Sex-dependent changes in metabolism and behavior, as well as reduced anxiety after eliminating ventromedial hypothalamic excitatory output

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ABSTRACT

Objectives: The ventromedial hypothalamic nucleus (VMH) regulates energy homeostasis as well as social and emotional behaviors. Nearly all VMH neurons, including those in the sexually dimorphic ventrolateral VMH (VMHvl) subregion, release the excitatory neurotransmitter glutamate and use the vesicular glutamate transporter 2 (Vglut2). Here, we asked how glutamatergic signaling contributes to the collective metabolic and behavioral responses attributed to the VMH and VMHvl.

Methods: Using Sfi1-Cre and a Vglut2 floxed allele, Vglut2 was knocked-out in SF-1 VMH neurons (Vglut2Sfi1-Cre). Metabolic and neurobehavioral assays were carried out initially on Vglut2Sfi1-Cre and Vglut2Sfi1-Cre mice in a mixed, and then in the C57BL/6 genetic background, which is prone to hyperglycemia and diet induced obesity (DIO).

Results: Several phenotypes observed in Vglut2Sfi1-Cre mice were largely unexpected based on prior studies that have perturbed VMH development or VMH glutamate signaling. In our hands, Vglut2Sfi1-Cre mice failed to exhibit the anticipated increase in body weight after high fat diet (HFD) or the impaired glucose homeostasis after fasting. Instead, there was a significant sex-dependent attenuation of DIO in Vglut2Sfi1-Cre females. Vglut2Sfi1-Cre males also display a sex-specific loss of conditioned-fear responses and aggression accompanied by more novelty-associated locomotion. Finally, unlike the higher anxiety noted in Sfi1Nestin-Cre mice that lack a fully formed VMH, both male and female Vglut2Sfi1-Cre mice were less anxious.

Conclusions: Loss of VMH glutamatergic signaling sharply decreased DIO in females, attenuated aggression and learned fear in males, and was anxiolytic in males and females. Collectively, our findings demonstrate that while glutamatergic output from the VMH appears largely dispensable for counter regulatory responses to hypoglycemia, it drives sex-dependent differences in metabolism and social behaviors and is essential for adaptive responses to anxiety-provoking stimuli in both sexes.

Keywords VMH; VGlut2; Sex-dependent obesity; Excitatory output; Anxiety; Male aggression

