Regulation of Glucose Tolerance and Sympathetic Activity by MC4R Signaling in the Lateral Hypothalamus

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Running title: LHA MC4R signaling regulates glucose metabolism

Abstract Word Count: 171  
Word Count: 4764  
Figures: 8  
Table: 0
ABSTRACT

Melanocortin 4 receptor (MC4R) signaling mediates diverse physiological functions including energy balance, glucose homeostasis and autonomic activity. While it is known that the lateral hypothalamic area (LHA) expresses MC4Rs and receives inputs from leptin-responsive arcuate POMC neurons, the physiological functions of MC4Rs in the LHA are incompletely understood. Here, we report that MC4R<sup>LHA</sup> signaling regulates glucose tolerance and sympathetic nerve activity. Restoring expression of MC4Rs specifically in the LHA improves glucose intolerance in obese MC4R-null mice without affecting body weight or circulating insulin levels. Fluorodeoxyglucose-mediated tracing of whole-body glucose uptake identify the interscapular brown adipose tissue (iBAT) as a primary source where glucose uptake is increased in MC4R<sup>LHA</sup> mice. Direct multi-fiber sympathetic nerve recording further reveals that sympathetic traffic to iBAT is significantly increased in MC4R<sup>LHA</sup> mice, which accompanied a significant elevation of Glut4 expression in iBAT. Finally, bilateral iBAT denervation prevents the glucoregulatory effect of MC4R<sup>LHA</sup> signaling. These results identify a novel role for MC4R<sup>LHA</sup> signaling in the control of sympathetic nerve activity and glucose tolerance independent of energy balance.
INTRODUCTION

The incidence and prevalence of obesity and associated disorders, such as diabetes and hypertension, are increasing worldwide (1). While there is a general consensus that the central nervous system (CNS) plays a key role in these processes (2; 3), the underlying neural substrates remain to be fully defined. Central melanocortin signaling pathway, mainly via action on the MC4Rs, is a key relay of arcuate proopiomelanocortin (POMC) and agouti-related peptide (AgRP) expressing neurons to coordinate long-term energy balance, glucose homeostasis and sympathetic nerve activity (SNA) (4). Loss of function of MC4R in both humans and rodents leads to the development of obesity, insulin resistance and diabetes (5; 6). MC4Rs are widely expressed in the CNS including certain hypothalamic nuclei implicated in the regulation of energy balance, glucose metabolism and sympathetic outflow, including the paraventricular nucleus, dorsomedial nucleus and lateral hypothalamic area (LHA) (7). Previous studies identified distinct roles of MC4R signaling in specific neural circuits (8-11), offering the possibility that discrete functions of MC4R signaling in specific CNS sites will allow the development of selective treatments for obesity and associated disorders. The LHA, which receives direct inputs from leptin-responsive arcuate POMC neurons (12) and expresses MC4Rs (7), regulates multiple physiological processes including food intake, reward-related behaviors and autonomic function (13; 14). However, the physiological role of MC4R signaling in the LHA is incompletely understood. We have previously shown that MC4R\textsubscript{LHA} neurons co-express the anorectic peptide neurotensin and functionally active form of the leptin receptor (LepR) and are responsive for leptin administration (15). These observations suggested a potential role for MC4R\textsubscript{LHA} neurons in metabolic regulation. Using a Cre-dependent reactivatable MC4R-null (MC4R-TB) mouse line (8), here we investigate the physiological function of MC4R\textsubscript{LHA} signaling with specific focus on energy balance, glucose metabolism and autonomic activity.
RESEARCH DESIGN AND METHODS

Animals. Mice with a transcriptional blocking (TB) cassette flanked by loxP sites placed in front of the MC4R gene (MC4R-TB) and back-crossed at least six generations onto the C57BL/6 line were previously reported (8; 16). The tdTomato reporter mice were purchased from the Jackson Laboratory (stock number 007909). Mice were housed in the University of Iowa vivarium in a temperature-controlled environment (lights on: 06:00–18:00) with ad lib access to water and food. To avoid the potentially confounding effects of female estrus cycle on metabolic profiling, only male mice were used for current study. All animal procedures were performed in accordance with the University of Iowa Institutional Animal Care and Use Committee guidelines.

Stereotaxic microinfusion of AAVs into the LHA

Stereotactic surgery was performed as previously described (15; 17). Briefly, 6-7 weeks old male mice were anesthetized with ketamine/xylazine (100:10 mg/kg, i.p.) and placed on a Kopf stereotaxic apparatus (Tujunga, CA). Following standard disinfection, ~1.0 cm incision was made to expose the skull and a small hole was drilled into the skull bilaterally at defined positions (AP -1.5 mm; ML +1.5 mm; DV -4.9 mm with 6 degree angle of injection arm), and Hamilton Microsyringe with small hub removable needle filled with virus was slowly inserted. Injection was made by pressing the plunger with 0.05 µl/min rate. After 10 minutes of waiting to ensure a full penetration of AAV into targeted area, the needle was removed and the incision was closed by wound clips. Mice were then kept on a warming pad until awake. At the end of study, brains were extracted and post-fixed in 4% paraformaldehyde and injection sites were confirmed for every case by observing the GFP signal blindly to physiological outcomes. Two out of the 59 MC4R-TB mice receiving AAV-Cre-GFP were excluded from final data analysis for complete mistargeting of LHA.

Quantification of feeding frequency and locomotor activity
Male mice at age of 13-15 weeks were tested for locomotor activity by using LABORAS (Metris, Netherlands), an advanced system for automated recognition of small laboratory animal behaviors, such as locomotion, grooming, eating, drinking, etc. Data was collected uninterrupted in the home cage over a 23-hour period of time and then analyzed via LABORAS software as previously reported (18).

**Energy balance and basal blood/plasma analysis**

After one week of full recovery from AAV microinfusion, mice were assigned *ad lib* access to either normal chow (#7913, Harlan-Teklad, Madison, WI) or high-fat diet (HFD, 42% kcal from anhydrous milk fat, TD.88137, Harlan-Teklad) as noted. Body weight and food intake were measured periodically on both HFD and regular chow up to 12 weeks after stereotaxic delivery of AAVs into the LHA. For blood biochemistry, a trunk blood sample was collected from 18 weeks old HFD-fed male mice. Sera were sent to the Vanderbilt University Mouse Metabolic Phenotyping Center for the measurement of blood hormones and cholesterol levels.

