelbaum and Stellar, 1954; Morrison et al., 1958). We now know that this region contains cells important for a wide variety

of behaviors and physiology. In this dissertation, I will present data from a modern lesioning technique that kills a specific population of cells in the lateral hypothalamus.

In the early twentieth century, the pioneering work of Dale and Loewi established that neurons communicate with surrounding cells by releasing chemicals (Valenstein, 2002). Over fifty such neurotransmitters have now been described. Some neurotransmitters, like GABA or glutamate, can be found all over the brain, released by neurons that serve very different functions. Other neurotransmitters, like agouti-related peptide (AgRP) or MCH, are released by clusters of neurons found only in small regions of the brain. Much has been learned in modern neuroscience by distinguishing groups of neurons based on their expression of particular neurotransmitters rather than their anatomical location. For example, in the arcuate nucleus of the hypothalamus two populations of neurons are intermixed that express AgRP and proopiomelanocortin (POMC) that respectively stimulate and inhibit feeding (Luquet et al., 2005; Zhan et al., 2013). In this dissertation, I distinguish a population of neurons based on their expression of the neurotransmitter MCH.

The neuropeptide melanin-concentrating hormone serves many roles

The neuropeptide 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 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). 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-knockout 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-knockout mice become more pronounced during a 24-h fast (Qu et al., 1996; Willie et al., 2008). MCH is also upregulated in leptin-deficient (*Lep*$^{ob/ob}$) mice (Qu et al., 1996).

Such mice (*Lep*$^{ob/ob}$) carry a spontaneous nonsense mutation in the leptin gene, resulting in obesity, hyperphagia, diabetes, and infertility (Friedman, 2002; Coleman, 2010). These phenotypes can be reversed by chronic delivery of recombinant leptin (Chehab et al., 1996; Friedman and Halaas, 1998). The neural circuitry underlying these phenotypes has been the subject of intense investigation (Elmquist et al., 2005; Myers et al., 2009; Israel and Chua, 2010). Genetic experiments have established that the lack of leptin signaling in several different neuronal populations in the hypothalamus and elsewhere in the brain contribute to various aspects of the metabolic phenotype (Elmquist et al., 2005; Israel & Chua, 2010; Myers et al., 2009). When AgRP neurons are ablated in *Lep*$^{ob/ob}$ mice, normal body weight and fertility are restored (Wu et al., 2012). AgRP neurons also express neuropeptide Y, which has the same orexigenic effects as AgRP but by a different mechanism (Cone, 2005). Because hypothalamic NPY-expressing neurons send projections to the vicinity of MCH-expressing neurons (Elias et al., 1998), which have electrophysiological responses to NPY (Van den Pol et al., 2004), the two neuronal populations may be part of the same circuitry mediating the phenotype of *Lep*$^{ob/ob}$ mice. In fact, knockout of MCH in these mice mildly improves their metabolic phenotype (Segal-Lieberman et al., 2003).

The MCHR1 is highly expressed in the nucleus accumbens shell and caudate-putamen (Pissios et al., 2008), and the functioning of the dopamine neural network has been examined in

several MCH-deficient models through several experimental approaches. Conclusions have varied, but MCH-deficient mice display signs of mostly hyperdopaminergia. The highly selective dopamine reuptake blocker GBR 12909 induces greater locomotion in MCH-knockout mice compared to wild-type controls (Pissios et al., 2008). On amphetamine, MCH-knockout mice sensitize to a dose that leaves wild-type mice unaffected (Pissios et al., 2008). Using MCHR1-knockout 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-knockout 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). Some of the differences in these results could be explained by variations in genetic backgrounds or gene targeting. Regardless, it will be valuable to explore how the dopamine system is modulated by the entire MCH neuron.

For a long time the regulation of blood glucose was thought to occur primarily in the periphery. The pancreas releases insulin and glucagon, which act on the liver, skeletal muscle and adipose tissue to modulate glucose storage, release, production and utilization. We now know that the central nervous system regulates all of these peripheral processes (Elmquist et al., 2005), but the identities of the involved neurons are still being discovered.

One class of neurons likely to be involved in the regulation of blood glucose is those that alter their firing properties when blood glucose changes. Such glucosensing occurs within discrete nuclei throughout the brain (Marty et al., 2007). MCH neurons are one such population, increasing their firing rates as extracellular glucose increases (Burdakov et al., 2005). One mechanism by which glucose excites neurons requires an ATP-dependent potassium (K(ATP)) channel (Levin, 2001). Glucose diffuses across the cellular membrane via a glucose transporter. Increasing intracellular glucose drives ATP production. Increasing intracellular

ATP closes the K(ATP) channel, depolarizing the cell. A particular mutation in this channel makes it insensitive to ATP-induced closure. By introducing this mutation specifically into MCH neurons, Kong *et al.* made MCH neurons congenitally insensitive to glucose (Kong et al., 2010). They found that such mice had altered glucose tolerance in adulthood, so glucosensing in MCH neurons is required for normal glucose homeostasis. It remains unclear, however, which neurotransmitters in MCH neurons effect their regulation of blood glucose.

Many of the roles for MCH described above come from studying mice in which the gene for MCH (*Pmch*) was inactivated. An important caveat to manipulations of *Pmch* is that this gene encodes a few other products. The propeptide from which MCH is cleaved also yields neuropeptides glutamate-isoleucine (EI) and glycine-glutamate (GE) (Risold et al., 1992). While the roles of neuropeptide GE have not been explored explicitly, neuropeptide EI may modulate the hypothalamic-pituitary-gonadal axis (Bittencourt and Celis, 2008). Furthermore, two gene products have been described that overlap *Pmch* in different reading frames – MCH-gene-overprinted-polypeptide (MGOP) and antisense-RNA-overlapping-MCH (AROM) (Toumaniantz et al., 1996; Borsu et al., 2000). Sequence analysis of AROM reveals four putative proteins produced through alternative splicing. The largest would likely localize to the nucleus and contains a DNA-binding motif (leucine zipper-like), an RNA-recognition motif, and an SR/SK-rich domain characteristic of spliceosome-associated factors. Recombinant mouse AROM binds *in vitro* to single stranded DNA and RNA, but lacking is direct evidence *in vivo* for the function of AROM proteins.

Additional indirect evidence comes from comparing MCH-knockout mice to MCH-receptor-knockout mice. The MCH-knockout mice that have been reported in the literature likely also lack MGOP and AROM. If these putative peptides or neuropeptides EI and GE played important roles in physiology and behavior, MCH-knockout mice would have a host of phenotypes not shared by mice lacking the MCH receptor. Such is not the case; MCH-knockout

mice are similar to MCH-receptor-knockout mice (Chen et al., 2002). Thus non-MCH products transcribed from *Pmch* (neuropeptides EI and GE) or that overlap *Pmch* (MGOP and AROM) likely play minor roles in physiology and behavior.

Neurons that release MCH may serve roles not revealed by existing genetic models.

In the early 20th century the pioneering work of Loewi and Dale demonstrated that neurons communicate via chemicals called neurotransmitters. For several decades afterwards, many neuroscientists believed that a given neuron releases only one neurotransmitter, referred to as Dale's principle. Interestingly, the first literature reference to a "principle of Dale" was made by Eccles in 1954, but neither Dale nor Eccles believed the single neurotransmitter idea to be true (Eccles et al., 1954). Rather, it was later scientists misinterpreting what Eccles wrote who attributed this idea to Dale (Strata and Harvey, 1999). Regardless of the origins of Dale's principle, evidence against it was well established by the late 1970's (Burnstock, 1976). We now know that many neurons release multiple neurotransmitters, most neurons that release a peptide neurotransmitter also release a fast-acting neurotransmitter, some neurons release multiple fast-acting neurotransmitters, and some even release both inhibitory and excitatory neurotransmitters (Kupfermann, 1991; Jonas, 1998; Noh et al., 2010). It is therefore not surprising that neurons expressing MCH also express GAD67, the major biosynthetic enzyme for GABA (Elias et al., 2008), and two secreted polypeptides – nesfatin and CART (Elias et al., 2001; Fort et al., 2008).

Although nesfatin was first discovered as a secreted peptide in 1994, it was not ascribed a function until 2006, being found in a screen for appetite-regulating molecules (Oh-I et al., 2006). Nesfatin is expressed in the lateral hypothalamus, but also in regions known to lack MCH cell bodies, including the periventricular (PVN), arcuate, supraoptic, and solitary tract

nuclei. Evidence for nesfatin's role in food intake comes from intracerebroventricular (ICV) injection; it decreases food intake. It is also upregulated in response to fasting, but only in the PVN. The receptor for nesfatin remains unknown, but it interacts with a G-protein-coupled receptor to increase intracellular calcium (Brailoiu et al., 2007). Its roles in physiology and behavior are being hotly pursued (Stengel & Taché, 2013). In the lateral hypothalamus, all MCH neurons express nesfatin, and most (85%) of nesfatin neurons express MCH (Fort et al., 2008). One role for nesfatin-MCH cells in this region may be the regulation of paradoxical sleep (also called REM sleep) (Jego et al., 2012; Vas et al., 2013). Nesfatin also modulates stress resilience, the hypothalamic-pituitary-gonadal axis during puberty onset and responses to glucose (García-Galiano et al., 2010; Bonnet et al., 2013; Emmerzaal and Kozicz, 2013). All of these processes are also modulated by MCH, though in several cases the effect of MCH seems to oppose that of nesfatin. Because studies of nesfatin have either injected it throughout the brain or knocked it out in all cells, the role of nesfatin released specifically from MCH neurons remains unclear.

CART was discovered in 1995 as a novel transcript upregulated by cocaine and amphetamine (4-5-fold in the striatum (Douglass et al., 1995)). Additional studies revealed that CART is expressed in the lateral hypothalamic area (LHA) , the arcuate nucleus (Arc), and several other hypothalamic nuclei (Elias et al., 2001). In the lateral hypothalamus, CART is expressed in two-thirds of MCH neurons, but in other nuclei, CART is expressed in neurons that coexpress dynorphin, neurotensin, or thyrotropin-releasing hormone (Elias et al., 2001). Although the receptor for CART remains unknown, CART satisfies the requirements to be a peptide neurotransmitter (Rogge et al., 2008). Many roles for CART have been described, including regulation of food intake and body weight, reward, stress resilience, pituitary hormone release, and anxiety (Rogge, Jones, Hubert, Lin, & Kuhar, 2008). As regards food intake, CART may inhibit food intake (whereas MCH increases food intake), but this may be due to CART neurons in the nucleus accumbens, rather than CART-expressing MCH neurons (Yang et al.,

2005). These CART-MCH neurons may project to regions of the brain distinct from CART-negative MCH neurons (Cvetkovic et al., 2004). As with nesfatin, little evidence exists to clarify what the CART released from MCH neurons may regulate distinct from CART released from other neurons.

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-knockout 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 masked other functions. 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).

RESULTS

The *Pmch*^{DTR} allele and diphtheria toxin methods

To explore the role of the entire MCH neuron in adult mice, we generated mice in which MCH neurons could be rapidly ablated in adulthood by targeting expression of the human DT receptor (DTR) to the *Pmch* locus. Unlike humans, mice are 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 green fluorescent protein (GFP) as a marker (Appendix B, Figure S2, panel A).

The cell bodies of MCH neurons reside throughout the lateral hypothalamus (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±1% of MCH-positive cells appeared to express GFP (n=4, Appendix A Fig. 1A). Among MCH-positive cells, some expressed GFP strongly, while 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 (Appendix A Fig. 2). Ten days after adult heterozygous *Pmch*$^{DTR/+}$ mice received injection of DT (50 ng/g, im), the LH contained only 2±1% of the number of MCH neurons counted in mice injected with saline (Appendix A Fig. 1A). Most sections from the lateral hypothalamus of DT-treated *Pmch*$^{DTR/+}$ mice lacked any MCH-positive cells, while similar sections from mice treated with saline 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±0.1% that of mice injected with saline (n=4/group), in agreement with previous results (Wu et al., 2012).

