Brief communication

MOLECULAR METABOLISM

Glutamate release mediates leptin action on energy expenditure

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ABSTRACT

Restricting energy expenditure is an adaptive response to food shortage. Despite being insulated with massive amount of fat tissues, leptin-deficient mice lose the ability to maintain their body temperature and develop deep hypothermia, which can be suppressed by exogenous leptin, suggesting an important role for leptin in energy expenditure regulation. However, the mechanism underlying the leptin action is not clear. We generated mice with disruption of glutamate release from leptin receptor-expressing neurons by deleting vesicular glutamate transporter 2 in these neurons, and found that these mice developed mild obesity purely due to reduced energy expenditure, exhibited bouts of rapidly reduced energy expenditure, body temperature and locomotion. In addition, these mice exhibited lower energy expenditure and body temperature in response to fasting and were defective in leptin-mediated thermogenic action in brown adipose tissues. Taken together, our results identify a role for glutamate release in mediating leptin action on energy expenditure.

Keywords Glutamate; Leptin; Energy expenditure; Hypothermia; Obesity

1. INTRODUCTION

Homeothermic animals are capable of adjusting thermogenesis to maintain a constant body temperature, which ensures that bodily system functions most efficiently [1]. However, when facing adverse environments such as fasting, these animals reduce energy expenditure (EE) and develop forms of hypothermia, for example, torpor, to reserve energy for survival [2]. Torpor is an adaptive response to fasting and cold, characteristic with bouts of rapidly reduced EE, body temperature and locomotion to save energy. Since torpor is a controlled process animals can recover from torpor without noticeable defects [3]. Previous studies have implicated a role for NPY in the brain, fibroblast growth factor (FGF) 21 from liver and ghrelin from gut in the regulation of EE and hypothermia [3–4]. However, the neural pathway underlying hypothermia and torpor regulation is not yet clear.

The role of leptin in body weight regulation has been well established. Part of the mechanism underlying obesity is reduced EE as a result of diminished thermogenic activity due to defective leptin action [5–6]. Leptin deficient mice show lower body temperature [7]. Conversely, leptin action in the hypothalamus increases body temperature through the sympathetic nervous input to the brown adipose tissue (BAT), the major thermogenic organ in rodents [8–9]. Consistent with the role of leptin in body temperature regulation and thermogenesis, mice with leptin-deficiency, although being insulated by large amounts of fat tissues, cannot defend their body temperature, develop hypothermia and enter torpor in response to fasting [10]. Importantly, administration of exogenous leptin prevents the entry of torpor in these mice [10], suggesting a critical role for leptin in torpor-related EE regulation. However, the neural pathway underlying leptin action in torpor-related EE regulation is not clear.

Leptin action is primarily mediated by isoform B leptin receptor (LepR) in the brain [11]. LepR-expressing neurons (LepR neurons) reside predominantly in the hypothalamus, but also in other brain regions including the brainstem nuclei. Within the hypothalamus, LepRs are abundant in the arcuate nucleus, ventromedial hypothalamus (VMN), dorsomedial hypothalamus (DMH), ventral premammillary (PMv), preoptic area (POA) and lateral hypothalamus [12,13]. Despite intensive research on LepRs in these discrete brain sites, the relative contribution of LepRs in these sites on body weight regulation is not clear, suggesting the complexity of leptin neural pathway. To reduce the level of complexity, Vong et al. used a novel approach by divining LepR neurons into broadly glutamatergic and GABAergic groups, and found that LepRs in GABAergic neurons mediate a major part of leptin action on body weight regulation while those in glutamatergic neurons mediate a small part [14]. We previously showed that GABA release from LepR neurons is required for both normal food intake and energy expenditure [15]. However, it remains untested whether glutamate release from LepR neurons mediates leptin action on energy balance regulation.

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2. MATERIALS AND METHODS

2.1. Materials

Leptin was purchased from Dr. Parlow from NHPP.

2.2. Animals

Generation of *LepR-iCre* mice, Ai9 mice and *Vglut2*$^{flox/flox}$ mice was described previously [16–18]. Study subjects were generated by mating *LepR-iCre:Vglut2*$^{flox/flox}$ mice with *Vglut2*$^{flox/flox}$ mice. *Vglut2-Ires-Cre* mice [14] , generously provided by Dr. Bradford Lowell, were crossed with Z/EG reporter mouse to visualize glutamatergic neurons. All animals and procedures were approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston or Pennington Biomedical Research Center. Mice were housed at 22–24 °C with a 14 h light/10 h dark cycle with standard mouse chow (Teklad F6 Rodent Diet 8664, 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat, Harlan Teklad, Madison, WI) and water provided ad libitum.

2.3. Body weight studies

Weekly body weight was monitored in controls and *LepR-iCre:Vglut2*$^{flox/flox}$ mice fed standard mouse chow (Teklad F6 Rodent Diet 8664, Harlan Teklad, Madison, WI) from 4 to 20 weeks of age and on the high-fat diet study (HFD, D12331 from Research Diets, NJ) from 6 weeks of age and maintained on HFD for 20 weeks. Body composition was measured at indicated times using an Echo-MRI machine (Echo MRI, Houston, Texas).

2.4. Energy expenditure and food intake measurements

Energy expenditure was measured by oxygen consumption using indirect calorimetry. Individual housed mice maintained on chow diet at 7–8 weeks old were placed at room temperature (22–24 °C) in chambers of a Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH). Daily food intake was measured for 6 days for mice, which have been individually housed for at least 1 week on either chow diet or high fat diet. Accumulated food intake was calculated based one first 3 days measured for food intake.