**Glucose and Pyruvate Tolerance Tests**

Seventeen to nineteen weeks old male mice were used for glucose and pyruvate tolerance tests. Mice were brought to the procedure room and handled for 5 days prior to testing to allow habituation to environment and reduce handling stress. On the testing day, mice were weighed and food was removed at 9:00 AM for 6 hour-fasting with free access to water. At 3:00 PM, blood glucose was measured at baseline (0’) followed by immediate intraperitoneal injection of D-glucose (1g/Kg) (G8270, Sigma Aldrich) or sodium pyruvate (2g/Kg) (P2256, Sigma Aldrich). Blood glucose level was then monitored at 15’, 30’, 60’ and 120’ time-point for each mouse.

**ICV cannulation**
Under continuous anesthesia, a small hole was drilled into the skull at defined point (AP -0.2 mm, ML +1.0 mm) and the ICV cannula with a specific length (0.23 mm below plastic pedestal, Plastic One Inc) was slowly inserted. The cannula was held in place with the appropriate amount of a glass ionomer luting cement anchoring with a small machine screw in the right-front of cannula, allowing 3-5 minutes for stiffening. Open skin wounds were adhered onto mounted glass ionomer luting cement by applying Vetbond Tissue Adhesive and the mouse was kept on a warmed surgical table until fully awake.

**Whole body FDG PET/CT imaging**

Whole body FDG scanning was performed in Small Animal Imaging Core (SAIC) at The University of Iowa as previously reported (19). Seventeen to nineteen weeks old, chow-fed male mice were used. Fasted animals (~14 hours) were anesthetized with 1.5% isoflurane, blood glucose was measured then received a tail vein injection of FDG (7.5 MBq +/-11.3). After a 60 minute awake uptake period the mice were imaged on the INVEON PET/CT (Siemens, Knoxville, TN). A 15 minute PET acquisition followed in the same workflow a CT for attenuation correction was acquired. Three days after initial scan, the same animals were PET imaged again. The same imaging protocol was followed except 30 min prior to FDG injection an intraperitoneal injection of Melanotan II (MTII, 2mg/Kg) (HC3902, BACHEM) were given. All images were reconstructed using 3D OP-MAP algorithm. Image analysis was completed using PMOD v3.2 (PMOD Technologies, Zurich Switzerland). Volumes of interest were drawn for iBAT, muscle, brain, kidney and heart and organ-specific uptakes of FDG were calculated from the scanned images and normalized change from baseline FDG uptake was compared between the groups.

**Direct multi-fiber recording of sympathetic nerve activity (SNA)**
Regional SNA was measured by direct multi-fiber recording of sympathetic nerve branches subserving the interscapular BAT (iBAT) and hindlimb muscles as previously described (20). Seventeen to nineteen weeks old, chow-fed male mice were used for SNA recording. Under anesthesia, catheterization of the carotid arteries and the jugular vein was performed for hemodynamic recording and maintenance of anesthesia with α-chloralose (25 mg/kg/h), respectively. A nerve fascicle to iBAT or hindlimb was carefully isolated under dissecting microscope. A bipolar platinum-iridium electrode (Cooner Wire) was suspended under the nerve and secured with silicone gel (Kwik-Cast, WPI). The electrode was attached to a high-impedance probe (HIP-511, Grass Instruments) and the nerve signal was amplified 105 times with a Grass P5 AC pre-amplifier. After amplification, the nerve signal was filtered at a 100- and 1000-Hz cutoff with a nerve traffic analysis system (model 706C, University of Iowa Bioengineering). Subsequently, the amplified and filtered nerve signal was routed to an oscilloscope (model 54501A, Hewlett-Packard) for monitoring the quality of the sympathetic nerve recording and to a resetting voltage integrator (model B600c, University of Iowa Bioengineering) that sums the total voltage output to a unit of 1 volt x sec per min before resetting to zero.

After 10 minutes recording of baseline activity, mice received intracerebroventricular infusion of MTII (2 µg). SNA was continuously monitored for 4 hours and change from baseline activity was calculated and compared between the groups. At the end of study, mice were received ICV infusion of 2 µl blue dye and brains were extracted to confirm correct ICV cannulation and AAV injection site.

**Bilateral iBAT sympathetic denervation**

Bilateral iBAT sympathetic denervation was performed as previously reported (21) with slight modification. Thirteen to fifteen weeks old, chow-fed mice were used for this study. On the day of surgery, each mouse was weighed and anesthetized with Isoflurane. When the proper state of anesthesia is reached, the mouse was shaved of hair on the nape region and secured on a warm surgical
table with a rectal probe to measure and maintain body temperature at 37.5 °C. Following a standard skin disinfection procedure, a lateral incision was made just below the two shoulder blades to expose the intrascapular fat pads. A blunt forceps was then used to clamp the caudal edge of the intrascapular fat pad and then retracted cranially over the head of the mouse. With this exposure, the bilateral sympathetic chain that innervates both right and left brown adipose tissue hemispheres was identified. All five branches of intercostal sympathetic nerves subserving the left and the right BAT fat pad were identified and carefully isolated, and the nerve branches between the root and BAT were sectioned out (Fig. 4A-C). After completion of bilateral BAT denervation, the intrascapular fat pad was then returned to its original position and secured in place to the surrounding thoracic tissue and muscle with absorbable 6.0 Vycril suture. A thin line of tissue adhesive (Vet-Bond) is applied along the edges of both skin flaps and the two edges of skin are held in place until the entire length of the incision in the nape area is completely closed. Mice were monitored for 7 days post operatively before subject to further experimental procedures.