1. INTRODUCTION

The murine ventromedial hypothalamus (VMH) is molecularly and functionally complex as evidenced by gene expression patterns [1] and phenotypes arising from numerous genetic lesions. Physiological and behavioral functions associated with the VMH include metabolic homeostasis, reproduction, social behaviors, anxiety, and female-specific energy expenditure, all of which are presumably mediated by distinct VMH neuronal subpopulations. Although complete annotation of these functionally distinct VMH neuronal subsets has yet to emerge, nearly all VMH neurons express two markers, steroidogenic factor 1 (SF-1 encoded by Nr5a1), and the vesicular glutamate transporter 2 (VGLUT2 encoded by Slc17a6). The prominent expression of Vglut2 in the VMH [2,3] suggests that excitatory, glutamatergic neurotransmission mediates multiple and diverse aspects of VMH functions. In addition, glutamatergic connections between the VMH and other metabolic brain centers, such as the arcuate nucleus have been documented [4,5]. To date, numerous genetic perturbations of the VMH have been reported, which target either VMH development or general signaling components affecting metabolism. For example, disrupting VMH
development by reducing or ablating SF-1 prenatally leads to obesity in both sexes on standard chow and accelerates diet-induced obesity (DIO) on high fat diet (HFD) [6–8]. Eliminating SF-1 postnatally, using the CamKII-Cre preserves the gross architecture of the VMH, but still promotes DIO as well as hyperglycemia in both fed and fasted states [6]. We previously reported that blocking development and migration of neurons to the sexually dimorphic ventrolateral VMH (VMHvl) subregion results in marked female-specific obesity on standard chow [9]. Several genetic lesions in SF-1 expressing neurons that manipulate metabolic signaling pathways are also reported to change DIO in both sexes. Reducing leptin sensitivity after deleting LepR, PI3K (p110α), or Ptpn1 with the Sfi1-Cre increases susceptibility to DIO [10–13]. On the other hand, deleting insulin signaling (Insr) using the same Sfi1-Cre prevents insulin-mediated inhibition of VMH neuronal activity and decreases DIO, at least in males [14]. Whether loss of these general signaling components in other SF-1 expressing tissues contributes to the observed metabolic phenotypes remains unclear. It is also unclear whether disrupting excitatory neurotransmission from all VMH neurons recapitulates the selective genetic lesions described above and leads to increased food intake and DIO. Tong et al. previously generated a VMH knockout of Vglut2 using Sfi1-Cre (Vglut2Sfi1-Cre) in a mixed genetic background [15]. Surprisingly, only a modest increase in weight gain is observed in Vglut2Sfi1-Cre males and females in response to a high-sucrose, high-fat diet (58% kcal fat). However, mutant mice exhibit lowered serum glucose in the fasted but not fed state, suggesting that loss of VMH excitatory output blunts the counterregulatory response to hypoglycemia. As mentioned above, the VMH also regulates fear and anxiety-like behaviors [16–18] as well as social behaviors that are regulated by the VMHvl, such as male aggression [19–21]. Inhibiting SF-1 neurons via pharmacogenetics decreases freezing to predator stimuli in both males and females, suggesting that the VMH is required for mounting appropriate defensive behaviors in both sexes [17]. In a CNS-specific knockout of SF-1 using the Nestin-Cre (Sfi1Nestin-Cre), anxiety-like behaviors are elevated as evidenced by fewer entries and less time spent in the open arm in the elevated plus-maze (EPM) assay [18]. However, as with the global Sfi1-Cre knockout, Sfi1Nestin-Cre mice show gross abnormalities in VMH architecture. Hence, it is unclear if the increased anxiety phenotype noted in the Sfi1Nestin-Cre mice arises from organizational rather than functional VMH deficits. To assess how blocking all excitatory VMH output might modulate the many physiological and behavioral functions associated with the VMH, we recreated the Vglut2Sfi1-Cre allele using Sfi1-Cre and a Vglut2 floxed allele (Vglut2Cre) [22]. As shown previously, targeted deletion of Vglut2 effectively blocks all synaptic release of glutamate from neurons that express this transporter protein [23]. Using multiple metabolic and behavioral assays, we examined the consequences of silencing glutamatergic signaling in both male and female mice bred in two genetic backgrounds. Analyses of these Vglut2Sfi1-Cre mice show that excitatory VMH output is an important factor in female metabolism, but surprisingly, less so for glucose homeostasis. Further, our results establish an essential role for VMH glutamatergic output in mounting adaptive behavioral responses to contextual and social cues.

2. MATERIALS & METHODS

2.1. Animals

Vglut2Cre mice in a mixed C57BL/6, Sv129J background were provided by Dr. R.D. Palmiter, (University of Washington) [22]. Sfi1-Cre mice were provided by Dr. J.K. Elmquist (University of Texas Southwestern Medical Center) [11] and subsequently backcrossed into the C57BL/6 background (Taconic Biosciences), which was confirmed by microsatellite analysis. Vglut2fl mice were generated and maintained on a mixed background or backcrossed for 10 generations into the C57BL/6 background. Experimental cohorts were obtained by crossing Sfi1Cre, Vglut2Cre with Vglut2Cre mice. We previously generated and characterized the Sfi1TadGFP reporter mice [24]. Mice were maintained on a 12 h light–dark cycle with ad libitum access to water and to either a standard chow diet (50:58, RatDiet, 4% fat) or a high fat diet (D12492; Research Diets, 60% fat), as indicated. All animal procedures were performed in accordance with the UCSF animal care committee’s regulations and the Ingraham lab IACUC protocol of record.

2.2. Tissue collection and processing

Mice were deeply anesthetized with 2.5% Avertin and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA). Brains were dissected and post-fixed overnight in 4% PFA. Fixed tissue was cryoprotected in 30% sucrose and embedded in OCT (Tissue-Tek). Cryosections (20 μm) were collected on glass slides and stored at −80 °C until processing.