2.5. Immunohistochemistry (IHC) and in situ hybridization (ISH) studies

The digoxigenin-labeled cRNA probes were generated against *Vglut2* mRNA, and ISH was performed as we previously described [15]. Briefly, free-floating brain sections were rinsed with diethylpyrocarbonate(-DEPC)-treated PBS, pH 7.0, for 30 min before being fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) in DEPC-PBS for 30 min. Tissues were then incubated with 3% H_2O_2 and then treated with proteinase K (2 μg/ml; Roche Applied Bioscience, Indianapolis, IN). After the fixation again in 4% paraformaldehyde, the sections were treated for 10 min in 0.1 M triethanolamine-HCl, pH 8.0. Then cRNA probes diluted in hybridization buffer (500 ng/ml) were applied to the tissues for overnight at 60 °C. Following rinsed with 0.3% Triton X-100, 0.15 M NaCl, 100 mM Tris–HCl, pH 7.5 (TNT buffer) for 10 min and blocked with 5% normal sheep serum in TNT buffer for 1 h, tissues were incubated with anti-digoxigenin antibody (1:200, Fab fragment; Roche Applied Bioscience) conjugated with horseradish peroxidase overnight at 4 °C. Probes were visualized with tyramide signal amplification kit (PerkinElmer, Inc., Wellesley, MA) after three thorough washes in TNT buffer. Sections were photographed with a TCS SP5 confocal microscope (Leica, Nussloch, Germany). To qualify cell numbers, in each mouse ($n=3$), three brain sections for ventromedial hypothalamus (VMH), and two sections for dorsomedial hypothalamus (DMH) and ventral premammillary (PMv) at the same rostrocaudal levels were chosen. All immunoreactive cells with clear profile were counted, and the numbers from all animals were summed and then averaged as the number per section.

For p-STAT3 (signal transducer and activator of transcription 3) and c-fos detection, animals (9–10 weeks old males) received daily handling and injections of saline for 1 week to reduced stress levels. On the testing day, for p-STAT3 experiment, single dose of leptin (2 μg/g body weight) were i.p. injected at 9 am after 24 h fasting and mice were perfused 45 min later; for c-fos experiments, animals were perfused 2 h post leptin (5 μg/g body weight) or saline injection. After fixation, brains were sliced into sections and immunostained. Phosphor-specific-(Tyr705) STAT3 rabbit antiserum (1:1000; Cell Signaling Technology, Danvers, MA) and rabbit anti-c-fos (Ab-5, 1:1000; Millipore, Billerica, MA) were used and followed by Alexa fluor 488 conjugated donkey antirabbit IgG (1:100; Invitrogen, Carlsbad, CA). Sections were taken with the TCS SP5 confocal microscope. To quantify cell numbers, in each mouse ($n=5$–7), three sections for paraventricular hypothalamus (PVH), DMH, and preoptic area (POA) at corresponding rostrocaudal levels were chosen. All immunoreactive cells with clear profile were counted, and the numbers from all animals were summed and then averaged as the number per section.

2.6. Quantitative PCR assay

RNA was extracted from hypothalamic, liver and brown adipose tissues, micro-punched from mice, using the Trizol Reagent (Invitrogen), and was reverse transcribed with RETROscript (Ambion) and amplified using SYBR green technology (Bio-Rad, Hercules, CA). The primers for real time PCR are as follows: POMC, 5′-AGAACGCCATCATCAAGAAC-3′ and 5′-AAGAGGCTAGAGGTCATCAG-3′; NPY, 5′-GTGGATCTCTTCTCTCACAGAGG-3′ and 5′-GCCCAAACACACGAGCAGAG-3′; FGF-21, 5′-CTCTAGGTTTCTTTGCCAACAG-3′ and 5′-AAGCTGCAGGCCTCAGGAT-3′; UCP-1; 5′-TGGAGGTGTGGCAGTGTTCA-3′ and 5′-GCTCTGGGCTTGCATTCTG-3′. Assays were linear over five orders of magnitude.

2.7. Body temperature and movement measurement

As previously reported [8], precalibrated sensitive transmitters (PDT-4000 G2 E-Mitter sensors, Respironics Inc. Murrysville, PA, USA) were used for performing telemetric measurements. For measuring interscapular BAT temperature and locomotor activity, mice were anesthetized with ketamine/xylazine and then implanted E-Mitters underneath the iBAT pad between the scapulae. For core body temperature monitoring, E-Mitters were placed into the peritoneal cavity. Mice were allowed for 1 week recovery before all data were collected. Signals emitted by the E-Mitter transponders were sensed by a receiver positioned underneath the animal's housing cage and analyzed using VitalView software (Respironics Inc.). Locomotor activity counts are recorded as gross motor activity. For all experiments, activity counts and temperature measurements were taken every 1 min. All mice were acclimated for at least 3 days and then data were collected for 24 h. Multiple series of data at the same collected time point and from the same genotype mice were summed and then averaged to get their mean temperature and movement.

2.8. Glucose homeostasis studies

Fed blood glucose levels were measured between 9 am and 10 am and fasted blood glucose levels were obtained 24 h after removing food at 9 am on the previous day. For glucose tolerance test study, individually housed animals with overnight fasting were administrated with i.p. glucose (1.5 g/kg), and glucose levels were measured at time (minutes) 0, 15, 30, 60 and 90 after glucose administration. Similarly, for insulin tolerance test, individually housed animals with 4-hour fasting were administrated with i.p. insulin (1 U/kg), and glucose levels were measured at time (minutes) 0,