**RNA Isolation and Real-Time PCR Analyses,**

Total RNA was isolated from snap-frozen iBAT and inguinal white adipose tissue (WAT) of 16-18 weeks old, chow-fed male mice and complementary DNA was generated by reverse transcription as previously described (22). Quantitative real-time PCR was performed with SYBR green method with standard protocol as previously described (22). The following primer sets were used to determine relative abundance and expression of UCP1, Cidea, PGC1a, β3-AR, Glut1 and Glut4: 5'-AAGCTGTGCGATGTCCATGT-3' and 5'-AAGCCACAAACCCTTTGAAAA-3' for UCP1, 5'-AGACAATGTGCTTCCAAAAAGAA3' 5'-GAAGAGATAAAGTTGTTGGTTGTCG-3' for PGC1a, 5'-GGTTCAGGCCGTGTTAAGG-3' and 5'-CGTCATCTGTGCAGCATAGG-3' for Cidea, 5'-TCCTTCTACCTCCCTCCT-3' and 5'-CGGCTTAGCCACAACGAACAC-3' for β3-AR, 5'-CGCCCCCCAGAAGGTAT-3' and 5'-CGATGCAGGTGTTCCAT-3' for Glut1, 5'-
TTGGCTCCCTTCAGTTTGGC-3' and 5'-CTACCCAGCCACGTTGCAT-3' for Glut4. The expression of 36B4 (5'-CACTGGTCTAGGACCGAGAAG-3' and 5'-GGTGCTCTGAAGATTTCG-3’) was used as an internal control gene.

**Insulin Sensitivity and Western Blotting Analysis**

Nineteen to twenty-one weeks old, overnight-fasted male mice were anesthetized with ketamine/xylazine (100:10 mg/Kg) and received retro-orbital bolus insulin (5 units). iBAT, soleus muscle and liver were then harvested at 10-minute and snap frozen in liquid nitrogen. Small piece of frozen tissues were homogenized in cold Radio-Immunoprecipitation Assay (RIPA) buffer (#89901, Thermo Scientific) containing cocktails of proteinase inhibitors (11836170001, Roche Diagnostics) and phosphatase inhibitors (04906845001, Roche Diagnostics) and kept on ice for 30 minutes. Samples were then centrifuged (13000 rpm) at 4 °C for 20 minutes and supernatant was taken as total protein extraction. Protein concentration was determined by BAC protein assay method (#23228, Thermo Scientific) and 20 ug of protein were separated by SDS-PAGE gel, transferred onto PVDF membrane and immunoblotted with pAKT (1/2000) (#4060, Cell Signaling), AKT (1/1000) (#9272, Cell Signaling), pERK (1/1000) (#9101S, Cell Signaling) and ERK (1/1000) (#9102, Cell Signaling) as previously reported (23). Membranes were then incubated with HRP-conjugated secondary antibody (Jackson Immuno Research) and signals were detected by chemiluminescence. Signal intensity was measured and analyzed by BioSpectrum 810 Imaging System (Upland, CA, USA). For each experiment, signal intensity of targeted proteins was normalized to total AKT or ERK signal intensity and then compared between the groups.

**Statistics**

GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) was used to perform all statistical analyses. Comparisons between the groups were made by two-way or one-way ANOVA.
with bonferroni post hoc analysis, or by Student's t-test as noted. P < 0.05 was considered to be statistically significant. Data are presented as mean ± SEM.
RESULTS

Restoration of MC4R<sup>LHA</sup> signaling improves impaired glucose tolerance in obese MC4R-TB mice without affecting food intake and body weight

To directly study the physiological function of MC4R<sup>LHA</sup> signaling, we used adeno-associated virus (AAV) to specifically deliver Cre-recombinase-GFP or GFP-alone (AAV-Cre-GFP and AAV-GFP) into the LHA of ‘reactivatable’ MC4R-TB mice to remove a transcription blocking (TB) cassette and restore endogenous expression of MC4Rs. To optimize the delivery volume of AAV-Cre-GFP specifically into the LHA, we first performed microinfusion of AAV-Cre-GFP into the LHA of tdTomato reporter mouse in which a loxP-flanked TB cassette prevents transcription of a ubiquitous promoter-driven tdTomato which can be re-expressed upon removal of TB cassette by Cre-mediated recombination. Two weeks after AAV-Cre-GFP microinfusion, a large number of tdTomato+ and GFP+ cells were observed in the LHA and nearly all of GFP+ cells were tdTomato+ (Fig. 1C), indicating that AAV-Cre-GFP is properly inducing Cre/loxP recombination in the LHA neurons with limited infection to surrounding structures (Fig. 1B, 1C). The volume and titer of AAV-Cre-GFP were optimized by repeated injections in tdTomato reporter mice (data not shown). Using 0.3µl/side at a titer of 1.18 X 10<sup>12</sup> GC/mL we were able to achieve a maximum coverage of dorsomedial LHA, where MC4R-positive neurons are mainly located (Fig. 1A), with minimized infection of adjacent hypothalamic nuclei. After validation and optimization of the AAV microinfusion protocol, AAV-Cre-GFP or AAV-GFP was bilaterally infused into the LHA of MC4R-TB and their wild-type (WT) littermates. In contrast to our hypothesis, restoration of MC4R<sup>LHA</sup> expression had no measurable effects on body weight (Fig. 1D), food intake (Fig. 1E), locomotion (Fig. 1F), fasting glucose (Fig. 1G) and insulin (Fig. 1H), body composition (Fig. 1L), or regional fat mass (Fig. 1M-O) when fed a chow diet. Subsequent assessment for glucose homeostasis, however, revealed that while both MC4R-TB groups were similarly obese (Fig. 1D), MC4R<sup>LHA</sup> mice displayed significantly improved glucose
tolerance compared to MC4R-TB (AAV-GFP) mice, especially at 15’ and 30’ time points (Fig. 1I, 1J). To further test whether improved glucose tolerance is associated with altered insulin levels, we also measured insulin levels at 0, 15 and 30 time points after intraperitoneal injection of glucose (1 g/Kg) and did not observe significant difference between two MC4R-TB groups (Fig. 1K). Since none of these parameters measured was significantly different between WT (AAV-GFP) (n=5) and WT (AAV-Cre-GFP) (n=5) groups, both groups of WT mice were combined and referred as control to increase statistical power.