Thus, two independent methods for quantifying the extent of ablation revealed that 98 to 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 (Appendix A Fig. 1B). We refer to these mice as MCH-deficient mutant mice. Upon injection of DT to adult homozygous *Pmch*$^{DTR/DTR}$ mice, all GFP-expressing neurons in the lateral hypothalamus were killed within 10 d (Appendix A Fig. 1B).

Over the years using DT, the lab has encountered a number of pitfalls with the method. DT is a secreted toxin of *Corynebacterium diphtheria*, from which commercial DT is purified. Some vendors have proven more reliable than others at producing DT that is free of side effects in wild-type mice. We have had good luck with DT from List Biological Laboratories, but problems with that obtained from Sigma-Aldrich. DT comes lyophilized in vials of 1 mg. We resuspend this in normal saline (0.9% sodium chloride) to give a stock solution of 1 mg/mL that we aliquot to avoid repetitive freeze-thaw cycles. Some members of the lab have resuspended in phosphate-buffered saline, but there has been some debate that these suspensions are more liable to make wild-type mice sick. Additionally, there seems to be some variability in biologic activity between different vials (i.e. different resuspensions), even when the vials share the same lot number. After resuspending a new vial of DT, I recommend injecting it into wild-type mice and comparing their body weights to vehicle-injected mice for at least two weeks. For dosing in adult mice, we generally gave two injections spaced one or two days apart of 50 ng DT per gram of body weight on each day. Sometimes a lower dose was needed because of toxicity to wild-type mice. I always confirmed *in vivo* that my chosen dose was effective at killing MCH neurons. As for route of delivery, I sometimes injected intramuscular (IM) and sometimes intraperitoneal (IP). For IM administration, I injected at 1 mL/kg body weight while for IP administration, I injected at 10 mL/kg. During the lab's initial experiences with DT, it was reported that the IP route sometimes produced unexpected deaths of wild-type mice but that the IM route did not

(Luquet et al., 2005). In my experience the two routes produced similar results. Finally, when injecting DT into obese mice, it was necessary to lower the dose per gram of body weight to avoid toxicity. Again, the dose should be determined empirically – the highest dose that did not affect body weight of control mice that do no express the DTR. When possible, the best experimental control is comparing mice expressing the DTR with littermates lacking the DTR after both received DT. For additional details on experimental protocols, see the methods sections from appendices A and B.

Adult ablation of MCH neurons produces leanness, hyperactivity.

Studies of MCH-knockout mice suggest MCH is haplosufficient (Shimada et al., 1998). Indeed, heterozygous *Pmch*$^{DTR/+}$ mice had body weights similar to wild-type littermates for at least the first two months of life. After DT injection, heterozygous *Pmch*$^{DTR/+}$ mice developed mild leanness over the next two weeks (Appendix A Fig. 3; two-way ANOVA; genotype×time; $F_{(13,182)}$=5.83, p<0.0001; n=8/group). The degree of leanness was similar to that reported for MCH-knockout mice (Jeon et al., 2006). Food intake, however, 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/day, respectively (mean±SEM). During the two weeks after DT injection, neuron-ablated mice tended to eat less than controls (14.2±0.4 and 15.1±0.3 kcal/day), but the difference was not significant (two-way ANOVA; genotype×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-knockout mice on a mixed genetic background (Shimada et al., 1998), these mice on other backgrounds eat normally (Kokkotou et al., 2005).

MCH-knockout 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 (Appendix A Fig. 4A-B). In both cases, this difference only occurred during the dark period; during the light period the genotypes were similarly active (Appendix A Fig. 4C-D; two-way ANOVA; gentoype×period of day; fed $F_{(1,13)}$=13.82, p=0.0026; fasted $F_{(1,13)}$=10.11, p=0.0073; Bonferroni *post hoc* analysis; n=7-8/group).

MCH-knockout mice spend less time sleeping 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, I 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-knockout mice (Appendix A Fig. 5A; two-way ANOVA; genotype×period of day; $F_{(1,13)}$=5.48, p=0.036; Bonferroni *post hoc* analysis; n=7-8/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 due to a decreased number of rest bouts, though average bout length also decreased slightly and neither reached statistical significance (Apendix A Fig. 5C,E). During a 24-h fast, the difference between genotypes in time spent at rest became more pronounced (Appendix A Fig. 5B; two-way ANOVA; genotype×period of day; $F_{(1,13)}$=4.94, p=0.045; Bonferroni *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 (Appendix A Fig. 5D,F; two-way ANOVA; genotype×period of day; number of bouts $F_{(1,13)}$=5.93, p=0.030; Bonferroni *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 per min active, while 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).

Young *Lep$^{ob/ob}$* Mice Survive Ablation of MCH Neurons.

To assess the contribution of MCH neurons to the phenotypes of leptin-deficient *Lep$^{ob/ob}$* mice, I bred *Pmch$^{DTR/+}$* mice with mice heterozygous for *Lep^{ob}*. By crossing double heterozygotes with *Pmch$^{DTR/+}$*, I generated *Lep$^{ob/ob}$*; *Pmch$^{DTR/+}$* mice and *Lep$^{ob/+}$*; *Pmch$^{DTR/+}$* controls and treated them with DT or saline. DT treatment did not alter food intake of *Lep$^{ob/ob}$; Pmch$^{DTR/+}$* mice, but it slightly reduced the rate at which male (but not female) *Lep$^{ob/ob}$; Pmch$^{DTR/+}$* mice gained weight (Appendix B Fig. 2A-D). Analysis of the brains from *Lep$^{ob/ob}$*; *Pmch$^{DTR/+}$* mice and the controls at the end of the experiment revealed that, in both cases, >95% of the MCH neurons were gone as measured by immunocytochemistry or quantitative PCR for *Pmch* transcripts (Appendix B Fig. S2). DT treatment of similarly aged control mice lacking the DT receptor had no effect on body weight, food intake or glucose tolerance. Older, more obese (35-45 g body weight) *Lep$^{ob/ob}$*; *Pmch$^{DTR/+}$* mice also survived DT treatment with a modest effect on body weight and improvement in glucose tolerance.

Lep$^{ob/ob}$ mice become diabetic as they gain weight, exhibiting hyperglycemia, hyperinsulinemia, and glucose intolerance (Dubuc, 1976). Ablation of MCH neurons in *Lep$^{ob/ob}$*; *Pmch$^{DTR/+}$* mice improved glucose tolerance in both sexes compared to saline-treated obese

controls (Appendix B Fig. 4C,D). This improvement in glucose tolerance was independent of a decrease in body weight.

The fertility of *Lep*$^{ob/ob}$; *Pmch*$^{DTR/+}$ mice that had been treated with DT was also tested; only 1 of 9 males sired a litter, but none (0/6) of the females became pregnant. Therefore, ablation of MCH neurons did not improve the fertility of *Lep*$^{ob/ob}$; *Pmch*$^{DTR/+}$ mice. Ablation of MCH neurons in *Lep*$^{ob/+}$*; Pmch*$^{DTR/+}$ mice did not disrupt fertility.

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-knockout 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, I first acclimated 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 (Appendix A Fig. 6A). The next day, after acclimatization, the same mice received GBR 12909 (20 mg/kg). This dose increased locomotion in both genotypes compared to 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 (Appendix A Fig. 6A; two-way ANOVA, genotype×time, GBR 12909 $F_{(36,288)}$=2.87, p<0.0001, n=5/group).

MCH-neuron-ablated mice were also tested for locomotor sensitization to repeated cocaine administration. Because this protocol has not been reported for MCH-knockout mice, we simultaneously tested homozygous *Pmch*$^{DTR/DTR}$ littermates. When naïve to cocaine, heterozygous *Pmch*$^{DTR/+}$ mice injected with DT 10 d prior to testing and homozygous *Pmch*$^{DTR/DTR}$ mice injected with saline (SAL) were both hypersensitive to the locomotor-

increasing effects of cocaine (20 mg/kg, ip) compared to wild-type littermates (Appendix A Fig. 6B; two-way ANOVA; genotype; $F_{(2,11)}$=4.27, p=0.043; Bonferroni's *post hoc* analysis; n=4-5/group). Upon repeated, daily injection of cocaine, wild-type mice showed a significant increase in locomotion on the third and fourth days, reaching a level equivalent to MCH-deficient mice; neither MCH-neuron-ablated mice nor MCH-deficient mutant mice sensitized to cocaine (Appendix A Fig. 6C; one-way ANOVA, $F_{(3,9)}$=13.1, p=0.0013; Boferroni's *post hoc* analysis, $^{**}p<0.01$ for comparison to day 1). MCH-neuron-ablated mice appeared indistinguishable from MCH-deficient mutant mice in their responses to cocaine.

Details of method for glucose tolerance testing

Blood glucose is readily altered by stress and by a variety of environmental factors. Consequently, I performed glucose tolerance tests under carefully controlled conditions. All mice being compared were tested on the same day, or pooled from no more than two consecutive days. With practice, it was possible to test 15 mice in one morning. Mice were singly housed for about two weeks in cages with Alpha-dri bedding (Shepherd Specialty Papers). Standard bedding is derived from corn cobs, which contain caloric value, and hungry mice may eat this bedding which could introduce variability into their "fasting" blood glucose. Alpha-dri is made from cellulose and provides no calories.

Mice were maintained on diet 12450B (Research Diets). This diet provides nutrition similar to standard lab chow. Originally, I used this diet because I was determining daily food intakes during the week before glucose tolerance testing. The pellets of D12450B are harder than standard chow and crumble less, allowing more accurate measurement of food intake. With regard to glucose tolerance, I would expect standard lab chow to yield similar results, but standard lab chow may contain unmeasured phytoestrogens that could alter results (Brown and

Setchell, 2001). To keep testing conditions similar, I maintained mice on D12450B for all glucose tolerance testing.

Between 4:00 PM and 6:00 PM on the day before a glucose tolerance test, I removed all food from each cage. The dark period of the light cycle lasted from 6:00 PM to 6:00 AM. In some experiments the mice were housed in a different facility where the dark period was from 7:00 PM to 7:00 AM. I began measuring blood glucose between 10:00 AM and 12:00 PM. The most reliable results seemed to occur when mice were housed and tested in room G619 of the 6th floor animal facility. This room is quiet and lacks both breeding and euthanasia.

To begin, each mouse was weighed and its tail nicked with an 18-g needle. This nick was sufficient to collect a drop of blood applied to a Freestyle Lite blood glucose monitor (Abbott Laboratories). I reported this blood glucose value as time zero. Each mouse was then injected with a pharmaceutical-grade dextrose solution certified to contain 250 mg dextrose (D-glucose) per milliliter (Hospira). This was injected at 4 μL/g body weight, intraperitoneal, to give a dose of 1 mg/kg. Injections were carefully timed to occur once every minute. To measure the injection volume more accurately than the usual disposable syringes, I used a Hamilton syringe of volume 250 μL (with a slip tip for disposable needles). Blood glucose was measured again 15, 30, 60, and 120 min after each mouse's glucose injection. Sometimes a measurement was also taken at 45 min. After the final blood glucose measurement, food was returned.

Adult ablation of MCH neurons improves glucose tolerance, even in 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 (Appendix A Fig 7A; Student's *t*-test; p=0.006; *n*=8/group). In contrast, MCH-deficient mice had normal glucose tolerance at this age

(Appendix A 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, I measured glucose tolerance in homozygous *Pmch*$^{DTR/DTR}$ mice injected with DT. Like heterozygotes, these mice showed improved AUC compared to homozygous littermates injected with SAL (Appendix A Fig. 7B; one-way ANOVA; $F_{(3,25)}$=6.85, p=0.0016; Bonferroni's *post hoc* analysis; n=6-8/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 (Appendix A Fig. 7C; Student's t test; p=0.45; n=7/group).

