Melanocortin 4 receptors in autonomic neurons regulate thermogenesis and glyceremia

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Whether melanocortin 4 receptors (MC4Rs) in extra-hypothalamic neurons, including cholinergic autonomic pre-ganglionic neurons, are required to control energy and glucose homeostasis is unclear. We found that MC4Rs in sympathetic, but not parasympathetic, pre-ganglionic neurons required to regulate energy expenditure and body weight, including thermogenic responses to diet and cold exposure and ‘beiging’ of white adipose tissue. Deletion of Mc4r genes in both sympathetic and parasympathetic cholinergic neurons impaired glucose homeostasis.

Distinct subsets of MC4Rs in the CNS exert segregated effects on the tissues and processes underlying energy balance, glucose homeostasis and cardiovascular function1–5. MC4Rs expressed outside the hypothalamus in pre-ganglionic cholinergic neurons of the sympathetic and parasympathetic nervous system are potential regulatory sites3–5 and may be crucial for energy and glucose homeostasis. Data6–9 further suggest that MC4Rs in sympathetic pre-ganglionic neurons may be required to stimulate thermogenesis in brown and white adipose tissue depots (BAT and WAT, respectively). The thermogenic properties of BAT and WAT are areas of interest to treat obesity and diabetes. However, the pathway(s) that govern these processes are not fully understood.

To study the requirement(s) for MC4Rs in the autonomic nervous system (ANS) for modulating energy and glucose homeostasis, including role(s) of BAT and WAT depots, we generated mice that lack Mc4r in pre-ganglionic autonomic neurons. We used Mc4rloxP/loxP mice4 and mice expressing Cre recombinase (cre) in choline acetyltransferase–positive neurons (Chat-cre)3 to target sympathetic and parasympathetic pre-ganglionic neurons. We used mice lacking Mc4r in Phox2B-positive neurons10 to assess MC4R function in autonomic control neurons, including the dorsal motor nucleus of the vagus (DMV). Phox2b is not expressed in sympathetic pre-ganglionic neurons. In situ hybridization and electrophysiological techniques similar to those used to confirm Mc4rloxP/loxP; Chat-cre3,4 mice revealed successful ablation of Mc4r in the DMV (Supplementary Fig. 1a,b) that did not impair normal biochemical, phenotypic properties, yet rendered cells insensitive to MC4R agonism (Supplementary Fig. 1c–f).

MC4R loss of function causes obesity in humans1,11,12 and animals1,12. Chow-fed Mc4rloxP/loxP; Chat-cre mice initially exhibited normal body weight, but diverged at 8 weeks of age and were ~40% heavier at 20 weeks of age (Fig. 1a). This obesity was intermediate versus Mc4r null mice and a result of higher lean and fat mass (Supplementary Fig. 2a). Body length was normal in Mc4rloxP/loxP; Chat-cre mice, whereas the snout-to-anus distance of Mc4r null mice was longer (data not shown). This is, to the best of our knowledge, the first MC4R mutation causing obesity that is not confounded by altered growth. We used metabolic cages in young mice (to minimize differences in body weight; Fig. 1a) to determine that Mc4rloxP/loxP; Chat-cre mice were obese as a result of low energy expenditure (EE), rather than hyperphagia (Fig. 1b and Supplementary Fig. 2b). As expected, Mc4r null mice were hypometabolic and hyperphagic (Fig. 1b and Supplementary Fig. 2b). In contrast, chow-fed Mc4rloxP/loxP; Phox2b-cre mice had no differences in body weight or body composition (Supplementary Fig. 2c,d). Collectively, these results indicate that MC4R signaling in cholinergic neurons, including sympathetic pre-ganglionic neurons, are required to maintain normal EE and body weight. MC4Rs in Phox2b-expressing neurons, including those in the DMV, however, are dispensable for normal body weight homeostasis.

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Growing evidence suggests that MC4Rs directly regulate glycemia. Consistent with this, we found that circulating glucose, insulin and glucagon were similarly elevated in young, body weight–matched, post-absorptive Mc4rloxP/loxP; Chat-cre mice and Mc4r null littermates (Fig. 2a–c). Only plasma insulin was elevated in Mc4rloxP/loxP; Chat-cre mice (Supplementary Fig. 3a–c). Intraperitoneal glucose tolerance tests revealed exaggerated glucose excursions and augmented plasma insulin responses in both groups, suggesting insulin resistance (Supplementary Fig. 3d–g). Using hyperinsulinemic-euglycemic clamps (Supplementary Fig. 3h–k), we confirmed this defect based on lower exogenous glucose infusion rates (GIRs) (Fig. 2d).