2.3. Immunofluorescence and in situ hybridization

Frozen brain sections were processed using standard procedures. Reporter GFP expression was detected by immunofluorescence using chicken anti-GFP antibody (1:2500; Aves Labs) and AlexaFluor 488-conjugated goat anti-chicken antibody (1:1000; Invitrogen). Detection of mRNA expression by in situ hybridization (ISH) was accomplished using probes against the carboxy-termini and 3’ UTR of Vglut1 and Vglut3 (nt:1589–2510 and nt:1511–1869, respectively); exon 2 of Vglut2 (nt:902–1230); and exons 9–18 of Gad67 (nt:1313–2267). DIG-labeled (Roche) riboprobes were transcribed according to the manufacturers specifications. ISH was performed using standard procedures, as previously described [1].

Double-label immunofluorescence in situ hybridization (ISH) was performed with the following modifications to standard IF/ISH protocols. Sections were permeabilized with 0.3% Triton X-100 (in PBS) for 10 min at room temperature. Vglut2 riboprobe was hybridized to the sections overnight at 72 °C. Sections were washed and blocked with 10% heat-inactivated donkey serum (HIDS) in buffer B1 (0.1 M Tris, pH 7.5; 0.15 M NaCl) for 3 h at room temperature. Primary antibodies (1:3000 Anti-Dig-AP, Roche; 1:2500 chicken anti-GFP) diluted in B1 with 2% HIDS were incubated overnight at 72 °C. Sections were washed and incubated with biotin-conjugated goat anti-chicken antibody (1:500) (Invitrogen) in B1 with 2% HIDS for 2 h at room temperature. After washing 3 × 5 min in buffer TNT (1 M Tris HCl, pH 7.5; 5 M NaCl; 0.05% Tween 20), 100% methanol was added for 10 min, followed by 100% ethanol for 10 min, and finally 0.1% H2O2 in 100% ethanol for 5 min.

2.4. Metabolic analyses

Male and female body weights were recorded weekly beginning at the time of weaning (3 wks of age). Fed and 24-hour fasting glucose levels were measured in 8–10 wk old mice on standard chow or after 6 wks on HFD. Glucose tolerance tests were performed after 6 wks on HFD. Mice were fasted overnight for 16 h and given glucose (2 g/kg) by IP injection. For both assays, blood was collected from the tail vein, and glucose levels measured using the OneTouch Ultra Glucometer (LifeScan). Measurements of metabolic parameters and indirect calorimetry were conducted by the UCSF Diabetes Center Metabolic Core facility using.
the Oxymax Comprehensive Laboratory Animal Monitoring System (CLAMS) chambers. Prior to data collection, mice were acclimated to individual housing for 1 wk and subsequently acclimated to CLAMS chambers for 3 days. Chambers were maintained at room temperature, and mice had *ad libitum* access to standard chow and water.

2.5. Behavioral analyses

Levels of anxiety in adult male and female mice were analyzed by the UCSF Gladstone Mouse Phenotyping Core Facility (http://labs.gladstone.ucsf.edu/behavioral/home). For all assays described below, adult Vglut2^{fl/fl} and Vglut2^{sf2t-Cre} control littersmates in the C57BL/6 background were used. Tests were started during the light phase between 9:00—11:00. Mice were allowed to recover for at least 1 week between each test. The order of testing for each experimental cohort is as follows:

2.6. Rotarod

Subjects were transferred to the room containing the rotarod apparatus 30 min prior to testing. During the test, the speed of the rotating rod was increased from 4 to 40 rpm at 8 rpm/min. Latency to fall was recorded by the experimenter. Vertical spacers on the rod allowed five mice to be tested simultaneously. Each mouse was tested 3 times with an inter-trial interval of 15—20 min. The rotarod was cleaned with 70% ethanol between testing sessions.

2.7. Open field assay

The arena was a clear plastic chamber (41 × 41 × 30 cm). Activity and position measurements in the arena were recorded electronically using two 16 × 16 photobeam arrays (San Diego Instruments). Mice were habituated to the testing room under normal light for 30 min. During the testing interval, mice were allowed to explore the arena for 15 min. The arena was cleaned with 70% ethanol between testing sessions.