Because MC4R-null mice are hypersensitive to a palatable HFD with marked hyperphagia and weight gain compared to chow-fed condition (24; 25), we generated another cohort of MC4R-TB mice by MC4R-TB homo X MC4R-TB het breeding paradigm and divided into two groups to receive either AAV-GFP or AAV-Cre-GFP microinfusion. After one week of recovery from surgery, mice were fed a HFD to assess if there is a diet-specific effect of MC4R_{LHA} signaling. Restoration of MC4R_{LHA} signaling did not affect weight gain (Fig. 2A) or food intake (Fig. 2B) on HFD. While both groups of mice are similarly obese, fasting glucose level was significantly lower in MC4R_{LHA} mice (Fig. 2D) without significant change in circulating insulin levels (Fig. 2C). Subsequent glucose tolerance test (GTT) again confirmed a significant improvement in glucose tolerance in MC4R_{LHA} mice (Fig. 1E, 1F). No differences were seen in fasting serum glucagon, leptin, free fatty acids, triglyceride, total cholesterol or dHDL levels (Fig. 2G-L). Collectively, these data demonstrate a role for MC4R_{LHA} signaling in the regulation of glucose tolerance without altering body energy balance or the levels of critical glucoregulatory hormones such as insulin and glucagon.

MC4R_{LHA} signaling increases glucose uptake in iBAT without affecting hepatic gluconeogenesis or insulin sensitivity
Improved glucose tolerance in MC4R<sup>LHA</sup> mice without a significant change in body weight or circulating insulin or glucagon levels led us to ask whether glucose disposal was increased in any tissue of MC4R<sup>LHA</sup> mice. To this end, we performed whole-body PET/CT scanning for fluorodeoxyglucose (FDG) uptake by which biodistribution of FDG can be quantified in a spatially resolved manner. While both groups of MC4R-TB mice were similarly obese (Fig. 3B), FDG uptake was significantly increased in interscapular brown adipose tissue (iBAT) of MC4R<sup>LHA</sup> mice (Fig. 3A, 3C) following MTII administration compared to control MC4R-TB (AAV-GFP) mice. A similar trend was noted in skeletal muscle but did not reach statistical significance (Fig. 3D). No difference in FDG uptake was found in brain (Fig. 3E), kidney or heart (data not shown).

To further test if MC4R<sup>LHA</sup> signaling also affects hepatic glucose production, pyruvate tolerance test (PTT) was performed as an indirect measurement of hepatic gluconeogenesis. Intraperitoneal injection of sodium pyruvate (2 g/Kg) in 6-hour fasted animals sharply increased blood glucose level within 15 minutes, which gradually returned to baseline levels by 2 hours in control mice (Fig. 4B, 4C). In contrast, glucose levels continuously increased in both groups of MC4R-TB mice and no significant difference was noted between the two groups during the 2-hour test (Fig. 4B, 4C), suggesting that restoring MC4R<sup>LHA</sup> signaling does not likely affect hepatic gluconeogenesis. Again, no difference of body weight was noted between two groups of MC4R-TB mice when performing GTT (Fig. 4A).

Additionally, to test the possibility that restoration of MC4R<sup>LHA</sup> may affect insulin signaling, insulin-induced phosphorylation of v-akt murine thymoma viral oncogene homolog 1 (AKT) at Ser<sup>473</sup> site (pAKT) and phosphorylation of extracellular-signal-regulated kinases (ERK) at Thr202 and Tyr204 sites (pERK) were also measured as markers of acute activation of insulin receptor signaling cascade. After confirming a significant induction of pAKT and pERK by retro-orbital insulin bolus (5 units) in iBAT of WT mice (Fig. 4D and 4H), the same procedure was used to test insulin signaling in iBAT, soleus muscle and liver. No significant difference was seen between the two MC4R-TB groups in any
of the tissues tested (Fig. 4E-G, 4I-K), although there was a trend toward increased pERK/ERK levels in soleus muscle that did not reach statistical difference (Fig. 4J).

**Reactivation of MC4R_{LHA} signaling restores blunted melanotan II-induced iBAT sympathetic nerve activation**

The observation that MC4R_{LHA} mice display improved glucose tolerance and increased FDG uptake in iBAT without affecting circulating insulin or peripheral insulin sensitivity suggested the possibility that MC4R_{LHA} signaling may facilitate glucose uptake in iBAT by increasing sympathetic nerve activity (SNA). Given the known role of the LHA in the regulation of sympathetic outflow and direct multisynaptic innervation of LHA MC4R-positive neurons to iBAT (26; 27), we tested the possibility that MC4R_{LHA} signaling mediates activation of SNA to iBAT induced by the melanocortin agonist melanotan II (MTII). While direct multi-fiber recording demonstrated that control mice showed significantly increased iBAT-SNA following infusion of MTII (Fig. 5A, B), the effect was completely abolished in MC4R-TB (AAV-GFP) mice (Fig. 5A, B). In contrast, MTII-induced iBAT SNA in MC4R_{LHA} mice was restored to control levels (Fig. 5A, B), indicating that reactivation of MC4R_{LHA} signaling is sufficient to rescue blunted sympathetic traffic to iBAT in obese MC4R-null mice.

Because we noted a trend toward increased FDG uptake in skeletal muscle, we also recorded lumbar nerve SNA to hindlimb skeletal muscle. As expected, control groups demonstrated a robust increase in lumbar SNA following ICV injection of MTII (Fig. 5C). MC4R-TB mice receiving AAV-GFP in the LHA displayed a significantly delayed and blunted response to MTII-induced lumbar SNA compared to WT mice with a modest increase in lumbar SNA observed after 180 minutes of recording. This blunted response of MTII-induced lumbar SNA is restored to WT level in MC4R_{LHA} mice (Fig. 5C). These results indicate that MC4R_{LHA} signaling mediates MTII-induced sympathetic activation to iBAT consistent with the increased glucose uptake observed in this tissue in MC4R_{LHA} mice. To further test whether altered iBAT SNA will affect body’s thermoregulation, we also measured core body
temperature in a separate cohort of conscious mice by quick inserting oiled rectal probe (Kent Scientific, CT). In contrast to earlier report (28), we found that obese MC4R-TB (AAV-GFP) mice have slightly reduced core body temperature (34.81 ± 0.21) compared to WT control mice (35.59 ± 0.22) and similar reduction was also observed in MC4R-TB (AAV-Cre-GFP) mice (34.80 ± 0.23), suggesting that LHA MC4R signaling has no significant effect on core body temperature.