Defects in intrascapular BAT (iBAT), inguinal WAT (iWAT) and skeletal muscle (gastrocnemius and soleus) glucose uptake contributed to reduced $R_g$ in Mc4rloxP/loxP; Chat-cre mice (Fig. 2f). Defective impairment in insulin-mediated suppression of endogenous glucose production ($R_g$) and stimulation of glucose disposal ($R_e$) were the causes of lower GIR in Mc4rloxP/loxP; Chat-cre mice (Fig. 2f). Defects in intrascapular BAT (iBAT), inguinal WAT (iWAT) and skeletal muscle (gastrocnemius and soleus) glucose uptake contributed to reduced $R_g$ in Mc4rloxP/loxP; Chat-cre mice (Fig. 2f). Defective impairment in insulin-mediated suppression of endogenous glucose production ($R_g$) and stimulation of glucose disposal ($R_e$) were the causes of lower GIR in Mc4rloxP/loxP; Chat-cre mice (Fig. 2f). Defective impairment in insulin-mediated suppression of endogenous glucose production ($R_g$) and stimulation of glucose disposal ($R_e$) were the causes of lower GIR in Mc4rloxP/loxP; Chat-cre mice (Fig. 2f).

We next challenged energy homeostasis in both Mc4rloxP/loxP; Chat-cre and Mc4rloxP/loxP; Phox2b-cre cohorts using high-fat/high-sucrose (HFHS) diet-induced obesity. Whole-body MC4R-deficient mice are more sensitive to diet-induced obesity. Time course analyses in Mc4rloxP/loxP; Chat-cre mice revealed that HFHS diet accelerated weight gain, resulting in obesity that was nearly comparable to Mc4r null littermates (Fig. 3a). Results from a metabolic cage procedure similar to that of prior work in which HFHS diet is introduced to naive, young (to minimize differences in body weight) Chow-fed adult mice revealed that both hyperphagia and failure to fully increase EE likely underlie obesity in Mc4rloxP/loxP; Chat-cre mice (Fig. 3b,c). These responses were similar to those of Mc4r null littermates (Fig. 3b,c), which are the predicted responses on the basis of prior work in other MC4R-deficient mice fed an obesogenic diet. We next challenged energy homeostasis in both Mc4rloxP/loxP; Chat-cre and Mc4rloxP/loxP; Phox2b-cre cohorts using high-fat/high-sucrose (HFHS) diet-induced obesity. Whole-body MC4R-deficient mice are more sensitive to diet-induced obesity. Time course analyses in Mc4rloxP/loxP; Chat-cre mice revealed that HFHS diet accelerated weight gain, resulting in obesity that was nearly comparable to Mc4r null littermates (Fig. 3a). Results from a metabolic cage procedure similar to that of prior work in which HFHS diet is introduced to naive, young (to minimize differences in body weight) Chow-fed adult mice revealed that both hyperphagia and failure to fully increase EE likely underlie obesity in Mc4rloxP/loxP; Chat-cre mice (Fig. 3b,c). These responses were similar to those of Mc4r null littermates (Fig. 3b,c), which are the predicted responses on the basis of prior work in other MC4R-deficient mice fed an obesogenic diet. We next challenged energy homeostasis in both Mc4rloxP/loxP; Chat-cre and Mc4rloxP/loxP; Phox2b-cre cohorts using high-fat/high-sucrose (HFHS) diet-induced obesity. Whole-body MC4R-deficient mice are more sensitive to diet-induced obesity. Time course analyses in Mc4rloxP/loxP; Chat-cre mice revealed that HFHS diet accelerated weight gain, resulting in obesity that was nearly comparable to Mc4r null littermates (Fig. 3a). Results from a metabolic cage procedure similar to that of prior work in which HFHS diet is introduced to naive, young (to minimize differences in body weight) Chow-fed adult mice revealed that both hyperphagia and failure to fully increase EE