2.8. Elevated plus-maze

The testing apparatus consisted of a plus-shaped maze (Hamilton—Kinder) with two open and two closed arms positioned 63 cm above the ground. Mice were transferred to the testing room and acclimated to dim lighting for 30 min. At the start of the test, subjects were placed in the intersection of the plus-maze. Mice were allowed to explore the maze for 10 min, while activity and location measurements were recorded electronically. The maze was cleaned with 70% ethanol between testing sessions.

2.9. Corticosterone assay

Tail vein blood was collected in the beginning of the light phase to determine baseline corticosterone levels in control and mutant mice. The following day, an acute stress was induced by 15 min of restraint in the Broome Rodent Restraint (Harvard Apparatus) or by a 5 min exposure to the elevated plus-maze. Following these stressors, a second blood sample was collected. Plasma hormone levels were quantified using the Corticosterone Enzyme Immunoassay (EA) Kit (Cayman Chemical), according to the manufacturer’s instructions.

2.10. Cued fear conditioning

Training and testing were performed in a sound attenuated clear, square chamber. Mice were transferred to the room containing the conditioning apparatus 30 min before training or testing. Initial levels of freezing were recorded of 5 min with no audible tone or shock presented. The next day, 3 min baseline freezing levels were recorded. The mice were then trained with three repetitions of a 30 s audible tone co-terminating with a 2 s foot shock. After each training trial, freezing levels were recorded for 90 s. On day 3, the context of the conditioning apparatus was altered (switched to a black, triangular, unlit chamber). After 2.5 min in the chamber, mice were presented with three 30 s exposures to the conditioned tone (100 s inter-tone interval). Freezing levels during each 30 s tone exposure were recorded.

2.11. Predator odor assay

Experimental mice were habituated to testing environment for 45 min prior to exposure of 30 μL of 10% solution of 2,3,5-trimethyl-3-thiazoline (TMT) (Sigma) placed on filter paper. Behaviors were videotaped in the dark cycle with red light while the experimenter was out of room. Total exposure was 5 min, and responses scored for the final 3 min. Time spent engaged in freezing, sniffing, and grooming were scored by an observer, who was blinded to the genotype of mice.

2.12. Resident intruder assay

Experimental mice were singly housed for 1 wk. Individual males from a socially housed cohorts were placed into the home cages of the resident Vglut2^{fl/fl} and Vglut2^{sf2t-Cre} male mice, and mice were allowed to interact for 15 min. Interactions were videotaped and scored offline. Attacks toward the intruder were scored twice by 2 independent observers, who were blinded to the genotype of mice.

2.13. Statistics

Data were analyzed using the GraphPad Prism statistical software package (version 6.0). Pairwise comparisons were made using unpaired, two-tailed *t*-tests. Comparisons involving multiple factors were made using 2-way ANOVA with repeated measures and Bonferroni post-test. Results were considered statistically significant when *p < 0.05*. Data are presented as means ± SEM. For all figures, * = *p < 0.05*, ** = *p < 0.01*, *** = *p < 0.005*, and **** = *p < 0.001*.

3. RESULTS

3.1. Loss of Vglut2 expression in Vglut2^{sf2t-Cre} mice does not alter gross organization of the VMH

As expected, SF-1 and Vglut2 are coexpressed throughout the rodent VMH (Figure 1A). We used SF1-Cre backcrossed into a pure C57BL/6 background [11,24] and Vglut2^{sf2t-Cre} [22,25] to conditionally eliminate VGLUT2 in neurons from the SF-1 lineage (Vglut2^{sf2t-Cre}). As demonstrated previously, eliminating exon 2 renders VGLUT2 non-functional [23]. Within the CNS, SF1-Cre-mediated loss of Vglut2 is selective for the VMH and does not diminish Vglut2 expression in other brain regions (Figure 1B). Furthermore, no compensatory upregulation of the other vesicular glutamate transporters, Vglut1 or Vglut3 is observed in the VMH of Vglut2^{sf2t-Cre} mice. Similarly, Vglut2^{sf2t-Cre} males and females show normal patterns of glutamate decarboxylase (Gad67) expression, which marks inhibitory neurons and is normally excluded from the VMH (Figure 1B). No differences in the pattern and intensity of major VMH projections were noted in Vglut2^{sf2t-Cre}, as illustrated by the prominent ascending fibers through the ventral supraoptic commissure (VSOC) (Figure 1C) [24]. Taken together, these data establish that the gross exterior boundaries and major efferent projections of the VMH remain intact after deleting Vglut2 in the VMH.