**The effect of MC4R<sub>LHA</sub> signaling on gene expression in iBAT and inguinal white adipose tissue (WAT)**

Activation of mitochondrial energy dissipation pathways in iBAT has previously been linked with improved glucose tolerance (29). SNA is known to regulate the expression of genes involved in mitochondrial energy dissipation, including UCP1, Cidea, PGC1a, β3-AR, Glut1 and Glut4 (30). Therefore, we measured mRNA level of these genes in iBAT to correlate changes of gene expression with SNA and FDG uptake. Gene expression profiling in iBAT revealed that significantly decreased expression of UCP1 and Cidea (Fig. 6A, 6B), but not PGC1a and β3-AR (Fig. 6C, 6D), in MC4R-TB mice were normalized in MC4R<sub>LHA</sub> mice. Notably, while Glut1 expression was similarly increased in both MC4R-TB groups (Fig. 6E), a 2-fold increase in Glut4 expression was only observed in MC4R<sub>LHA</sub> mice (Fig. 6F). None of these transcriptional changes were observed in inguinal WAT (Fig. 7A-F), indicating an iBAT-specific effect of SNA by MC4R<sub>LHA</sub> signaling. Significantly increased expression of Glut4 in iBAT may, at least in part, explain the facilitated glucose uptake in iBAT and the ameliorated hyperglycemia observed after restoration of MC4R<sub>LHA</sub> signaling.

**iBAT denervation blocks improved glucose tolerance in MC4R<sub>LHA</sub> mice**

In order to directly demonstrate that SNA to iBAT is required for the improvement in glucose tolerance, another cohort of MC4R<sub>LHA</sub> mice was generated and confirmed to have improved glucose tolerance without an effect on body weight (data not shown). One week after this GTT, mice
underwent bilateral iBAT denervation by removal of five intercostal sympathetic nerve branches subserving iBAT (Fig. 8A-C) as previously described (21), which resulted in significantly decreased UCP1 mRNA level compared to sham control group in WT male mice, confirming effective iBAT denervation (Fig. 8D). One week after recovery from surgery, all three groups of mice were subjected to GTT again. While there was no significant change in body weight during one week of recovery period (Fig. 8E), the improved glucose tolerance seen in MC4R<sup>LHA</sup> mice was lost following iBAT sympathetic denervation (Fig. 8F, 8G), confirming that iBAT SNA driven by MC4R<sup>LHA</sup> signaling is required to improve glucose intolerance in obese MC4R-TB mice.
DISCUSSION

In present study we report a previously unappreciated role of MC4R\textsuperscript{LHA} signaling in the regulation of glucose metabolism through the regulation of sympathetic outflow to iBAT. This effect may be in part mediated by upregulated expression of Glut4 in iBAT. Interestingly, while genes that are known to promote energy dissipation during excess feeding, such as UCP1 and Cidea, were upregulated in iBAT of MC4R\textsuperscript{LHA} mice, no body weight effect was observed in either chow- or HFD-fed mice. This result is consistent however with a previous report that UCP1-deficient mice are cold-sensitive but do not develop obesity under normal housing conditions (31). Our findings suggest that the energy balance and glucoregulatory roles of iBAT can be dissociated. Consistent with this possibility, a thyroid hormone β-selective agonist and a β3-AR agonist have been shown to increase SNA and thermogenesis in iBAT without increasing glucose uptake (32). These observations have led us to speculate that MC4R\textsuperscript{LHA} signaling-mediated sympathetic outflow may differentially innervate a portion of iBAT that affects glucose utilization without altering energy expenditure or blunting the severe obesity seen in MC4R-null mice.

While the primary effect that we detected for MC4R\textsuperscript{LHA} signaling was on iBAT, we did also observe a potential role for skeletal muscle as well. A trend toward increased FDG uptake was noted in MC4R\textsuperscript{LHA} mice and restoration of MC4R\textsuperscript{LHA} signaling did show a delayed response to MTII in lumbar SNA. Although these observed effects of MC4R\textsuperscript{LHA} signaling on muscle was less profound compared to iBAT, a potentially important contribution of muscle for the glucoregulatory effect of MC4R\textsuperscript{LHA} signaling should be considered as there is a significantly larger volume of muscle compared to iBAT in the humans. It is also possible that our ability to detect significant uptake of FDG by PET/CT imaging in muscle could be limited by the challenging spatial resolution of a diffuse organ such as muscle. The mechanism of MTII-mediated increase in lumbar SNA is unclear. One possibility is that because MTII
can also act on the melanocortin-3 receptor (MC3R), which may account for the residual lumbar SNA response to MTII. Further work is needed to test this possibility.

While we show that restoration of MC4R\textsuperscript{LHA} signaling does not affect circulating insulin levels as well as bolus insulin-activated signaling cascades in iBAT and liver, the role of insulin in the glucoregulatory effect of MC4R\textsuperscript{LHA} signaling should also be considered as Glut4-mediated transport of glucose is insulin dependent (33) and there was a trend toward increased pERK activation in muscle. Additionally, it is important to note that most of these measures in the present study were performed in chow fed mice, and insulin-resistance in muscle of MC4R-null mice is markedly worse under HFD feeding conditions (34; 35). It is possible that a protective effect of MC4R\textsuperscript{LHA} activity on insulin signaling in muscle may only emerge under the severe insulin resistance that occurs during HFD feeding. Nevertheless, future work with hyperinsulinemic-euglycemic clamp in HFD fed mice may be needed to clarify the role of insulin in the glucoregulatory effect of MC4R\textsuperscript{LHA} signaling. It is somewhat surprising to see that restoration of MC4R\textsuperscript{LHA} signaling reduces glucose excursion during GTT and fasting glucose levels in HFD fed mice but without a concomitant reduction in insulin levels. In fact there was a trend towards higher insulin levels during GTT in the LHACMC4R group when the lower glycemic levels would predict lower insulin levels. This finding may indicate that there is a degree of protection against obesity-related beta-cell dysfunction conferred by MC4R\textsuperscript{LHA} activity. Whether such protection might be mediated by the demonstrated improvement of glucose disposal in other tissues, by modulating factors such as glucagon-like peptide 1 or circulating nutrients (36), or by more direct action of MC4R\textsuperscript{LHA} activity on islets is unclear.