DISCUSSION

To study the role of the entire MCH neuron, we chose the well-established paradigm of identifying function by removing components. To kill genetically identified cells, one option is to express a toxin from the targeted gene locus. Such an approach results in cell death early in development. It has been demonstrated, however, that loss of some hypothalamic neurons in early life results in attenuated phenotypes compared to the sudden loss of the same neurons in adulthood (Luquet et al., 2005). Furthermore, we hypothesized that developmental changes in response to the absence of MCH signaling in early life might have contributed to the phenotypes of knockout mice lacking MCH or its receptor. During development, MCH is expressed early (Risold et al., 2009), at a time when other neural circuits have yet to be established. MCH could be important for the differentiation and development of other neurons. Alternatively, other neural circuits have the opportunity to compensate developmentally for the absence of MCH. To avoid such compensation, we chose a method for killing MCH neurons that would allow normal expression of the MCH-neuron-phenotype until adulthood.

Our method targeted expression of the DTR to MCH neurons to delay cell death until injection of DT. This method removed almost all MCH neurons from the lateral hypothalamus within two weeks of injection. This reduction is greater than one would have expected, because 11% of MCH neurons appeared to lack GFP (and, therefore, lack DTR). We know, however, 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 two weeks. The phenotypes of MCH-neuron-

ablated mice did not support my original hypothesis that developmental compensation attenuates the phenotypes of MCH-knockout mice.

Food intake, energy balance, activity

Although MCH is often cited as orexigenic, 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 two 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-knockout mice reported hypophagia (Shimada et al., 1998). When these MCH-knockout 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 orexigenic effect of MCH release from MCH neurons may be countered by anorexigenic effects from nesfatin-1 and CART. This possibility is consistent with my observation that ablating MCH neurons in adult mice leaves food intake 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 MCH neurons down-regulate activity during a fast primarily by releasing a product of the *Pmch* gene. Previous studies of mice deficient in MCH signaling revealed that knockout of MCH differs from knockout of MCHR1; more specifically, MCH-knockout mice sleep less, but MCHR1-knockout mice sleep more (Adamantidis et al., 2008; Willie et al., 2008). Willie et al. (2008) speculated that the phenotype of MCH-knockout mice reflects loss of neuropeptides EI and GE in addition to MCH. Ablation of

MCH neurons yielded a phenotype similar to knockout of MCH. Because neuropeptides EI and GE are expressed from the *Pmch* gene and we placed the DTR under control of the *Pmch* promoter, MCH-neuron-ablated mice also lack neurons that express neuropeptides EI and GE. Therefore, our results confirm the phenotype of MCH-knockout mice, while enhancing the idea that neuropeptides EI and GE regulate locomotion and patterns of rest and activity. Furthermore, because ablating MCH neurons altered patterns of locomotor activity to a degree similar to knockout of MCH, the products of *Pmch* appear to be the key neuromodulators released by MCH neurons to regulate these behaviors.

Leptin-deficient mice

Loss of leptin or its receptor results in severe, early-onset obesity associated with hyperphagia, diabetes, hypothermia and infertility in rodents and humans (Farooqi & O'Rahilly, 2009; Jeffrey M Friedman, 2002). When AgRP/NPY neurons are ablated in leptin-deficient $Lep^{ob/ob}$ mice, normal body weight and fertility are restored (Wu et al., 2012). The brain region where MCH neurons reside receives projections from hypothalamic AgRP/NPY-expressing neurons (Elias et al., 1998), and MCH neurons respond electrophysiologically to NPY *in vitro* (Van den Pol et al., 2004). Therefore, we hypothesized that these two neuronal populations may be part of the same circuitry mediating the phenotype of $Lep^{ob/ob}$ mice. If this were true, we would expect that ablation of MCH neurons would dramatically reverse the leptin-deficient phenotype, similar to ablation of AgRP neurons. However, ablation of MCH neurons had no effect on feeding or fertility, but it did slightly improve body weight in male mice and glucose tolerance in both sexes, in agreement with previous results (Segal-Lieberman et al., 2003). Thus, MCH neurons seem to lie outside of the AgRP-neuron circuit that influences leptin's effects on food intake and fertility.

Responses to psychostimulants

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-knockout 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-knockout mice compared to 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-knockout 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 upon 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-knockout mice sensitize to a dose that leaves wild-type mice unaffected (Pissios et al., 2008). Using MCHR1-knockout 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-knockout mice have been reported both to have no difference in sensitivity or conditioned place preferece (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-knockout mouse. Some were

maintained on a mixed background of 129Sv×C57Bl/6, while 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.

Blood glucose homeostasis

In regulating the behaviors discussed above, MCH neurons rely predominantly on MCH. In contrast, I 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 that are 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 previously ablated using a different strategy (Alon and Friedman, 2006), my 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 my 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-knockout 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 an opposite 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 two 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-sensing by MCH neurons. Because of the increasing prevalence of childhood obesity and an upsurge in early-onset type 2 diabetes in America, 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.

Conclusions

In this work, my goals were twofold: first, to clarify how development might influence the roles of MCH itself and, second, to test the roles of non-MCH neurotransmitters that may be released from MCH neurons. To accomplish these goals, I used mice that expressed the human DTR under control of the endogenous *Pmch* gene promoter. By injecting DT into adult mice, I killed MCH neurons abruptly, extensively, and specifically, whereupon I examined phenotypes described for MCH-knockout mice. I also tested the regulation of blood glucose, in which the literature hinted that MCH neurons might play a role independent of MCH. To test whether the loss of MCH explained the phenotypes of MCH-neuron ablated mice, I injected DT into mice homozygous for the $Pmch^{DTR}$ allele, which are null for MCH. Regarding body weight, locomotion, and responses to psychostimulants, I found that MCH-neuron ablation was similar to knockout of MCH. For these phenotypes, I conclude that MCH does not play a special role in development, and development does not compensate for the loss of MCH. In other words, the phenotypes described in MCH-knockout mice reflect accurately the functions that MCH plays during adulthood, and MCH is the most important signaling molecule released by MCH neurons for regulating these phenotypes.

However, for glucose tolerance, MCH-neuron ablation produced a response different from the knockout of MCH. Furthermore, this effect was duplicated by ablating MCH-deficient MCH neurons; the increased clearance of blood glucose after MCH-neuron ablation is not due to the absence of MCH. I conclude that MCH neurons regulate blood glucose by releasing neurotransmitters other than MCH.

FUTURE DIRECTIONS

The concentration of glucose in the blood is tightly regulated and reflects a balance between many peripheral processes, including gluconeogenesis, glycogen synthesis or breakdown, insulin and glucagon secretion, uptake by skeletal muscle, lipolysis and thermogenesis (Marino et al., 2011). These processes are regulated by both branches of the autonomic nervous system under the control of several central nuclei, including the LH, PVH, DMH, VMH, Arc, PBN and the dorsal vagal complex (Marty et al., 2007). In the quest to develop better interventions for controlling blood glucose in diabetes and other pathologies, it will be useful to know greater detail about the circuit through which MCH neurons regulate blood glucose. To which nuclei do they project? Which of their neurotransmitters are important there? Which peripheral processes do they alter? Answering these questions would comprise my future efforts.

What peripheral processes regulating blood glucose do MCH neurons modulate?

To better understand the mechanism of improved glucose tolerance, I would measure blood insulin during a glucose tolerance test (GTT) and blood glucose during an insulin tolerance test (ITT). To test glucose-induced insulin release, blood samples (50 μL) would be larger than previously needed for measuring blood glucose. These would be taken from the tail every 30 min for two hours following a bolus of glucose (IP). If insulin levels do not differ between experimental and control mice during the GTT, then the improvement in glucose tolerance may be due to increased sensitivity to insulin action. If insulin levels during the GTT are higher in the experimental mice, then insulin secretion from the pancreas may be enhanced. Taking 50 μL of blood from the tail stresses mice, which can alter glucose homeostasis. For this reason, I would measure blood glucose as a positive control for the changes in glucose tolerance after MCH-neuron ablation. If the glucose results are not reproducible under these testing conditions, I

would try alternative sampling sites and/or limiting the insulin blood draw to one time point during the GTT.

Sensitivity to insulin could be tested by measuring glucose levels during an ITT, because fasting blood glucose is unchanged by ablation of MCH neurons. If ablation of MCH neurons enhances insulin action, then the experimental mice will have lower blood glucose, particularly during the earlier time points. Because different mouse models and strains have different tolerances to insulin, I would optimize the dose of insulin to lower blood glucose but avoid a ceiling effect.

The results of these experiments could be used to direct future experiments into the peripheral regulation of blood glucose. Such experiments could examine hepatic glucose production and insulin sensitivity (through clamps) or tissue uptake of glucose (through radioactive tracing).

Do MCH neurons modulate glucose homeostasis by releasing GABA?

My results suggest that MCH neurons regulate glucose tolerance by releasing a neurotransmitter other than MCH. Candidates known to be coexpressed in MCH neurons include GABA, nesfatin-1 and CART. Of these three, GABA is understood the best, and the Palmiter lab already possesses mice that would allow elimination of GABA release from MCH neurons.

My approach would rely on Cre-loxP technology. The *Slc32a1*^{lox} allele has loxP sites flanking the second exon of the gene encoding the vesicular GABA transporter (VGAT). In mice homozygous for this allele, neurons expressing Cre recombinase cannot release GABA (Tong et al., 2008). *Pmch-Cre* BAC transgenic mice express Cre recombinase from the *Pmch* promoter/enhancer (Kong et al., 2010). Currently, I am crossing these two lines of mice to produce *Pmch-Cre; Slc32a1*$^{lox/lox}$ mice, which should not be able to release GABA from MCH neurons. Such mice have not yet been described in the literature.

Because mice whose MCH neurons cannot sense glucose from birth have impaired glucose tolerance (Kong et al., 2010), I would predict that *Pmch-Cre; Slc32a1*$^{lox/lox}$ mice have impaired glucose tolerance compared to *Slc32a1*$^{lox/lox}$ littermates that lack Cre recombinase. Because glucose tolerance is influenced by adiposity, mice would be weighed once per week from weaning until ten weeks of age. At this time, mice would be tested for glucose homeostasis as described previously (GTT, insulin measurement, and ITT). Because ablation of MCH neurons produces mild leanness similarly to knockout of MCH, body weight seems likely to remain normal after loss of GABA release from MCH neurons. If glucose tolerance is not changed in *Pmch-Cre; Slc32a1*$^{lox/lox}$ mice, then another of the non-MCH neurotransmitters released by MCH neurons may be regulating blood glucose homeostasis. Future studies to explore these alternative transmitters could use Cre-loxP technology to similarly target deletion of nesfatin and CART.

The line of *Pmch-Cre* mice that I would use has already been characterized (Kong et al., 2010). Expression of Cre recombinase was specific, did not affect glucose tolerance by itself, and did not occur in the germline. I would confirm that *Pmch-Cre* expression is working as expected under my experimental conditions and would screen mice for ectopic Cre activity in tail DNA.

Do MCH-neuron projections to the paraventricular nucleus modulate glucose homeostasis?

MCH neurons project to many nuclei throughout the brain, with subpopulations projecting to different targets (Croizier et al., 2011). Which of these targets is responsible for MCH-neuron modulation of glucose homeostasis remains unknown. The nucleus that I would investigate first is the paraventricular nucleus of the hypothalamus (PVN). Neurons in the PVN project to autonomic preganglionic nuclei and may help regulate blood glucose and insulin concentrations

(Melnick et al., 2011). MCH neurons project to neurons in the PVN that project polysynaptically to the liver and other peripheral targets (Stanley et al., 2010). I hypothesize that MCH neurons regulate blood glucose through their projections to the PVN.

To kill MCH neurons that project to the PVN, I would inject into the PVN of adult *Pmch-Cre* mice a retrogradely transported virus that drives Cre-dependent expression of the human DTR and also expresses a fluorescent marker independently from Cre. I would use 0.5 μL injections of high titer virus that have proven useful for other applications (Darvas et al., 2011). For controls, I would inject Cre-negative littermates with this same virus. After two weeks of recovery, the mice would receive systemic DT injections, as before. Ten days after DT injection, I would test glucose tolerance then confirm the extent of MCH-neuron ablation. Before performing the DT injections, a cohort of *Pmch-Cre* mice would be injected with this virus and then sacrificed to confirm injection coordinates. I would also confirm that DTR expression is restricted to MCH neurons.