3.2. Eliminating Vglut2 in the VMH does not change fasting blood glucose and unexpectedly attenuates diet-induced obesity in females

Control and mutant mice were initially maintained in a mixed genetic background. When fed standard chow (4% kcal fat), no significant differences in body weight were observed between Vglut2^{fl/fl} and...
Vglut2Sf1-Cre males or females (Figure 2A, left panel). When placed on HFD (60% kcal fat) beginning at 10 wks of age, weight gain was equivalent in Vglut2Sf1-Cre males compared to control littermates (Figure 2B, left panel). Vglut2Sf1-Cre females gained slightly less weight over time on HFD with 2-way repeated measures ANOVA revealing a significant interaction between genotype and time on HFD ($F_{7,98} = 3.116, p = 0.0052$). Surprisingly, neither Vglut2Sf1-Cre mutant males nor females exhibited significant differences in fasting blood glucose levels on standard chow or on HFD (Figure 2A, B, right panels). These data differ from the slight increase in DIO and the impaired glucose homeostasis observed in separately derived Vglut2Sf1-Cre mice [15].

Given these discrepant results, we generated congenic Vglut2fl/fl and Vglut2Sf1-Cre mice in the C57BL/6 background, a strain that exhibits increased weight gain and hyperglycemia on HFD [26,27]. Similar to the mixed background, body weights between male Vglut2Sf1-Cre and Vglut2fl/fl C57BL/6 mice were equivalent on standard chow. However, we observed a modest, but significant, decrease in female Vglut2Sf1-Cre body weights (Figure 3A). Other metabolic parameters were analyzed in Vglut2Sf1-Cre C57BL/6 mice using CLAMS chambers. The decreased weight of Vglut2Sf1-Cre females correlated with a non-significant trend towards increased heat generation during both the light and dark periods (Figure 3B). Vglut2Sf1-Cre females had normal expression of the thermogenic uncoupling protein 1 (Ucp1) in brown adipose tissue (BAT) at both warm and cold temperatures (Figure S1A). Further analysis of the CLAMS data revealed no significant alterations in ambulatory activity, food intake, oxygen consumption, respiratory exchange ratio, or lean mass between female Vglut2Sf1-Cre and Vglut2Sf1-Cre mice on standard chow (Figures 3C and S1B).

As expected, DIO was readily apparent in the C57BL/6 mice on HFD compared with the mixed background, especially in females (Figures 3D and 2B). When compared to Vglut2Sf1-Cre controls, weight gain in Vglut2Sf1-Cre females was notably lower when placed on HFD at 10 wks of age (Figure 3D); however both genotypes consumed equivalent amounts of HFD (Figure S1C). In contrast, C57BL/6 mutant males showed no body weight differences on HFD. Consistent with the female-specific resistance to DIO, glucose clearance was improved in Vglut2Sf1-Cre females but not in mutant males as measured by an intraperitoneal glucose tolerance test performed at 16 wks of age (Figures 3C and S1D). The unexpected body weight reduction in Vglut2Sf1-Cre females suggests that normal weight gain and sensitivity to DIO in females depends on glutamatergic output from the VMH. It should be noted that no gross deficits in reproduction were noted in Vglut2Sf1-Cre females on both the mixed and C57BL/6 backgrounds, suggesting that the hypothalamic-pituitary-gonad axis controlling reproductive physiology was not impaired in these mutant mice.

3.3. Reduced anxiety-like behaviors in male and female Vglut2Sf1-Cre mice

While conducting metabolic studies in which mice were housed individually, we noted a difference in the location of home cage nests built.
Figure 2: Fasting glucose levels on HFD are unaffected in Vglut2fl/fl on a mixed genetic background. Weekly body weight measurements of male (M) and female (F) Vglut2fl/fl and Vglut2Sfi1-Cre mice from 3 to 18 weeks of age. (A) Body weights of mice on standard chow (left panel) with corresponding blood glucose levels in each cohort measured pre- (Pr, solid bars) and post- (Po, hatched bars) a 24-hour fast (right panel). (B) Body weights of mice on standard chow and placed on HFD (arrow) at 10 weeks (left panel) with corresponding blood glucose levels measured as above and after HFD for 6 wks (right panel). Data are presented as means ± SEM. Statistical significance was determined by 2-way repeated measures ANOVA and Bonferroni post-test. Number of animals is indicated in each panel.