While the current study uses a combination of Cre-lox technology and advanced physiological measures to assess the function of MC4R\textsuperscript{LHA} signaling, several limitations should be noted. Our study uses the ‘reactivation’ model in which MC4R expression is restored in the LHA of adult mice. This method is well suited to determine biological sufficiency, but cannot determine if there is redundancy
in MC4R function. Indeed, a role for MC4R signaling in the regulation of autonomic activity has been reported for several additional brain sites (37; 38). Additionally, we attempted to control for ‘off-target’ effects of Cre-recombinase expression by infusing both AAV-GFP and AAV-Cre-GFP wild-type groups. However, we cannot exclude the possibility that Cre expression affects FDG uptake as we did not include WT mice as controls due to cost. Many of the methods and assessments used here are technically challenging, labor intensive, and expensive, which limits the statistical power of certain assessments. While the primary outcome measures are of large effect size, we cannot exclude that possibility that we were unable to discern subtle differences in secondary outcome measures. Finally, the relevance to human studies should be noted. Many studies have proposed increasing activity of BAT to treat metabolic disorders, such as obesity and diabetes (39). Our study is consistent with this approach, although it does suggest that the effects of BAT activity on body weight and glucose tolerance may be dissociable depending on the method utilized. However, any approach targeting SNA or BAT activity in humans may have limited therapeutic potential due to side effects. Even so, understanding the neural circuitry that improves glucose tolerance will be important to design optimal treatments.

In summary, our results expand the understanding of how MC4R signaling differentially regulates a diverse set of physiological functions. Isolating the function of this neural circuit within the LHA also improves our understanding of how specific hypothalamic circuits control glucose tolerance. The results represent a novel mechanism by which brain MC4R signaling regulates glucose homeostasis independent of energy balance and may help in the development of novel strategies for the treatment of diabetes.
ACKNOWLEDGMENTS.

We would like to thank Drs. Joel Elmquist (UT Southwestern Medical Center, Dallas, TX) and Bradford Lowell (Beth Israel Deaconess Medical Center, Boston, MA) for use of MC4R-TB mouse line. This work was funded by the following grants: National Institute of Health (MH084058-01A1 to M.L.; HL084207 to K.R.; R01 DK097820 to A.W.N.), the American Heart Association (Postdoctoral Fellowship 12POST9120037 and Scientist Developmental Grant 14SDG20140054 to H.C.; Established Investigator Award 14EIA18860041 to K.R.), Brain and Behavior Research Foundation (formerly NARSAD) Young Investigator Award to H.C. and the University of Iowa Fraternal Order of Eagles Diabetes Center Pilot Grant to M.L. H.C., K.R. and M.L. designed the experiments. D.A.M. and H.C. performed most critical experiments and analyzed data. L.N.M., T.Y., J.J. and M.K. assisted stereotaxic surgery, mouse breeding and genotyping, and Western blotting. M.R.A. and S.A.W. performed PET/CT scanning for fluorodeoxyglucose uptake experiment. L.L.B.P. analyzed fluorodeoxyglucose uptake data. H.C. wrote the manuscript and D.A.M., K.R., A.W.N. and M.L. reviewed/edited manuscript. H.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflicts to declare.
Reference


FIGURE LEGENDS

Figure 1. Reactivation of MC4R<sup>LHA</sup> signaling improves glucose tolerance in chow-fed mice without affecting food intake and body weight.

(A-C) Representative images show the location of MC4R-GFP neurons in dorsomedial part of LHA (A) where AAV-Cre-GFP microinfusion was made (B). AAV-Cre-GFP was validated in tdTomato reporter mouse by observing Cre-GFP (green) and tdTomato (red) fluorescent signals (C). After stereotaxic delivery of AAV into the LHA of young adult male mice, body weight gain (D), cumulative food intake (E), locomotor activity (F), basal blood glucose (G) and insulin (H) levels, GTT (I) and its area under curve (AUC) analysis (J), insulin secretion response to IP glucose (1 g/Kg) (K), body composition (L), the mass of regional fat pads including BAT (M), perigonadal WAT (N) and perirenal WAT (O) were measured in chow-fed mice. [for D-J and L-O, n=10, 7, 12 for control, MC4R-TB (AAV-GFP), MC4R-TB (AAV-Cre-GFP), respectively; for K, n=9, 7, 8 for control, MC4R-TB (AAV-GFP), MC4R-TB (AAV-Cre-GFP), respectively]. *p<0.05, **p<0.01 by one-way or two-way ANOVA with bonferroni post hoc. Data are presented as mean ± SEM.

Figure 2. Reactivation of MC4R<sup>LHA</sup> signaling improves hyperglycemia and glucose tolerance in HFD-fed mice without affecting food intake or body weight.

After stereotaxic delivery of AAV into the LHA of young adult male MC4R-TB mice, body weight gain (A), cumulative food intake (B), basal blood glucose (C) and insulin (D) levels, GTT (E) and its AUC analysis (F), blood glucagon (G), leptin (H), FFA (I), triglyceride (J), cholesterol (K), dHDL (L) levels were measured in HFD-fed mice. [n=9, 17 for MC4R-TB (AAV-GFP), MC4R-TB (AAV-Cre-GFP), respectively]. *p<0.05, **p<0.01 by two-way ANOVA with bonferroni post hoc or by Student's t-test. Data are presented as mean ± SEM.
Figure 3. Restoration of MC4R<sub>LHA</sub> signaling increases FDG uptake in iBAT.

(A) Representative images showing FDG uptake in iBAT at baseline or after MTII administration. (B) Body weight of each group of mice used in FDG PET/CT scanning. (C-E) The change of FDG uptake (normalized SUV) by MTII in iBAT (C), muscle (D) and brain (E) [n=6, 6 for MC4R-TB (AAV-GFP), MC4R-TB (AAV-Cre-GFP), respectively]. *p<0.05 by Student's t-test. Data are presented as mean ± SEM.

Figure 4. Restoration of MC4R<sub>LHA</sub> signaling does not affect hepatic gluconeogenesis and peripheral insulin signaling.