I predict that ablation of MCH neurons that project to the PVN will improve glucose tolerance, similarly to ablation of all MCH neurons. If the neurons that project to the PVN do not modulate glucose tolerance, then I would see no change in glucose tolerance between ablated mice and controls. This result would rule out the importance of MCH-neuron projections to the PVN as regulators of blood glucose. Other candidate nuclei include the ventromedial hypothalamus, nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus, and sympathetic premotor areas in the ventral medulla (Zheng et al., 2005). For additional controls, I would repeat the above experiments with one of these alternative candidates (the NTS) and also with the shell of the nucleus accumbens, which is well innervated by MCH neurons but not linked to the regulation of blood glucose.

We do not know whether the same MCH neurons that project to the PVN also project to other nuclei. If some other nucleus is critical for MCH-neuron regulation of glucose tolerance

and the MCH neurons projecting to the PVN also project to this other nucleus, then my strategy will alter glucose tolerance even though the PVN is not the critical target. If I see a change in glucose tolerance in the experiment above, I would do double-label tracer experiments to ask whether MCH neurons projecting to the PVN also project to other candidate nuclei. Understanding the structure of this MCH neurocircuitry would lay the groundwork for the many future studies that would be needed to dissect the function of these widely projecting neurons.

Closing Remarks

I began this dissertation by posing a quest shared broadly by the field of neuroscience – to understand how the brain controls physiology and behavior. All beings are constantly bombarded by an ever-changing external and internal milieu. To survive, an organism must choose appropriately among a variety of possible responses. In multicellular organisms, the most complex coordination of disparate tissues is provided by the brain. Humans are capable of exceedingly complex behaviors driven towards goals whose attainment may be many years away. Of course, with mice as my model, the most perplexing mysteries of the human condition remain unassailable, but plenty of challenge remains in the neural underpinnings of murine behavior and physiology.

With such lofty goals in the back of my mind, I targeted for study a population of neurons based on their shared expression of one peptide neurotransmitter. In an earlier era, I might have chosen instead one region of the brain. The division of the brain into neurotransmitter systems has certainly provided a wealth of knowledge beyond what would have been achievable with anatomy alone. Today, with the explosion of genetic information comprising whole genomes, the neurotransmitter scheme of division has been expanded to include a dizzying array of genetic biomarkers throughout the brain. These markers can be selectively manipulated through amazing transgenic technologies. Neuroscientists are rushing

to target their favorite biomarkers with photo-activated ion channels, designer receptors coupled to G proteins and other technologies to control *in vivo* the activity of neurons. Yet, how well do genes demarcate the functional units of the brain? Will we someday move from genetic divisions to functional ones? Only time will tell. And only with much effort.

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APPENDIX A: ABLATION OF NEURONS EXPRESSING MELANIN-CONCENTRATING HORMONE (MCH) IN ADULT MICE IMPROVES GLUCOSE TOLERANCE INDEPENDENT OF MCH SIGNALING.

Behavioral/Cognitive

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 μl/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 μl/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 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 μm 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.

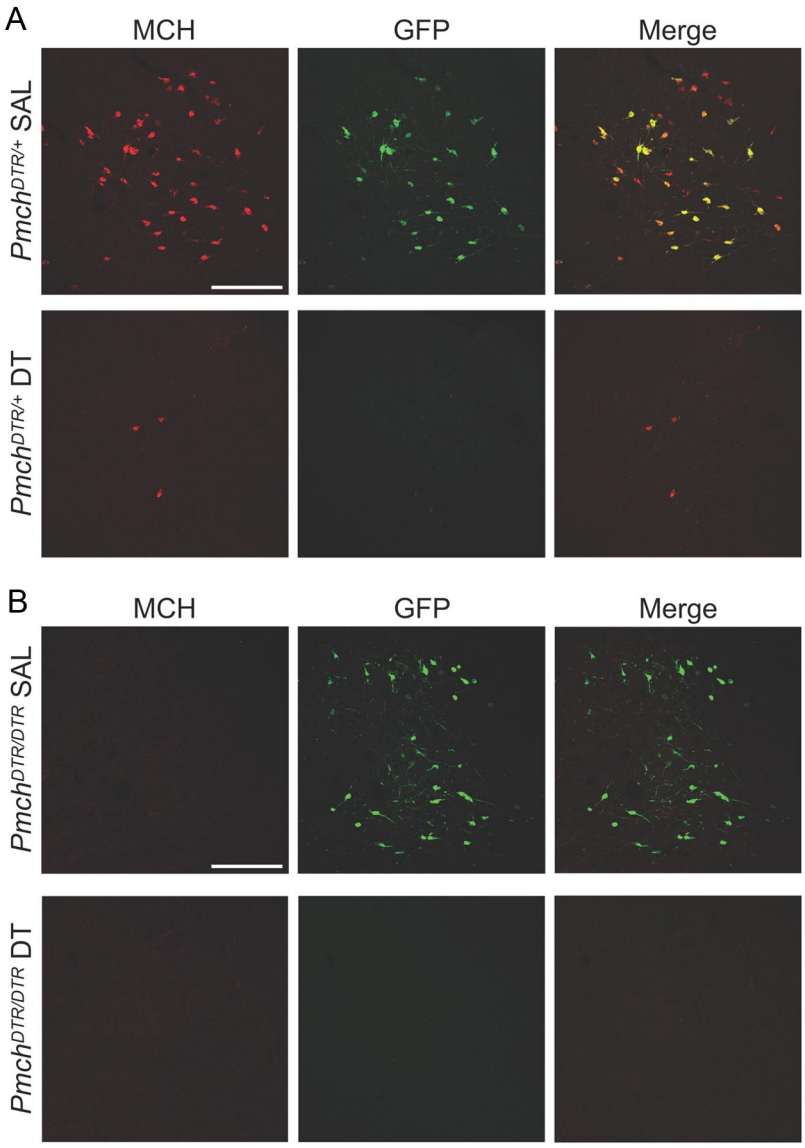

Figure 1. Immunohistochemical characterization of *Pmch*^{DTR} allele expression in the LH. ***A***, As revealed by GFP expression (green), the *Pmch*^{DTR} allele was expressed in most neurons that expressed MCH (red) of heterozygous *Pmch*$^{DTR/+}$ mice injected with SAL (top). After injection with 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 μm.

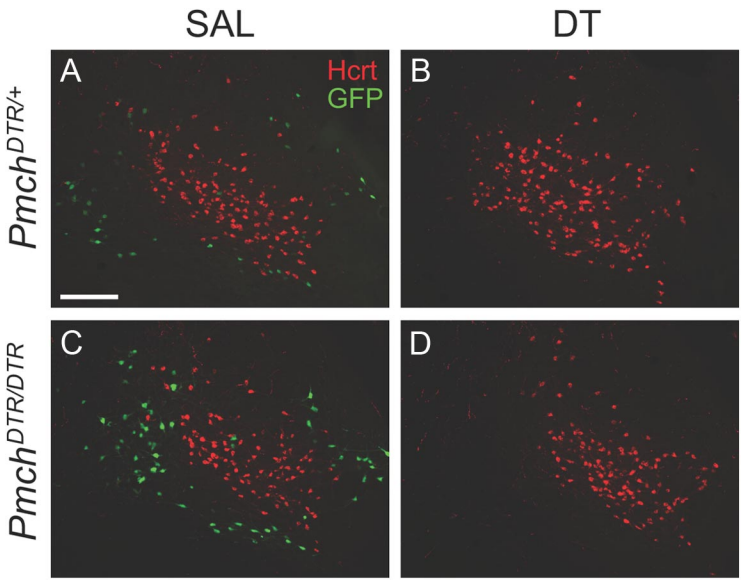

Figure 2. Specificity of *Pmch*^{DTR} allele expression. ***A***, ***C***, As revealed by GFP expression (green), the *Pmch*^{DTR} allele was not expressed in neighboring neurons that express hypocretin (Hcrt, red), nor is the pattern of hypocretin staining affected by injection of DT (***B***, ***D***).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

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 ± 1% of MCH-positive cells appeared to express GFP ($n = 4$; Fig. 1*A*). 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 ± 1% of the number of MCH neurons counted in mice injected with SAL (Fig. 1*A*). 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 ± 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. 1*B*). 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. 1*B*).

Adult ablation of MCH neurons produces leanness, hyperactivity

Studies of MCH knock-out mice suggest that MCH is haplosufficient (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 × 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 ± 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 × 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. 4*A*,*B*). In both cases, this difference only occurred during the dark period; during the light period, the genotypes were similarly active (Fig. 4*C*,*D*; two-way ANOVA; genotype × 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. 5*A*; two-way ANOVA; genotype × 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. 5*C*,*E*). During a 24 h fast, the difference between genotypes in time spent at rest became more pronounced (Fig. 5*B*; two-way ANOVA; genotype × 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. 5*D*,*F*; two-way ANOVA; genotype × 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).

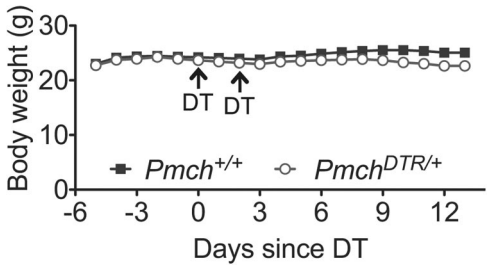

Figure 3. Body weights after MCH-neuron ablation in adult mice. In the 2 weeks after injection of DT, heterozygous $Pmch^{DTR/+}$ mice gained body weight more slowly than wild-type littermates that also received DT ($n = 8$ per group).

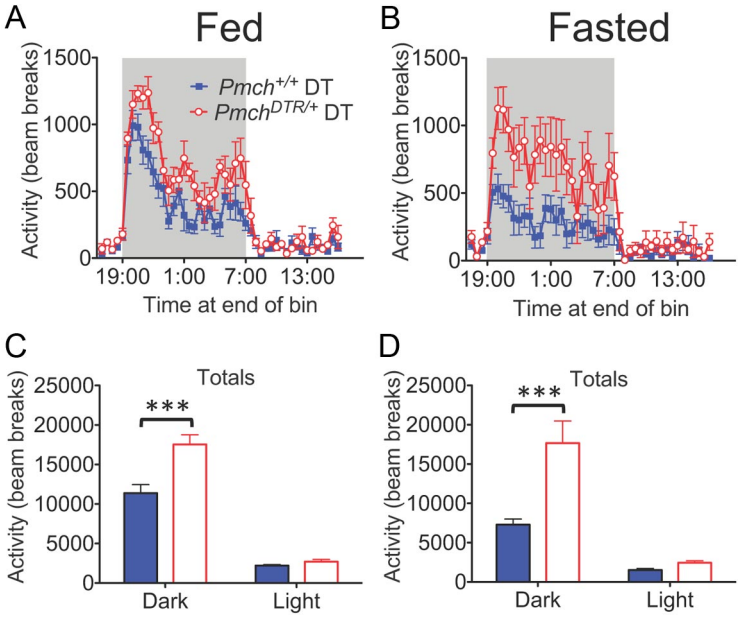

Figure 4. Locomotor activity of MCH-neuron-ablated mice when fed and fasted. ***A***, Ten days after injection of DT, total activity over 30 min intervals was recorded in the home cage with *ad libitum* access to chow. Data were averaged over 2 consecutive days. ***B***, Shortly before the dark period (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).

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. 6*A*). 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. 6*A*; two-way ANOVA, genotype × 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. 6*B*; 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. 6*C*; 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.