by Vglut2fl/fl and Vglut2Sfi1-Cre mice (Figure 4A). Nearly all Vglut2fl/fl control mice (96%) followed a stereotypic pattern and built their nests in the corner of the cage. In contrast, the majority of control mice (96%) followed a stereotypic pattern and built their nests by Vglut2fl/fl and Vglut2Sfi1-Cre mice (62%) chose a central nest location. Based on these preliminary observations, anxiety-like behaviors in mutant and control mice were formally assayed in open field (OF) and elevated plus-maze (EPM) tests. Although the nesting phenotype was initially observed in mutant mice on the mixed background, the following behavioral tests were exclusively performed in C57BL/6 Vglut2fl/fl and Vglut2Sfi1-Cre mice. The C57BL/6 background is known to have intermediate levels of anxiety-like behavior and therefore allows detection of both anxiolytic and anxiogenic phenotypes [28]. An initial rotarod test confirmed that motor skills were equivalent between genotypes (data not shown). However, in an OF test both male and female Vglut2Sfi1-Cre mice exhibited significantly more ambulatory movement in the center of the field than control Vglut2fl/fl mice (Figure 4B). We also observed higher total ambulatory activity for Vglut2Sfi1-Cre males within the short OF test (15 min) (Figure 4B). Because total ambulatory movement was unchanged in mutant Vglut2Sfi1-Cre males when monitored over a 4 day period (Figure S1E), the increased movement in the OF test suggests a greater exploratory drive for mutant males when initially placed in a novel environment.

Exploratory and anxiety-like behaviors were also examined using the EPM assay. Entries into the anxiety-promoting open arm, time spent in the open arm, and distance covered in the open arm were analyzed across two consecutive intervals (0–5 and 5–10 min). Similar to the OF assay, Vglut2Sfi1-Cre males, but not females exhibited a higher level of exploratory drive and/or activity in the initial interval (0–5 min), as measured by the total distance traversed in the EPM (Figure 4C, last panel). During the final 5-min interval (5–10 min), both male and female Vglut2Sfi1-Cre mice displayed higher levels of all three open arm parameters relative to controls (Figure 4C). Despite the sustained open arm activity observed in mutants, total distance traveled was equivalent between genotypes. A significant interaction between genotype and time was observed for open arm entries (males: F1,22 = 17.33, p < 0.0005; females: F1,25 = 5.751, p < 0.05); this EPM behavior is thought to best reflect altered anxiety levels [29]. The increased open arm activity in the Vglut2Sfi1-Cre mice is consistent with their increased time in the center of the OF, and together these results demonstrate that loss of glutamatergic output from the VMH is anxiolytic in both males and females. To confirm that Vglut2Sfi1-Cre mice could mount an appropriate physiological response to stressors, serum corticosterone levels were measured after 5 min in the EPM or 15 min of acute restraint. Despite lowered anxiety-like behavior in both the OF and EPM assays, corticosterone levels increased appropriately in Vglut2Sfi1-Cre mice (Figure 4D). For both wild type and mutant genotypes, two-way repeated measures ANOVA revealed a significant main effect of stressor only (EPM: F1,21 = 228.8, p < 0.0001; acute restraint: F1,21 = 420.13, p < 0.0001). These data show that the hypothalamic-pituitary-adrenal (HPA) axis is fully intact and unaffected by loss of glutamatergic transmission from the VMH.