likely underlie obesity in Mc4rloxP/loxP; Chat-cre mice (Fig. 3b,c). These responses were similar to those of Mc4r null littermates (Fig. 3b,c), which are the predicted responses on the basis of prior work in other MC4R-deficient mice fed an obesogenic diet. We next challenged energy homeostasis in both Mc4rloxP/loxP; Chat-cre and Mc4rloxP/loxP; Phox2b-cre cohorts using high-fat/high-sucrose (HFHS) diet-induced obesity. Whole-body MC4R-deficient mice are more sensitive to diet-induced obesity. Time course analyses in Mc4rloxP/loxP; Chat-cre mice revealed that HFHS diet accelerated weight gain, resulting in obesity that was nearly comparable to Mc4r null littermates (Fig. 3a). Results from a metabolic cage procedure similar to that of prior work in which HFHS diet is introduced to naive, young (to minimize differences in body weight) Chow-fed adult mice revealed that both hyperphagia and failure to fully increase EE likely underlie obesity in Mc4rloxP/loxP; Chat-cre mice (Fig. 3b,c). These responses were similar to those of Mc4r null littermates (Fig. 3b,c), which are the predicted responses on the basis of prior work in other MC4R-deficient mice fed an obesogenic diet. We next challenged energy homeostasis in both Mc4rloxP/loxP; Chat-cre and Mc4rloxP/loxP; Phox2b-cre cohorts using high-fat/high-sucrose (HFHS) diet-induced obesity. Whole-body MC4R-deficient mice are more sensitive to diet-induced obesity. Time course analyses in Mc4rloxP/loxP; Chat-cre mice revealed that HFHS diet accelerated weight gain, resulting in obesity that was nearly comparable to Mc4r null littermates (Fig. 3a). Results from a metabolic cage procedure similar to that of prior work in which HFHS diet is introduced to naive, young (to minimize differences in body weight) Chow-fed adult mice revealed that both hyperphagia and failure to fully increase EE likely underlie obesity in Mc4rloxP/loxP; Chat-cre mice (Fig. 3b,c). These responses were similar to those of Mc4r null littermates (Fig. 3b,c), which are the predicted responses on the basis of prior work in other MC4R-deficient mice fed an obesogenic diet.
Figure 3  Deletion Mc4r in pre-ganglionic cholinergic neurons promotes obesity and impairs cold tolerance. (a) Body weight in mice with intact MC4R signaling (Mc4rloxP/loxP), selective deletion of Mc4r in cholinergic, pre-ganglionic neurons (Mc4rloxP/loxP; Chat-cre) or ectopic ablation of Mc4r (Mc4r null) fed high-fat/high-sucrose (HFHS) after 8 weeks of age (n = 9, 12 and 9, respectively). *P < 0.05, **P < 0.01 versus Mc4rloxP/loxP mice; †P < 0.05 versus Mc4rloxP/loxP; Chat-cre mice using one-way ANOVA followed by Tukey’s post hoc analyses. (b,c) EE (b) and food intake (c) in Chow-fed 7–8-week-old mice introduced to HFHS diet 3 d before metabolic cage assessment (n = 8, 9 and 7, respectively). Dashed lines in b denote linear regression of body weight versus body weight using ANCOVA. *P < 0.05, **P < 0.01 versus Mc4rloxP/loxP mice; †P < 0.05 versus Mc4rloxP/loxP; Chat-cre mice using one-way ANOVA followed by Tukey’s post hoc analyses. (d) Basal (23 °C) and cold (6 °C)-induced body temperature determined by remote telemetry in Chow-fed Mc4rloxP/loxP, Mc4rloxP/loxP; Chat-cre and Mc4r null littermate mice (n = 8, 9 and 7, respectively) over 24 d. *P < 0.05, **P < 0.01 versus Mc4rloxP/loxP mice; †P < 0.05 versus Mc4rloxP/loxP; Chat-cre mice using one-way ANOVA followed by Tukey’s post hoc analyses. Cold exposure experiments were performed twice. All mice were male and data are presented as means ± s.e.m.