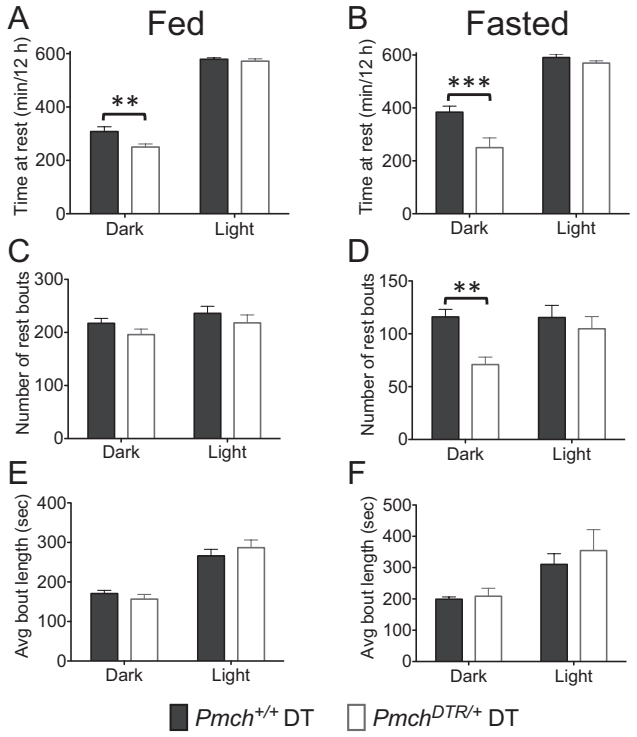

Figure 5. Analysis of time spent at rest in MCH-neuron-ablated mice when fed and fasted. ***A***, During the dark period heterozygous $Pmch^{DTR/+}$ mice fed *ad libitum* spent less time at rest than wild-type littermates, but during the light period resting times were similar (**$p < 0.01$; $n = 7–8$ 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 (**$p < 0.01$).

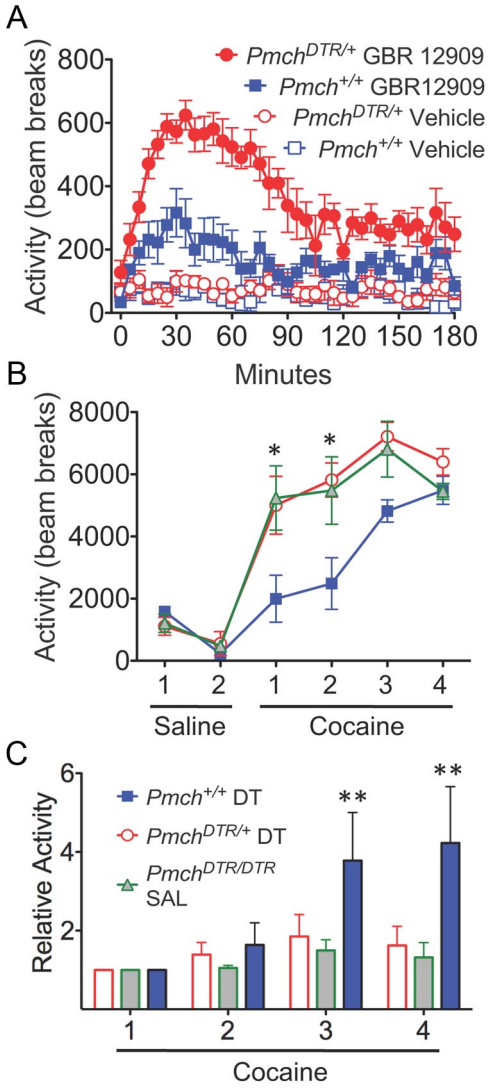

Figure 6. Locomotor responses to psychostimulants. ***A***, Ten days after DT injection, mice were injected intraperitoneally with vehicle and monitored for locomotor activity for 3 h. The next day the same mice were injected with the dopamine reuptake inhibitor GBR 12909 (20 mg/kg, i.p.). Data are represented 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.

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. 7*A*; Student's *t* test; $p = 0.006$; $n = 8$ per group). In contrast, MCH-deficient mice had normal glucose tolerance at this age (Fig. 7*B*; 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. 7*B*; 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. 7*C*; 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 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 orexigenic, 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 orexigenic effect of MCH release from MCH neurons may be countered by anorexigenic 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.

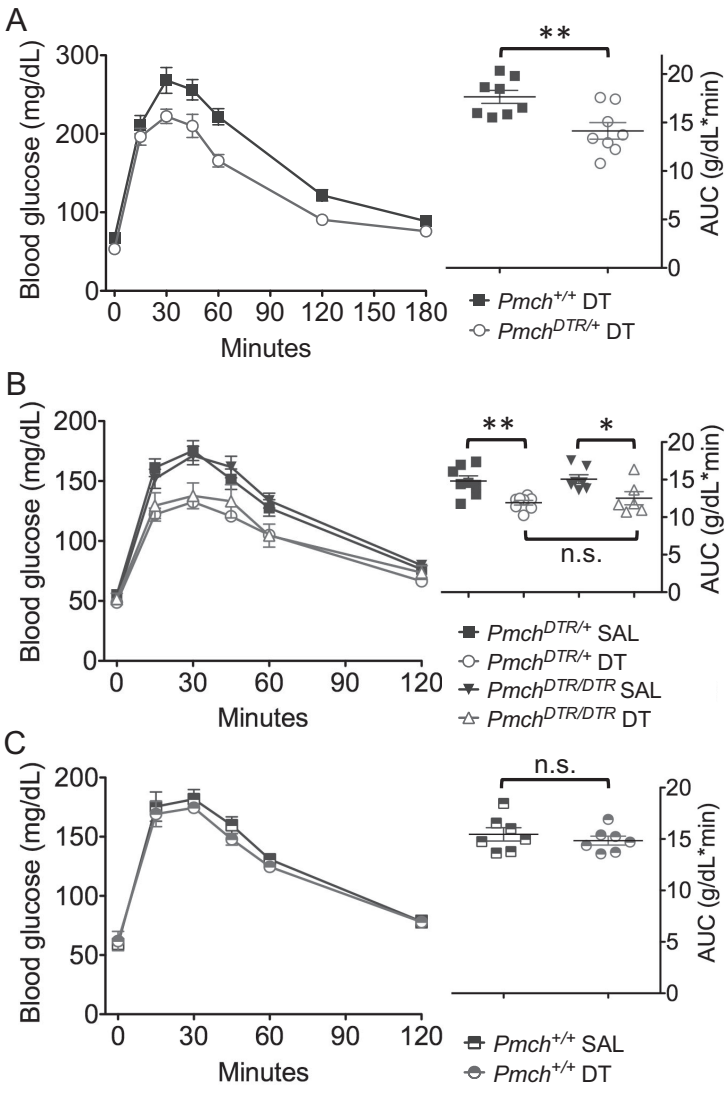

Figure 7. Glucose tolerance tests. ***A***, Ten days after DT injection, heterozygous $Pmch^{DTR/+}$ mice showed improved AUC during glucose tolerance test compared with wild-type littermates injected with DT (D-glucose, 2 g/kg, i.p.; $^{**}p < 0.01$; $n = 8$ per group). These data have been replicated 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 (D-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.

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APPENDIX B: ABLATION OF NEURONS EXPRESSING AGOUTI-RELATED PROTEIN, BUT NOT MELANIN CONCENTRATING HORMONE, IN LEPTIN-DEFICIENT MICE RESTORES METABOLIC FUNCTIONS AND FERTILITY.

Ablation of neurons expressing agouti-related protein, but not melanin concentrating hormone, in leptin-deficient mice restores metabolic functions and fertility

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Contributed by Richard D. Palmiter, December 12, 2011 (sent for review November 15, 2011)

Leptin-deficient ($Lep^{ob/ob}$) mice are obese, diabetic, and infertile. Ablation of neurons that make agouti-related protein (AgRP) in moderately obese adult $Lep^{ob/ob}$ mice caused severe anorexia. The mice stopped eating for 2 wk and then gradually recovered. Their body weight fell to within a normal range for WT mice, at which point food intake and glucose tolerance were restored to that of WT mice. Remarkably, both male and female $Lep^{ob/ob}$ mice became fertile. Ablation of neurons that express melanin-concentrating hormone (MCH) in adult $Lep^{ob/ob}$ mice had no effect on food intake, body weight, or fertility, but resulted in improved glucose tolerance. We conclude that AgRP-expressing neurons play a critical role in mediating the metabolic syndrome and infertility of $Lep^{ob/ob}$ mice, whereas MCH-expressing neurons have only a minor role.

diphtheria toxin | norepinephrine | neuropeptide Y | gonadotropin releasing protein | kisspeptin

The spontaneous nonsense mutation in the leptin gene that generated $Lep^{ob/ob}$ mice results in obesity, hyperphagia, diabetes, and infertility (1, 2). These phenotypes can be reversed by chronic delivery of recombinant leptin (3, 4). The neural circuitry underlying these phenotypes has been the subject of intense investigation (5–7). Mice lacking leptin have elevated levels of neuropeptide Y (NPY) and melanin-concentrating hormone (MCH) in the hypothalamus (8, 9), and genetic experiments have demonstrated that $Lep^{ob/ob}$ mice lacking either of these peptides have an improved metabolic phenotype (10, 11).

Because hypothalamic NPY-expressing neurons send projections to the vicinity of MCH-expressing neurons (12), which have electrophysiological responses to NPY (13), the two neuronal populations may be part of the same circuitry mediating the phenotype of $Lep^{ob/ob}$ mice. NPY-expressing neurons in the arcuate nucleus also express agouti-related protein (AgRP), which exerts the same orexigenic effects as NPY but by a different mechanism (14).

We take advantage of the restricted expression of AgRP and MCH to hypothalamic neurons by targeting the human diphtheria toxin (DT) receptor to the genes encoding those neuropeptides, thereby allowing their selective ablation by administration of DT (15). Ablation of AgRP-expressing neurons (AgRP neurons) in adult mice results in severe anorexia and death in ~6 d (15, 16). In this study, we applied this same methodology to MCH-expressing neurons (MCH neurons) and asked whether ablation of either of these neuronal populations in adult $Lep^{ob/ob}$ mice is beneficial (as predicted from the constitutive loss of the neuropeptides) or lethal (as predicted from the loss of AgRP neurons in a WT background).

Results

Young $Lep^{ob/ob}$ Mice Do Not Survive Ablation of AgRP Neurons. $Lep^{ob/+}$;$Agrp^{DTR/+}$ mice were bred with $Lep^{ob/+}$ mice to generate $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice, and $Lep^{ob/ob}$;$Agrp^{+/+}$ or $Lep^{ob/+}$;$Agrp^{DTR/+}$ controls. After treatment of young male and female mice weighing ~27 g with DT, all of the mice carrying the $Agrp^{DTR}$ allele (whether $Lep^{ob/ob}$ or $Lep^{ob/+}$) gradually stopped feeding and lost 20% of their body weight during the next 6 d and would have succumbed, whereas mice without the $Agrp^{DTR}$ allele maintained their feeding and body weight (Fig. 1). Statistical analyses of our results are reported in the figure legends. Analysis of the brains of DT-treated mice at the end of the experiment revealed a virtual absence of AgRP neurons in $Agrp^{DTR/+}$ mice, as measured by immunocytochemistry or quantitative PCR for *Agrp* transcripts (Fig. S1). We conclude that the absence of leptin does not prevent ablation of AgRP neurons by DT and does not protect these young mice from severe anorexia.

Young $Lep^{ob/ob}$ Mice Survive Ablation of MCH Neurons. Mice with the DT receptor targeted to the *Pmch* locus, which encodes MCH, were produced as described in *Materials and Methods*. Young $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice and $Lep^{ob/+}$;$Pmch^{DTR/+}$ controls were treated with DT or saline. DT treatment did not alter food intake of $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice, but it slightly reduced the rate at which male (but not female) $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice gained weight (Fig. 2). Analysis of the brains from $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice and controls at the end of the experiment revealed that in both groups of mice, >95% of the MCH neurons were gone, as measured by immunocytochemistry or quantitative PCR for *Pmch* transcripts (Fig. S2). DT treatment of similarly aged control mice lacking the DT receptor had no effect on body weight, food intake, or glucose tolerance. Older, more obese (35–45 g body weight) $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice also survived DT treatment, with a modest effect on body weight and improvement in glucose tolerance. This experiment reaffirms that DT-mediated ablation of neurons with the attendant gliosis does not promote anorexia; thus, the effects of AgRP neuron ablation are likely specific to that neuronal population.