(A) Body weight of each group of mice used in PTT. (B) PTT (2g/Kg) and (C) its AUC analysis [n=10, 9 and 11 for control, MC4R-TB (AAV-GFP), MC4R-TB (AAV-Cre-GFP), respectively]. Representative western blotting image of bolus insulin (5 units)-mediated induction of pAKT (D) and pERK (H) in iBAT of WT mice. (E-G) Immunoblotting quantification of an induction of pAKT by bolus insulin in iBAT (E), soleus muscle (F) and liver (G) (n=5-6/group). (I-K) Immunoblotting quantification of an induction of pERK by bolus insulin in iBAT (I), soleus muscle (J) and liver (K) (n=7-9/group). *p<0.05, ***p<0.001 by one-way or two-way ANOVA with bonferroni post hoc. Data are presented as mean ± SEM.

Figure 5. Reactivation of LHA MC4R signaling normalizes impaired SNA response in to MTII in iBAT and muscle of MC4R-null mice.

(A) Representative images showing iBAT SNA recording at baseline and at 4<sup>th</sup> hour of ICV MTII. (B) Four-hour time course of MTII-induced changes of iBAT SNA from baseline activity [n=10, 7, 7 for control, MC4R-TB (AAV-GFP) and MC4R-TB (AAV-Cre-GFP), respectively]. (C) Four-hour time
course of MTII-induced changes of lumbar SNA from baseline activity [n=10, 6, 6 for control, MC4R-TB (AAV-GFP) and MC4R-TB (AAV-Cre-GFP), respectively]. *p<0.05, **p<0.01 by two-way ANOVA with bonferroni post hoc [comparison between MC4R-TB (AAV-GFP) and MC4R-TB (AAV-Cre-GFP) groups]. Data are presented as mean ± S.E.M.

**Figure 6. The effect of restoration of MC4R<sub>LHA</sub> signaling on iBAT gene expression.**

Whole iBAT tissues was collected from chow-fed 16-18 weeks old male mice (n=6/group), snap frozen in liquid nitrogen and processed for total mRNA extraction. Quantitative PCR was performed to determine relative abundance of expression of UCP1 (A), Cidea (B), PGC1a (C), β3-AR (D), Glut1 (E) and Glut4 (F). *p<0.05, ***p<0.001 by one-way ANOVA with bonferroni post hoc. Data are resented as mean ± SEM.

**Figure 7. The effect of restoration of MC4R<sub>LHA</sub> signaling on inguinal WAT gene expression.**

Whole iBAT tissues was collected from chow-fed 16-18 weeks old male mice [n=6 for each group of WT (AAV-Cre-GFP), MC4R-TB (AAV-GFP) and MC4R-TB (AAV-Cre-GFP) mice], snap frozen in liquid nitrogen and processed for total mRNA extraction. Quantitative PCR was performed to determine relative abundance of expression of UCP1 (A), Cidea (B), PGC1a (C), β3-AR (D), Glut1 (E) and Glut4 (F). *p<0.05, ***p<0.001 by one-way ANOVA with bonferroni post hoc. Data are resented as mean ± SEM.

**Figure 8. Bilateral iBAT denervation prevents the glucoregulatory effect of MC4RLHA signaling.**

(A) Representative image showing five branches of intercostal nerves before denervation. (B) Five
branches of intercostal nerves were sectioned out as shown. (C) Representative image showing after surgical procedure of iBAT denervation. (D) iBAT denervation in WT male mice significantly decreased UCP1 mRNA expression in iBAT compared to sham control WT mice. (E) Body weight of each group of mice after one week of iBAT denervation. (F) GTT and (G) its AUC analysis after iBAT denervation. *p<0.05, **p<0.01, ***p<0.001 by student’s t-test or one-way ANOVA with bonferroni post hoc. Data are resented as mean ± SEM.
A

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B

Body weight (g)

C

BAT

D

P=0.103

E

AFDQ uptake (normalized SUV)

99x47mm (300 x 300 DPI)
Diabetes

151x112mm (300 x 300 DPI)
Diabetes
Diabetes
Diabetes

E

F

G

129x71mm (300 x 300 DPI)
Figure 1D: Two-way ANOVA reveals there is significant effect of genotype over time \[ F(2, 26) = 40.79, p<0.0001 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p<0.0001 \) or MC4R-TB (AAV-Cre-GFP) \( p<0.0001 \) groups, but not between two MC4R-TB groups \( p > 0.999 \), at the end of study.

Figure 1E: Two-way ANOVA reveals there is significant effect of genotype over time \[ F(2, 26) = 14.16, p<0.0001 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p<0.0001 \) or MC4R-TB (AAV-Cre-GFP) \( p<0.0001 \) groups, but not between two MC4R-TB groups \( p > 0.999 \), at the end of study.

Figure 1F: There were statistically significant differences between group means as determined by one-way ANOVA \[ F(2, 20) = 5.594, p=0.0118 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p<0.05 \) or MC4R-TB (AAV-Cre-GFP) \( p<0.05 \) groups, but not between two MC4R-TB groups \( p > 0.999 \).

Figure 1G: There were statistically significant differences between group means as determined by one-way ANOVA \[ F(2, 26) = 10.38, p=0.0005 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p=0.0006 \) or MC4R-TB (AAV-Cre-GFP) \( p=0.0082 \) groups, but not between two MC4R-TB groups \( p=0.4372 \).

Figure 1H: There were statistically significant differences between group means as determined by one-way ANOVA for fed condition \[ F(2, 26) = 4.638, p=0.0252 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p=0.0312 \) or MC4R-TB (AAV-Cre-GFP) \( p=0.0411 \) groups, but not between two MC4R-TB groups \( p=0.8994 \).

Figure 1I: There were statistically significant differences between group means as determined by one-way ANOVA for fed condition \[ F(2, 26) = 18.70, p<0.0001 \]. Bonferroni post hoc test indicated that, in addition to a significant difference was between control and MC4R-TB (AAV-GFP) \( p<0.0001 \) or MC4R-TB (AAV-Cre-GFP) \( p=0.0024 \) groups, there was also a significant difference between two MC4R-TB groups \( p=0.0291 \).