Reduced in HFHS-fed Mc4rloxP/loxP; Chat-cre iBAT (Supplementary Fig. 4d). This was not the case in HFHS-fed Mc4rloxP/loxP; Phox2b-cre mice (Supplementary Fig. 4e). We used remote telemetry probes to assess body temperature at 23 °C and in response to cold (5 d at 6 °C). Basal body temperature was not reduced in Mc4rloxP/loxP; Chat-cre (Fig. 3d). However, Mc4rloxP/loxP; Chat-cre mice were unable to engage adaptive thermogenesis, and body temperature in cold-exposed Mc4rloxP/loxP; Chat-cre mice declined rapidly to Mc4r null values within 4 h (Fig. 3d). Analyses of iBAT histology and uncoupling protein 1 (UCP1) protein content after 120 h at 6 °C further confirmed the observed impaired thermogenic responses in Mc4rloxP/loxP; Chat-cre mice, which were on par with defects in Mc4r null littermates (Supplementary Figs. 5a and 6). In addition, iWAT in cold-exposed Mc4rloxP/loxP; Chat-cre mice failed to show evidence of ‘beiging’ based on gene expression (Supplementary Fig. 5c) or histology for UCP1 (Supplementary Fig. 5d).

Hence, selective loss of MC4R in the sympathetic pre-ganglionic neurons deleteriously and independently affects both processes, whereas deletion in the vagal motor neurons results in hyperinsulinemia and modest insulin resistance. MC4Rs in sympathetic pre-ganglionic neurons are required for diet- and cold-induced thermogenesis in iBAT and ‘beiging’ of iWAT. Functional iBAT in adult humans14–18 and the ability of WAT9,20 to exhibit thermogenic properties characteristic of BAT have recently been shown. However, our data cannot prove that defects in iBAT and/or the ability to ‘beige’ iWAT contribute to low EE and/or hyperglycemia in mice lacking MC4Rs in sympathetic pre-ganglionic neurons. Nonetheless, sympathetic nervous system-mediated mechanisms regulate an array of tissues, including the pancreas, liver, BAT and WAT. In contrast, MC4R-mediated control via the DMV appears to be restricted to pancreatic β cells and circulating insulin. Outlining these functional connections may help develop more selective strategies to target MC4Rs or related pathways. Caution is warranted, however, as this approach could cause pathological sympathetic nervous system side effects.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

E.D.B. designed and performed all experiments except for gene expression and immunoblotting, analyzed the data, and wrote the manuscript. T.L. designed and performed all experiments except gene expression and immunoblotting, analyzed the data, and edited the manuscript. X.K. analyzed gene expression in adipose tissue. E.D.B. designed and performed all experiments except for gene expression and immunoblotting, analyzed the data, and reviewed the manuscript. L.V. and D.P.O. designed and developed the Mc4rloxP/loxP mice. D.P.O. designed and developed the Chat-cre mouse. K.W.W. and J.-W.S. performed electrophysiologic experiments. Z.D., S.L. and C.E.L. assisted with experiments. P.E.S. designed experiments and edited the manuscript. B.B.L. and J.K.E. supervised development of the mouse models, designed experiments and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Animal care. Mc4rloxP/loxP, Chat-cre and Phox2b-cre mice on a C57BL/6J background have been previously described.14 Mice were group housed (1–5 mice per cage) in a barrier facility at 23 °C unless otherwise noted. Mice were provided Harlan Teklad 2016 chow diet and water ad libitum unless otherwise noted. HFHS (Research Diets D12331) diet, if applicable, was removed and refilled weekly. Mice losing >10% of body weight during the acclimation period for metabolic cage studies (see below) or post-surgery before clamp studies (see below) were not studied. Body composition was measured using NMR (Bruker Mini-Spec).

Hormone measurements. All measurements were done in duplicate. Insulin was measured using a commercial ELISA kit (Crystal Chem). Glucagon was measured via radioimmunoassay at the Vanderbilt University Mouse Metabolic Phenotyping Core.

Metabolic cages. Experiments were performed in a temperature-controlled room containing 36 TSE metabolic cages maintained by University of Texas Southwestern Animal Resources personnel. 1 week before study, mice were singly housed to acclimate to new housing. 3 d before study, mice were transported to the room containing metabolic cages to acclimate to a new environment. HFHS diet, if applicable, was also introduced at the beginning of this acclimation period. After 3 d of acclimation, cages were connected to TSE system for a total of 5 d. Days 2–4 were used for data analyses.

Cold exposure. Body temperature was assessed using biotelemetry probes (IPTT-300, BioMedic Data Systems) injected under anesthesia 1 week prior. Mice were housed in cold chambers maintained at 6 °C by University of Texas Southwestern Animal Resources personnel.

Clamp studies. Experiments were done with the assistance of the University of Texas Southwestern Mouse Phenotyping Core.

Protein extraction and western blot analysis. Protein was extracted from BAT by using RIPA buffer (Boston BioProducts) supplemented with complete protease inhibitor cocktail (Roche). For western blot analyses, 60 mg of protein was subjected to SDSPAGE under reducing conditions, transferred and blotted with the antibody to UCP1 (Abcam, ab10983).

Histology. Tissues were dissected and fixed in formalin for 48 h at 4 °C followed by 50% ethanol. BAT histology was performed with assistance from the University of Texas Southwestern Histology Core. iWAT was performed with assistance from the Harvard Histology Core.

Analysis of gene expression by quantitative PCR. Total RNA was extracted from tissues with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. 1 μg total RNA was converted into first-strand cDNA with oligo(dT) primers as described by the manufacturer (Clontech). PCR was performed in an Mx3000P Q-PCR system (Stratagene) with specific primers and SYBR Green PCR Master Mix (Stratagene). The relative abundance of miRNAs was standardized with 36B4 mRNA as the invariant control.

Statistics. Sample sizes were determined using prior experience and power calculations designed to detect P < 0.05 with a 15% variance. There were no methods to randomize mice to experimental groups. There were also no methods to blind investigators to genotype during experiments. Pre-established criteria for excluding data points were data two s.d. outside the mean or any data obtained from mice that died or lost >10% of body weight due to metabolic cage acclimation or clamp studies.

A Supplementary Methods Checklist is available.