Moderately Obese $Lep^{ob/ob}$;$Agrp^{DTR/+}$ Mice Survive AgRP Neuron Ablation. We previously demonstrated that mice can adapt to the loss of AgRP neurons, but this adaptation takes ~10 d (16). Thus, we tested the hypothesis that older, more obese $Lep^{ob/ob}$ mice also might be able to adapt by using their greater energy reserves. Moderately obese (35–40 g body weight) $Lep^{ob/ob}$;$Agrp^{+/+}$ and lean (20–25 g) $Lep^{ob/+}$;$Agrp^{DTR/+}$ controls were treated with DT. The lean control mice gradually stopped eating, lost 20% of their body weight, and became moribund over the subsequent 6 d, in agreement with previous results. However, both male and female $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice weighing 35–40 g ceased eating within 6 d, and did not eat over the next 12 d. During that time, their body weight fell to ~22–25 g. They then began to eat again at a rate similar to that of WT mice, and their body weight gradually increased but remained within the normal range (Fig. 3). The obese mice ($Lep^{ob/ob}$;$Agrp^{+/+}$)

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The authors declare no conflict of interest.

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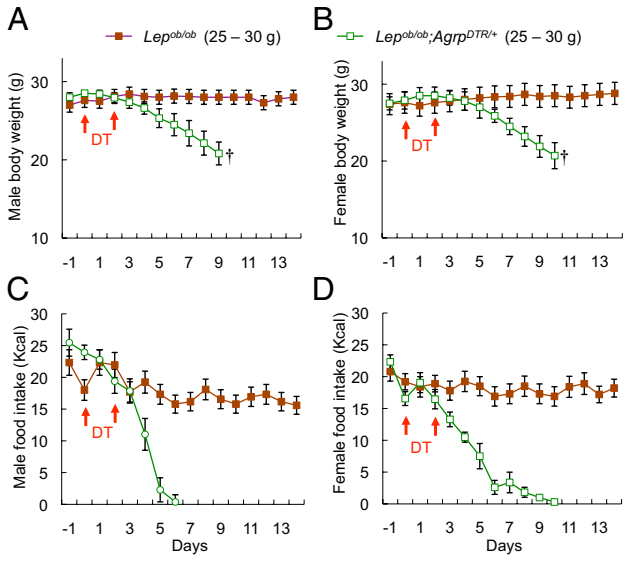

Fig. 1. Ablation of AgRP neurons in young, leptin-deficient mice leads to anorexia. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) $Lep^{ob/ob}$ mice (~6 wk old, 25–30 g) and $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice (~6 wk old, 25–30 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between the two groups (days 5–9). †Mice removed from the study because they became moribund.

that did not express the DT receptor were unaffected by DT administration (Fig. 3).

Improved Glucose Homeostasis After Ablation of AgRP or MCH Neurons. $Lep^{ob/ob}$ mice become diabetic as they gain weight, exhibiting hyperglycemia, hyperinsulinemia, and glucose intolerance (17). By 26 d after ablation of AgRP neurons in female and male $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice weighing 35–40 g, fasting blood glucose levels had returned to normal. Their response to glucose tolerance testing was not statistically different from that of $Lep^{ob/+}$ controls and significantly better than that of saline-treated $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice (Fig. 4 *A* and *B*).

Ablation of MCH neurons in the $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice also improved glucose tolerance in both females and males compared with saline-treated obese controls (Fig. 4 *C* and *D*). This improvement was independent of a decrease in body weight, unlike that occurring after ablation of AgRP neurons.

Restoration of Fertility to $Lep^{ob/ob}$ Mice After Ablation of AgRP but Not MCH Neurons. Male $Lep^{ob/ob}$ mice occasionally sire a litter when they are young, but female $Lep^{ob/ob}$ mice are infertile (3, 6). The fertility of DT-treated mice was tested by placing individual males and females with WT mice of the opposite sex and recording the birth and size of litters. Both male (5/5) and female (6/6) $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice weighing 35–40 g became fertile after ablation of AgRP neurons; some of the males sired several litters, and some females bore more than one litter within 4 mo after DT treatment (Table 1). Based on these data, we calculate that the hypothalamic-pituitary-gonadal axis becomes active within 10–16 d after the first DT injection in female leptin-deficient mice and 11–23 d in male leptin-deficient mice (*Discussion*). Table 1 also shows that the $Lep^{ob/ob}$;$Agrp^{DTR/+}$ males used in these breeding experiments had a mean body weight of 31.4 g, compared with ~55 g in untreated male $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice of the same age. Thus, the ablated mice remained relatively lean for several months.

The fertility of $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice that had been treated with DT was tested as well. Only one of nine males sired a litter, but none of the six females became pregnant. Therefore, ablation of MCH neurons did not improve the fertility of $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice. Ablation of MCH neurons in $Lep^{ob/+}$;$Pmch^{DTR/+}$ controls did not disrupt fertility.

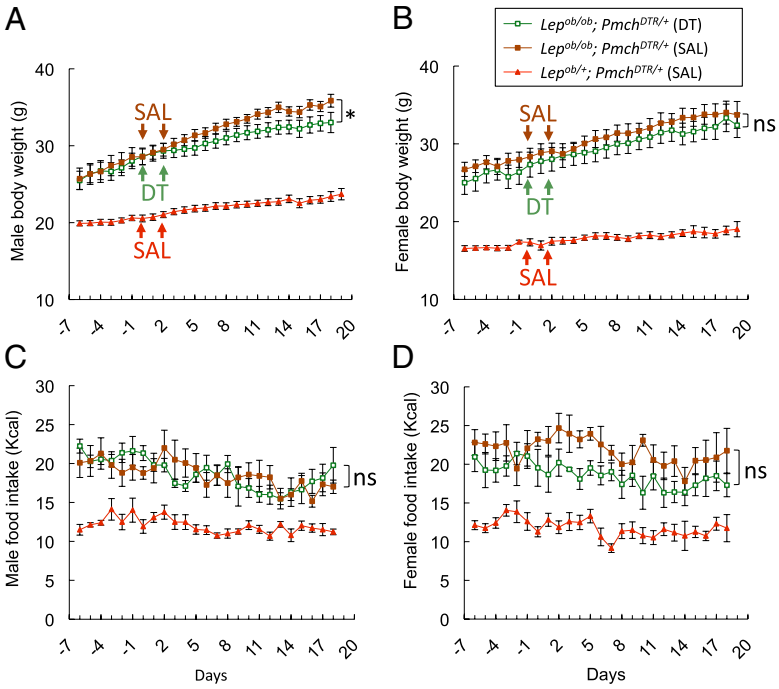

Fig. 2. Body weight and food intake after ablation of MCH neurons in $Lep^{ob/ob}$ mice. (*A* and *B*) Body weight was measured in obese male (*A*) and female (*B*) $Lep^{ob/ob}$; $Pmch^{DTR/+}$ and lean $Lep^{ob/+}$; $Pmch^{DTR/+}$ mice that had been injected with either saline (SAL) or DT on the days indicated by arrows. (*C* and *D*) Daily food intake by the mice described in *A* and *B*. Error bars represent SEM (n = 9–11 per group). *P = 0.03 for the interaction of time and DT treatment in two-way repeated-measures ANOVA. NS, not significant. $P > 0.05$.

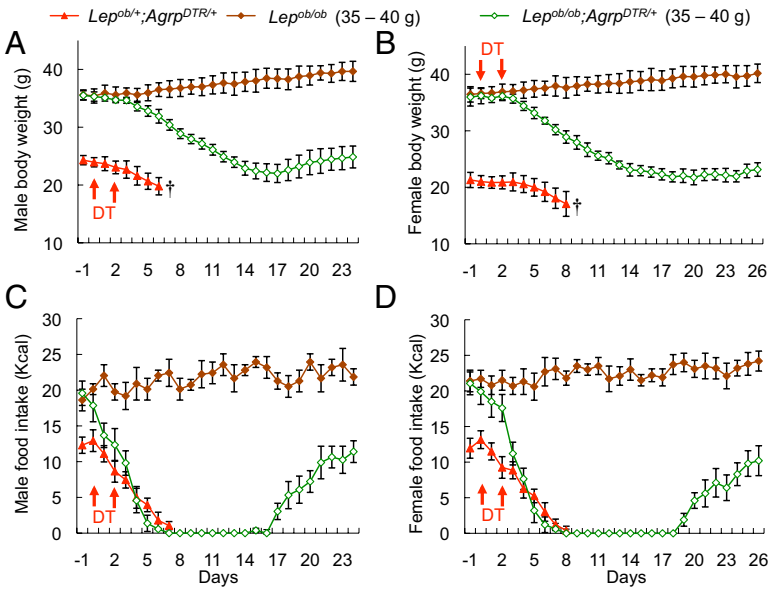

Fig. 3. Moderately obese leptin-deficient mice survived acute ablation of AgRP neurons and exhibited normal body weight with significantly reduced food intake. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) mice from three groups (1) $Lep^{ob/+};Agrp^{DTR/+}$ mice (~8 wk old, 20–25 g); (2) $Lep^{ob/ob}$ mice (~8 wk old, 35–40 g); and (3) $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~8 wk old, 35–40 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between group 2 and group 3 (days 5–24). †Mice removed from study because they became moribund.

Severely Obese $Lep^{ob/ob}$ Mice Do Not Survive Ablation of AgRP Neurons. We also ablated AgRP neurons in $Lep^{ob/ob}$ mice that weighed > 40 g; however, unlike the moderately obese mice, these >40 g mice became moribund despite their greater energy reserves. Like the lighter male and female $Lep^{ob/ob};Agrp^{DTR/+}$ mice, they stopped eating after ~7 d and began to lose weight; however, they appeared to be unhealthy, and thus the experiment was terminated (Fig. 5 *A*–*D*). One distinguishing feature of these older, more obese mice is that their body temperature plummeted to slightly above ambient temperature as they became moribund; in comparison, the body temperature of the $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing <40 g dropped slightly but then returned to that of the $Lep^{ob/ob}$ mice (Fig. S3). $Lep^{ob/ob};Agrp^{DTR/+}$ mice weighing >40 g and that were pair-fed with the group that received DT survived and did not exhibit a drop in body temperature. Thus, the drop in body temperature in the severely obese mice appeared to be a consequence of AgRP neuron ablation, not of reduced food intake.

We suspected that decreased output of the sympathetic nervous system (SNS) might contribute to the mortality of these severely obese mice. Thus, before ablation of AgRP neurons in $Lep^{ob/ob}$; $Agrp^{DTR/+}$ mice weighing ~55 g, we implanted osmotic minipumps to deliver norepinephrine, one of the principle neurotransmitters of the SNS, to potentially promote thermogenesis by brown adipose tissue, stimulate vasoconstriction to conserve heat, and enhance lipolysis (18). This norepinephrine treatment prevented the severe drop in body temperature (Fig. 5*F*) and prolonged the survival of the obese mice after AgRP neuron ablation (Fig. 5*E*) compared with control obese mice that received only the vehicle.