3.4. Loss of Vglut2 in the VMH impairs fear conditioning and aggression in males

VMH neurons are part of the circuit linking the amygdala to the periaqueductal grey, both of which are essential for eliciting normal
emotional behavior. Therefore, we asked if learned or innate responses to fear-provoking stimuli are affected in Vglut2sf1-Cre female mice on a pure C57BL/6 genetic background. A) Weekly body weights of adult male (M) and female (F) Vglut2ff and Vglut2sf1-Cre mice on standard chow (NC). Indicated metabolic parameters measured in adult female Vglut2ff and Vglut2sf1-Cre mice on standard chow using CLAMS chambers. B) Hourly heat production during a 24-hour period. C) Average hourly heat production, ambulatory activity, food intake, oxygen consumption (VO2), and respiratory exchange ratio (RER) during the same 24-hour period as shown in panel B. D) Weekly body weights of male (M) and female (F) Vglut2ff and Vglut2sf1-Cre mice fed HFD beginning at 10 weeks of age (arrow). E) Glucose tolerance test in adult (16 wks old) female Vglut2ff and Vglut2sf1-Cre mice on HFD for 6 wks. Data are presented as means ± SEM. For panels A, D, and E, the given p values represent the main effect of genotype as determined by 2-way repeated measures ANOVA, and for each time point results from Bonferroni post-test are indicated with * = p < 0.05; ** = p < 0.01; *** = p < 0.005; **** = p < 0.001. Number of animals is indicated in each panel.

4. DISCUSSION

Here, we created a conditional knockout of VGLUT2 in SF1-expressing cells to examine how excitatory VMH neurotransmission influences metabolism and behavior. Unexpectedly, Vglut2sf1-Cre mice fail to show increased susceptibility to DIO or impaired glucose homeostasis. Rather we find sex-dependent resistance to DIO that is accompanied by improved glucose tolerance in Vglut2sf1-Cre males. Conditional loss of VGLUT2 in the VMH also leads to a profound anxiolytic effect in both sexes and reduces aggression and conditioned-fear in males. Our results suggest that glutamatergic signaling in the VMH is largely dispensable for glucose homeostasis, but essential for sex-specific energy storage and for adaptive behavioral responses to anxiety and fear provoking stimuli.
4.1. Blocking VMH glutamatergic output results in female-specific resistance to DIO

The reduced body weight of congenic Vglut2Sfi-Cre C57BL/6 female mice, even on standard chow, demonstrates that glutamatergic output from the VMH is an essential determinant of female energy balance. Although the VMH is a well-known regulator of metabolic homeostasis, this result differs from the increased sensitivity to DIO when leptin signaling in the VMH is ablated using Sfi-Cre [11]. These divergent metabolic phenotypes may reflect silencing of excitatory output from all VMH neurons (this study) versus disturbing a single signaling pathway in a subpopulation of VMH neurons. Clearly, the ability to genetically target and lesion different VMH subpopulations is needed for a complete functional understanding of the VMH and energy balance. Indeed, it is often overlooked that use of the Sfi-Cre line are quite different in our study and could potentially affect both DIO [26] and glucose homeostasis by influencing the degree of the hypoglycemic insult needed to reveal a defective counterregulatory response. For our analyses, the Sfi-Cre line was backcrossed on to the C57BL/6 background and exhibits robust and specific Cre-mediated recombination throughout the VMH.
including the VMHvl [24]. Thus while VMH neurons are reported to control the counterregulatory response to hypoglycemia [31] and receive input from glucoregulatory CCK-expressing neurons [32], we conclude that under our experimental conditions glutamatergic output from the VMH does not appear to be the primary determinant of glucose homeostasis.

The sex-dependent metabolic phenotype observed in Vglut2Sf1-Cre mice adds to the growing body of literature showing that the VMH is a critical regulator of energy balance and body weight in females. This aspect of VMH function is dependent on estrogen receptor alpha (ERα) expression in the VMHvl [24,33,34] or the number of ERα-expressing VMHvl neurons [9] leads to female-specific impairment of energy expenditure and increased body weight [9]. Surprisingly, the reduced body weight in the female Vglut2Sf1-Cre mice, the origins of which remain unclear, mimics the metabolic consequence of elevated estrogen signaling. This result undermines the simple assumption that estrogen signaling potentiates VMH neurotransmitter output, suggesting instead that ERα signaling inhibits a circuit that involves glutamatergic VMH output, and that otherwise promotes energy storage. Because this brain region is tightly linked to reproductive behavior [36], we further speculate that estradiol-mediated regulation of glutamatergic VMH neurons in females maximizes fuel reserves in states of overnutrition (HFD) and ultimately improves reproductive fitness in times of undernutrition.