Figure 1K: Two-way ANOVA reveals there is significant effect of genotype over time \[ F(2, 21) = 40.79, p<0.0001 \]. Following bonferroni post hoc test indicated that, for each of time point, the significant difference was between control and MC4R-TB (AAV-GFP) or MC4R-TB (AAV-Cre-GFP) groups, but not between two MC4R-TB groups.

Figure 1L: Two-way ANOVA reveals there is significant effect of genotype over time \[ F(2, 26) = 20.12, p<0.0001 \]. Bonferroni post hoc test indicated that, for each parameter, the significant difference was between control and MC4R-TB (AAV-GFP) or MC4R-TB (AAV-Cre-GFP) groups, but not between two MC4R-TB groups.

Figure 1M: There were statistically significant differences between group means as determined by one-way ANOVA \[ F(2, 26) = 22.790, p<0.0001 \]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \( p<0.0001 \) or MC4R-TB (AAV-Cre-GFP) \( p<0.0001 \) groups, but not between two MC4R-TB groups \( p>0.9999 \).
Figure 1N: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 26) = 55.39, p<0.0001\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p<0.0001\) or MC4R-TB (AAV-Cre-GFP) \(p<0.0001\) groups, but not between two MC4R-TB groups \(p>0.9999\).

Figure 1O: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 26) = 30.27, p<0.0001\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p<0.0001\) or MC4R-TB (AAV-Cre-GFP) \(p<0.0001\) groups, but not between two MC4R-TB groups \(p>0.9999\).

Figure 4A: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 27) = 123.5, p<0.0001\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p<0.0001\) or MC4R-TB (AAV-Cre-GFP) \(p<0.0001\) groups, but not between two MC4R-TB groups \(p>0.9999\).

Figure 4C: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 27) = 9.54, p=0.0008\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p=0.0007\) or MC4R-TB (AAV-Cre-GFP) \(p=0.0271\) groups, but not between two MC4R-TB groups \(p=0.5189\).

Figure 4E: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 15) = 3.667, p=0.0218\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p=0.0311\) or MC4R-TB (AAV-Cre-GFP) \(p=0.0421\) groups, but not between two MC4R-TB groups \(p=0.9275\).

Figure 4F: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 15) = 3.325, p=0.0265\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-Cre-GFP) \(p=0.0121\) groups, but not between control and MC4R-TB (AAV-GFP) \(p=0.0711\) or between two MC4R-TB groups \(p=0.6325\).

Figure 4G: There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 14) = 4.166, p=0.0124\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p=0.0288\) or MC4R-TB (AAV-Cre-GFP) \(p=0.021\) groups, but not between two MC4R-TB groups \(p=0.937\).

Figure 4I: There were no statistically significant differences between group means as determined by one-way ANOVA \([F(2,21) = 1.53, p =0.2397]\).

Figure 4J: There were no statistically significant differences between group means as determined by one-way ANOVA \([F(2,21) = 2.990, p =0.072]\).

Figure 4K: There were statistically significant differences between group means as determined by one-way ANOVA \([F(2, 21) = 6.036, p=0.0085]\). Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) \(p=0.023\) or MC4R-TB (AAV-Cre-GFP) \(p=0.0216\) groups, but not between two MC4R-TB groups \(p>0.9999\).
Figure 5B: Two-way ANOVA reveals there is significant effect of genotype over time \( [F(2, 21) = 8.823, p=0.0017] \). Bonferroni post hoc test indicated that the significant difference was between MC4R-TB (AAV-GFP) and control (p<0.0001) or MC4R-TB (AAV-Cre-GFP) (p=0.0016) groups, but not between control and MC4R-TB (AAV-Cre-GFP) groups (p=0.4794), at the end of study.

Figure 5C: Two-way ANOVA reveals there is significant effect of genotype over time \( [F(2, 19) = 4.901, p=0.0192] \). Bonferroni post hoc test indicated that the significant difference was between MC4R-TB (AAV-GFP) and control (p=0.0102) or MC4R-TB (AAV-Cre-GFP) (p=0.0158) groups, but not between control and MC4R-TB (AAV-Cre-GFP) groups (p>0.9999), at the end of study.

Figure 6A: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 20) = 7.820, p=0.0012] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p=0.0463).

Figure 6B: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 20) = 46.27, p<0.0001] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p<0.0001).

Figure 6C: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 20) = 75.88, p<0.0001] \). Bonferroni post hoc test indicated that there is no significant difference between two MC4R-TB groups (p>0.9999).

Figure 6D: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 20) = 1087, p=0.0002] \). Bonferroni post hoc test indicated that there is no significant difference between two MC4R-TB groups (p>0.9999).

Figure 6E: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 19) = 11.94, p<0.0001] \). Bonferroni post hoc test indicated that there is no significant difference between two MC4R-TB groups (p>0.9999).

Figure 6F: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(3, 20) = 23.34, p<0.0001] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p<0.0001).

Figure 7A: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(2, 15) = 88.38, p<0.0001] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p>0.9999).

Figure 7B: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(2, 15) = 151.7, p<0.0001] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p>0.9999).

Figure 7C: There were statistically significant differences between group means as determined by one-way ANOVA \( [F(2, 15) = 60.97, p<0.0001] \). Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p>0.9999).
**Figure 7D:** There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 15) = 107.8, p<0.0001\]. Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p>0.9999).

**Figure 7E:** There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 15) = 7.983, p=0.0044\]. Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p=0.6796).

**Figure 7F:** There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 15) = 44.80, p<0.0001\]. Bonferroni post hoc test indicated that there is a significant difference between two MC4R-TB groups (p>0.9999).

**Figure 8E:** There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 26) = 117.7, p<0.0001\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) (p<0.0001) or MC4R-TB (AAV-Cre-GFP) (p<0.0001) groups, but not between two MC4R-TB groups (p>0.9999).

**Figure 8G:** There were statistically significant differences between group means as determined by one-way ANOVA \[F(2, 26) = 10.36, p=0.0005\]. Bonferroni post hoc test indicated that the significant difference was between control and MC4R-TB (AAV-GFP) (p=0.0005) or MC4R-TB (AAV-Cre-GFP) (p=0.0119) groups, but not between two MC4R-TB groups (p=0.5922).