Discussion

Loss of leptin or its receptor results in a severe, early-onset obesity phenotype associated with hyperphagia, diabetes, hypothermia, and infertility in rodents and humans (2, 19). Genetic experiments have established that the lack of leptin signaling in several different neuronal populations in the hypothalamus and elsewhere in the brain contribute to various aspects of the metabolic phenotype (5–7). AgRP neurons express leptin receptors, but their selective removal from AgRP neurons has minimal effects on body weight or metabolism and does not perturb fertility (20). The dramatic reversal of the metabolic phenotype of $Lep^{ob/ob}$ mice and the restoration of fertility described here suggest that these deleterious effects of leptin deficiency are mediated to a large extent by AgRP neurons. $Lep^{ob/ob}$ mice constitutively lacking MCH also have an

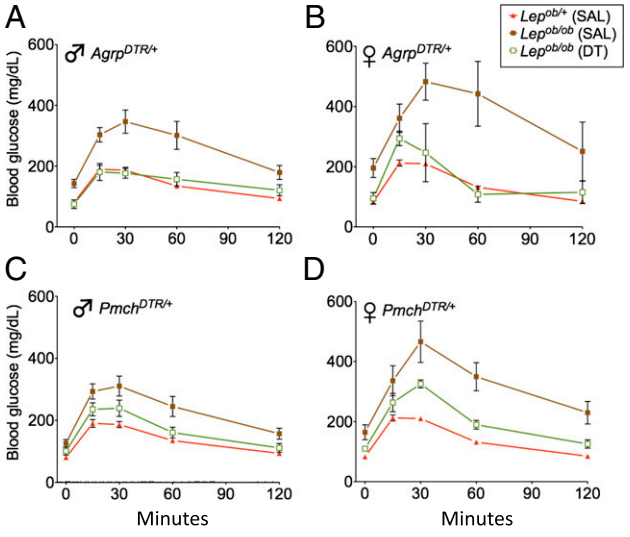

Fig. 4. Glucose tolerance tests after ablation of AgRP or MCH neurons. (*A* and *B*) Obese $Lep^{ob/ob}$ and lean $Lep^{ob/+}$ mice carrying the $Agrp^{DTR}$ allele were injected with either saline (SAL) or DT at 2 wk before glucose tolerance testing. Both males (*A*) and females (*B*) were tested. Error bars represent mean ± SEM (n = 5–11 per group). Area under the curve (AUC) measurements revealed significantly lower values in mice that received DT compared with obese $Lep^{ob/ob}$ controls ($P < 0.002$, one-way ANOVA with Dunnett's posttest), but not significantly greater values than those in leptin-replete, lean mice. (*C* and *D*) Glucose tolerance tests were performed as in *A* and *B*, but with the $Pmch^{DTR}$ allele instead of the $Agrp^{DTR}$ allele present. Error bars represent mean ± SEM (n = 3–8 per group). $P < 0.004$ for AUC analyses.

Table 1. Restoration of fertility in $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice after ablation of AgRP neurons

	DT treatment starts		Mating starts		When first litter born			When second litter born		
Sex	Day	Body weight, g	Day	Body weight, g	Day	Body weight, g	Litter size	Day	Body weight, g	Litter size
Male	1	38.6	43	24.4	78	28.1	5	106	32.8	6
	1	37.4	43	24.7	66	27.5	3	88	30.8	7
	1	35.4	43	26	73	28.5	4	99	32.2	7
	1	35.5	39	22.3	76	25.6	6	103	28.7	7
	1	35.1	39	23.9	66	28.1	6	93	32.5	7
	Average	36.4		24.3	71.8	27.6	4.8	97.8	31.4	6.8
	SD	±1.5		±1.3	±5.6	±1.2	±1.3	±7.3	±1.7	±0.4
Female	1	35.4	39	25.1	64	ND	3	90	ND	5
	1	37.2	39	28.6	68	ND	3	94	ND	5
	1	35.9	39	27.4	70	ND	4	93	ND	6
	Average	36.2		27.0	67.3		3.3	92.3		5.3
	SD	±0.9		±1.8	±3.1		±0.6	±2.1		±0.6

Results shown are for those mice (35–40 g; males and females) where a complete record of body weight and breeding record were obtained. Three additional females were fertile. After a litter was born, the original mates and pups were removed, and fresh mates were provided to score continued fertility for a total of 120 d after DT treatment. One of the males sired a third litter within 120 d.

improved metabolic phenotype (11), similar to that of mice lacking NPY (10). Thus, we anticipated that ablating MCH neurons in adult $Lep^{ob/ob}$ mice also would attenuate the metabolic phenotype and provide evidence that AgRP and MCH neurons could be part of the same circuitry mediating the deleterious effects of leptin deficiency. However, ablation of MCH neurons had no effect on feeding or fertility, but did slightly improve body weight in male mice and glucose tolerance in both sexes, in agreement with previous results (11). Thus, the beneficial effects of ablating AgRP neurons appear to be largely independent of their potential regulation of MCH neurons.

We initially assumed that $Lep^{ob/ob}$ mice weighing 35–40 g survived AgRP neuron ablation because these mice could live off their energy reserves, thereby allowing sufficient time for adaptation to occur. However, $Lep^{ob/ob}$ mice with greater energy reserves (weight >40 g) succumbed. We noticed that the heavier $Lep^{ob/ob}$ mice demonstrated a dramatic drop in body temperature at ~1 wk after ablation that was not observed in the leaner mice. Perhaps the phenotype of $Lep^{ob/ob}$ mice becomes more severe with increasing age or body weight and eventually becomes irreversible. The output of the sympathetic nervous system is important for stimulating lipolysis and regulation of body temperature (21, 22) and deteriorates with leptin deficiency (21). Thus, we hypothesized that the inability to mobilize energy reserves and escape from torpor when AgRP neurons are ablated in older $Lep^{ob/ob}$ mice might account for their morbidity. Consistent with this hypothesis, chronic infusion of norepinephrine during the ablation period prevented the severe loss of body temperature and allowed the more obese $Lep^{ob/ob}$ mice to survive longer. Body temperature was maintained in the obese $Lep^{ob/ob}$ mice that were pair-fed with the AgRP neuron-ablated group and survived, indicating that the loss of AgRP neurons exacerbates the dysregulation of sympathetic nervous system with severe obesity. The loss of AgRP, NPY, and/or GABA signaling by AgRP neurons may lead to excessive activity of postsynaptic cells in the paraventricular nucleus or other upstream neurons that regulate sympathetic output (23, 24).

The restoration of fertility in $Lep^{ob/ob}$ mice after AgRP ablation is remarkable. We focused on females because they are completely infertile, whereas young male $Lep^{ob/ob}$ mice manifest limited fertility, especially on a calorie-restricted diet (3). Leptin replacement (3) or treatment with gonadotropins and progesterone (25) can partially restore fertility to female $Lep^{ob/ob}$ mice. We anticipated seeing some effects of AgRP neuron ablation based on a previous observation of improved glucose regulation and fertility in $Lep^{ob/ob}$ mice lacking NPY (10); however, because NPY is widely expressed, we could not attribute these effects to loss of NPY signaling from AgRP neurons. Diet-induced obesity impairs fertility (26); thus, the loss of body weight induced by AgRP neuron ablation might help restore fertility in $Lep^{ob/ob}$ mice (26). However, other explanations are possible as well, given that fertility can be maintained in obese $Lep^{ob/ob}$ mice without reducing body weight by impairing STAT3 signaling through the leptin receptor (27).

Fertility depends on the pulsatile release of gonadotropin-releasing hormone (GnRH) into the portal system to stimulate follicle-stimulating hormone and luteinizing hormone production and release by the pituitary, resulting in the stimulation of oocyte maturation and ovulation (28). The neuropeptide kisspeptin stimulates GnRH release (29), and loss of kisspeptin signaling impairs fertility (30; but see ref. 31). Infertility of $Lep^{ob/ob}$ mice is associated with low expression of kisspeptins, low circulating gonadotropins, and dysregulation of GnRH release, but leptin does not appear to act directly on the neurons that produce kisspeptin or GnRH (32–34). Thus, the ability of leptin to hasten puberty and restore fertility to $Lep^{ob/ob}$ mice might be mediated by its actions on neurons that regulate kisspeptin- and/or GnRH-releasing neurons. AgRP neurons are not necessary for fertility (35); nevertheless, their hyperactivity might prevent fertility, as occurs in $Lep^{ob/ob}$ mice (36–38). Elevated release of AgRP, NPY, and/or GABA by AgRP neurons may be responsible for suppression of the gonadotropic axis (10, 16).

Murine gestation takes ~20 d, and it takes at least 14 d for folliculogenesis to give rise to oocytes ready to ovulate (39); therefore, adequate gonadotropin production in females probably commences ~34 d earlier than the birth of pups. Production of mature sperm starting from spermatagonial stem cells takes ~35 d (40); thus, adequate gonadotropin production in males probably occurs ~55 d before the birth of pups. Therefore, we estimate that hypothalamic-pituitary-gonadal axis activation occurs within 10–16 d after the first DT injection in female leptin-deficient mice and within 11–23 d in male leptin-deficient mice.

Ablation of AgRP neurons in adult mice leads to severe anorexia, resulting in starvation within 1 wk (15). Our previous experiments demonstrated that this lethal phenotype results from the loss of GABA signaling in the parabrachial nucleus and resulting hyperactivity of neurons in this brain region (16), rather than from the resulting activation of melanocortin signaling (41). Adult mice can adapt to the loss of AgRP neurons and resume feeding if the hyperactivity within the parabrachial nucleus is prevented either pharmacologically or genetically during a critical period of ~10 d (16). Ablation of AgRP neurons before they become functionally mature also allows adaptation (15). Here we demonstrate another paradigm that allows adaptation. Mice with sufficient energy reserves and the ability to mobilize these reserves can survive on

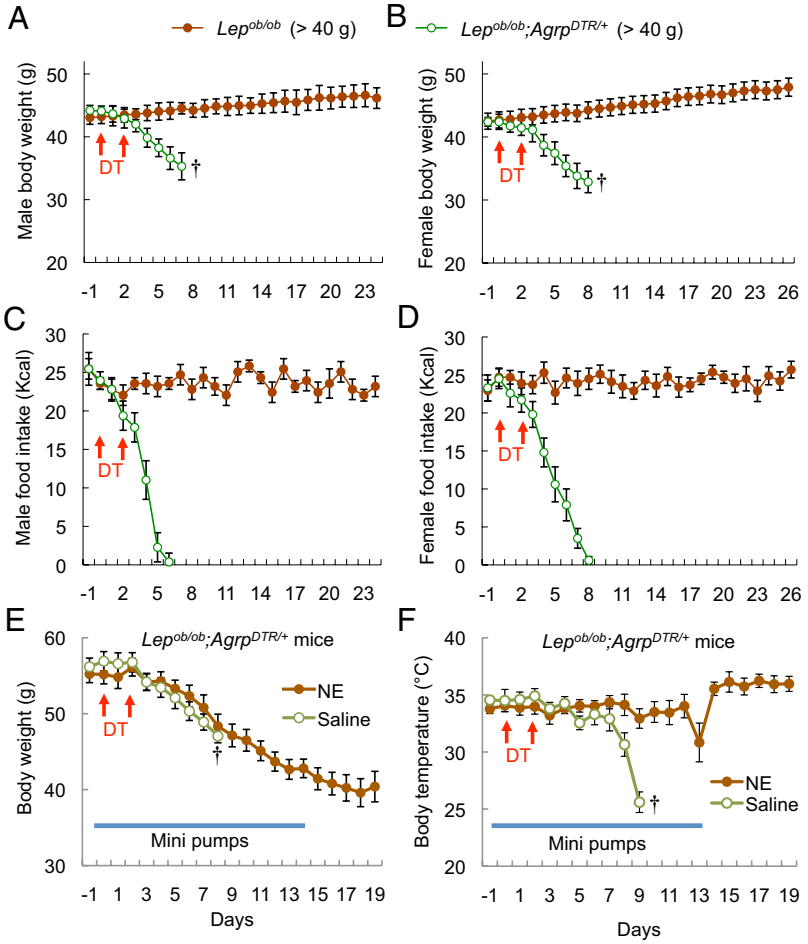

Fig. 5. Severely obese leptin-deficient mice do not survive ablation of AgRP neurons due to hypothermia that can be prevented by chronic treatment with norepinephrine. (*A* and *B*) Body weight of DT-treated male (*A*) and female (*B*) $Lep^{ob/ob}$ mice (~10 wk old, >40 g) and $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice (~10 wk old, >40 g). (*C* and *D*) Daily food intake by the mice described in *A* and *B*. All mice were raised on standard chow pellets. Error bars represent SEM. $P < 0.01$, ANOVA on body weight and food intake between the two groups (days 4–8). (*E*) Body weight of DT-treated $Lep^{ob/ob}$;$Agrp^{DTR/+}$ mice (~14 wk old, ~55 g) subjected to chronic administration of norepinephrine (1.5 mg/kg/d) through a 14-d osmotic pump implanted s.c. (*F*) Body temperature of the mice described in *E*. †Mice removed from the study because they became moribund.

them while the adaptation process occurs. Because AgRP neurons are hyperactive in $Lep^{ob/ob}$ mice, we suspect that loss of GABA signaling after their ablation also leads to hyperactivity within the parabrachial nucleus, but the mice can endure the complete anorexia of 12-d duration by mobilizing their adipose reserves. After the $Lep^{ob/ob}$ mice adapt to the loss of AgRP neurons, their phenotype resembles that of normal mice, as reflected by their feeding, body weight, glucose metabolism, and fertility.

Materials and Methods

Animals and DT Treatment. All animal experiments were approved by the University of Washington's Animal Care and Use Committee. $Lep^{ob/+}$ mice on a C57BL/6 background were obtained from Jackson Laboratories. $Agrp^{DTR}$ mice were produced and bred onto a C57BL/6 genetic background as described previously (15). $Pmch^{DTR}$ mice were generated by PCR amplifying a 360-bp region 5′ of the initiation codon using primers that introduced a unique Kpn1 site at the initiation codon. This fragment was cloned into a targeting vector that contained *PgkDTa* and *HSV-TK* genes for negative selection and *frt*-flanked *SvNeo* for positive selection. The 5′ arm was extended to ~9 kb by insertion of an *XbaI* fragment, after which a 4-kb 3′ arm was added. After linearization, the targeting construct was electroporated into G4 ES cells. Two positive colonies (out of 44) were identified by Southern blot analysis of ES cell DNA cut with *Kpn1* using a probe just outside of the 5′ arm. One clone that was injected in C57BL/6 hosts gave germline transmission. These mice were bred with FLper mice to remove the *frt*-flanked *SvNeo* gene. Mice carrying the $Pmch^{DTR}$ allele were subsequently identified by PCR using one primer in *Pmch* promoter and one primer in *hDTR*. These mice were backcrossed to C57BL/6 mice for more than six generations before being bred with $Lep^{ob/+}$ mice. $Lep^{ob/ob}$;$Agrp^{DTR/+}$ and $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice were generated by double-heterozygote crosses.

Before behavioral experiments, mice were maintained on standard laboratory chow (5053; Lab Diet) that was available ad libitum. When the $Lep^{ob/ob}$;$Agrp^{DTR/+}$ and $Lep^{ob/ob}$;$Pmch^{DTR/+}$ mice were ~6 wk old (weighing 20–25 g), ~8 wk old (weighing 35–40 g), or ~10 wk old (weighing 40–45 g), they were individually housed and switched to another standard chow diet (D12450B; Research Diets) for 7 d before being injected with DT. To ablate AgRP or MCH neurons, DT was injected twice per mouse (i.m., 2 d apart; List Biologicals). Body weight and food intake were recorded daily for several weeks. The DT dose was determined based on body weight at the time of injection, with 50 μg/kg for mice weighing <40 g and 40 μg/kg for mice weighing >40 g. For some experiments, temperature sensor chips (IPTT-300; Bio Medic Data Systems) were inserted s.c. to allow daily recording of body temperature. Experiments were terminated if the body weight of leptin-deficient mice fell to 80% of that of age-matched $Lep^{ob/+}$ littermates or if the leptin-deficient mice appeared moribund.

Fertility was assessed by housing each male DT-treated mouse with two C57BL/6 females (2 mo old) and each DT-treated female with a WT C57BL/6 male (2 mo old). The dates on which litters were born, litter sizes, and body weights of the parents were recorded. Original C57BL/6 mates and litters were removed before fresh mates were provided to score continued fertility.

Minipump Delivery of Norepinephrine. At 3 d before DT administration, osmotic pumps (Alzet model 1002; Durect) were loaded with norepinephrine (30 mM; Sigma-Aldrich) and ascorbate (2%; Sigma-Aldrich) to minimize oxidation and implanted under the skin along with a temperature-monitoring chip (IPTT-300;

Bio Medic Data Systems). Norepinephrine was delivered continuously at a rate of 1.5 mg/kg/day for 14 d until the pumps were depleted.

Glucose Tolerance Tests. At various times before and after DT treatment, glucose tolerance testing was performed by fasting mice overnight for 16 h, then sampling blood from the tail vein at 0, 15, 30, 60, and 120 min after the i.p. injection of D-glucose (1 g/kg). Blood glucose was measured with a FreeStyle Lite glucometer (Abbott Laboratories).

Determining the Extent of AgRP or MCH Neuron Ablation. At the end of the experiment, mice were perfused transcardially with 4% paraformaldehyde and brains were collected to evaluate the extent of AgRP or MCH neuron ablation by one of two methods. In one method, frozen sections (25 μm thick) containing appropriate hypothalamic regions were immunostained with antibodies against AgRP (rabbit anti-AgRP, 1:2,000 dilution; Phoenix Pharmaceuticals), NPY (rabbit anti-NPY, 1:1,000 dilution; Peninsula Laboratories), or MCH (rabbit anti-MCH, 1:1,000 dilution; Phoenix Pharmaceuticals), followed by Cy2- or Cy3-conjugated secondary antibodies (1:300 dilution; Jackson ImmunoResearch). Then the number of positive cells per section (five sections per mouse) was compared with that in control mice. Alternatively, the hypothalamus was dissected from fixed brain, RNA was isolated using a High Pure FFPE RNA Micro Kit (Roche) and reverse-transcribed with a Brilliant II One-Step SYBR Green QRT-PCR Kit (Stratagene) according to the manufacturer's protocol, and quantitative real-time PCR (Mx3000P; Stratagene) was performed using primers specific for *Agrp* or *Pmch* mRNA, with *Actb* as an internal control. The abundance of *Agrp* or *Pmch* mRNA was calculated relative to that of *Actb* and compared with that in untreated control mice.

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Supporting Information

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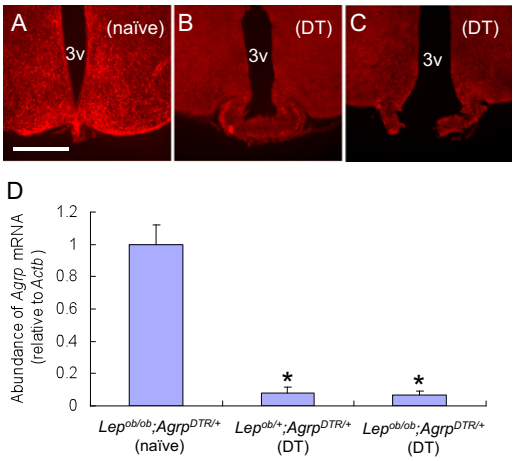

Fig. S1. Agouti-related protein (AgRP)-expressing neurons in adult leptin-deficient mice carrying the $Agrp^{DTR}$ allele are ablated by diphtheria toxin (DT) treatment. (*A–C*) Representative pictures of anti-AgRP immunostaining in the arcuate nucleus (ARC) of naive $Lep^{ob/ob};Agrp^{DTR/+}$ mice without DT treatment. (Scale bar: 400 μm for *A–C*.) (*B*) Representative pictures of anti-AgRP immunostaining in the ARC of $Lep^{ob/+};Agrp^{DTR/+}$ mice injected with DT (50 ng/g body weight, two i.m. injections given 2 d apart). (*C*) Representative pictures of anti-AgRP immunostaining in the ARC of $Lep^{ob/ob};Agrp^{DTR/+}$ mice treated with DT. (*D*) Quantification of the *Agrp* transcript levels in the ARC of mice as described in *A–C*. The relative abundance of *Agrp* mRNA was measured and normalized to that of the *Actb* gene (encoding β-actin) by quantitative RT-PCR. $n = 4–6$ per group; $^*P < 0.01$, ANOVA.

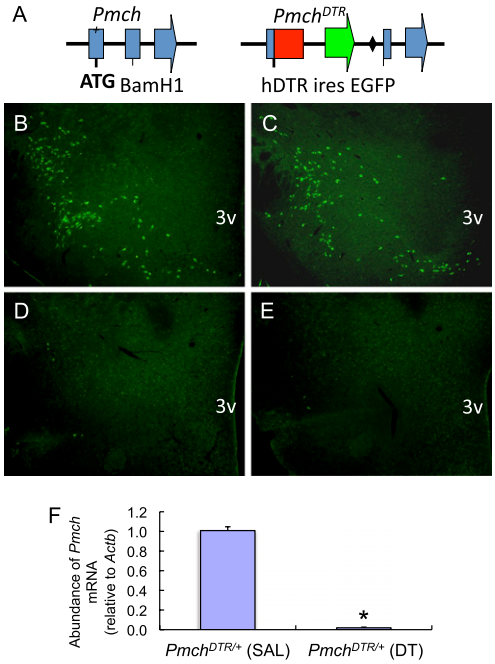

Fig. S2. Ablation of melanin-concentrating hormone (MCH)-espressing neurons after DT injection. (*A*) The $Pmch^{DTR}$ allele (*Right*) expresses the human DT receptor (hDTR) from the *Pmch* promoter, followed by an internal ribosome entry site (ires) and EGFP. These transgenes replace parts of the first and second exons of the WT *Pmch* allele (*Left*) between the initiation codon, ATG, and a BamH1 site. (*B–E*) Injection of DT (top of panels), but not vehicle (saline; bottom of panels), into adult mice carrying the $Pmch^{DTR}$ allele abolishes anti-MCH immunostaining in the lateral hypothalamus. The mice in *B* and *D* are leptin-replete ($Lep^{+/+}$), whereas those in *C* and *E* are leptin-deficient ($Lep^{ob/ob}$). 3v, third ventricle. (*F*) Quantification of *Pmch* transcript levels between $Pmch^{DTR/+}$ mice receiving either DT or vehicle [saline (SAL)]. Error bars represent mean ± SEM ($n = 3$ per group). $^*P < 0.0001$, unpaired *t* test.

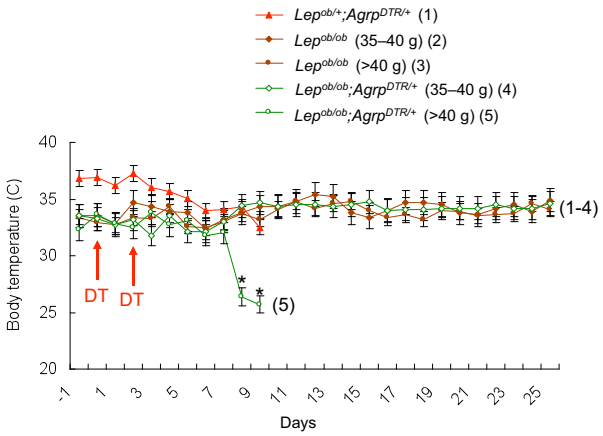

Fig. S3. Ablation of AgRP neurons leads to severe hypothermia in 10-wk-old, but not younger, leptin-deficient mice. Body temperature was recorded daily (6:00 PM) in DT-treated male mice from five groups: (1) $Lep^{ob/+};Agrp^{DTR/+}$ mice (~8 wk old, 20–25 g); (2) $Lep^{ob/ob}$ mice (~8 wk old, 35–40 g); (3) $Lep^{ob/ob}$ mice (~10 wk old, >40 g); (4) $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~8 wk old, 35–40 g); and (5) $Lep^{ob/ob};Agrp^{DTR/+}$ mice (~10 wk old, >40 g). *$P < 0.01$, ANOVA between groups 3 and 5; n = 6–8 per group.