4.2. Glutamatergic output from the VMH controls innate and learned behavior

Our data also reveal that glutamatergic VMH output contributes greatly to excitatory signals and neural circuits that modulate emotional behaviors, including those associated with anxiety-like behavior, fear, and sex-specific social behaviors. Indeed, we find that glutamatergic signaling from the VMH is required for male aggression. Ablating VMHvl neurons dramatically inhibits male aggressive behaviors [21]. Conversely, artificially activating VMHvl neurons triggers male aggressive behaviors [20]. As predicted from these studies, we find that disrupting the excitatory VMH output decreases the amount and extent of attack behavior when male Vglut2Sf1-Cre mice are presented with a resident intruder. That Vglut2Sf1-Cre males, but not females, exhibit lowered behavioral responses to a conditioned fear-provoking stimulus and exhibit increased novelty-associated locomotion but have no metabolic phenotype expand on the theme that the VMH mediates important sex-differences. Moreover, these data imply that neural circuits controlling fear and anxiety-like behaviors and metabolic responses are not tightly coupled.

Similar to the anxiolytic phenotype described here, acute, postnatal ablation of Sf1-positive neurons, which would also extinguish VMH glutamatergic output, significantly decreases anxiety-like behavior in the EPM [37]. The anxiolytic phenotype observed in Vglut2Sf1-Cre mice differs from the anxiogenic phenotype noted in Sf1Nestin-Cre mutants. However, the latter genetic lesion not only eliminates SF-1 in the VMH, but also disrupts the overall integrity of the VMH. Taken together, we conclude that the differences in anxiety-like behaviors observed in Vglut2Sf1-Cre and Sf1Nestin-Cre mice result from impaired VMH neuronal function versus disrupted VMH organization, respectively. We further demonstrate that circulating corticosterone, which mediates the physiological response to stress upon activation of the HPA axis is unaffected in Vglut2Sf1-Cre mutants. Interestingly, Sf1Nestin-Cre mutants exhibit normal circadian fluctuations in corticosterone [18]. Thus, the role of the VMH in anxiety-like behavior appears distinct from regulation of the HPA axis.

The periaqueductual grey (PAG) is a prominent downstream target of VMH projections [21,24,38], and regulates behaviors associated with anxiety and fear in response to glutamate. Consistent with the anxiolytic effect observed in mutant mice that have lost excitatory VMH output, delivering a glutamate receptor antagonist, 2-amino-7-phosphonoheptanoic acid, directly to the PAG in rats decreases anxiety-like behavior in the EPM [39]. Silencing VMH or PAG activity in mice using designer receptors exclusively activated by designer drugs (DREADDs) also impairs behavioral responses to fear-inducing stimuli, such as a predator or an aggressive conspecific [17]. Local application of a glutamate receptor agonist, N-methyl-D-aspartate, to the PAG in rats increases anxiety-like behavior in the EPM [40]. Collectively, we suggest that glutamatergic output from the VMH provides at least some of the excitatory input to the PAG that drives emotional behaviors associated with anxiety and/or fear.

Results from our behavioral assays indicate that not all responses to fearful stimuli are disrupted after inhibiting VMH glutamate release. For
instance, female mutant and control mice display equivalent levels of freezing to a predator odor, and both male and female Vglut2<sup>Sf1-Cre</sup> mice had normal freezing responses to foot shock during fear conditioning. However, during testing, male Vglut2<sup>Sf1-Cre</sup> mice had a weaker conditioned response, indicating impairment in the formation or the retention of the association between auditory tone and foot shock. Together, the lack of a uniform response to different anxiogenic and fear-provoking stimuli in Vglut2<sup>Sf1-Cre</sup> mutants is broadly consistent with the idea that distinct neural circuits mediate behavioral responses to different sources of anxiety and/or fear [41].

4.3. Conclusions

In summary, our study establishes that excitatory glutamatergic output from the VMH is required for optimal energy storage in females and appropriate behavioral responses to novel or fear-evoking stimuli in both sexes. The phenotypes of Vglut2<sup>Sf1-Cre</sup> mice reported here are consistent with emerging evidence that the VMH, specifically the VMHvl, regulates sex-dependent metabolic responses and social behaviors. Clearly, additional work is needed to define the precise anatomical and molecular pathways in the VMH needed to generate the complex emotional and metabolic responses associated with this neuroendocrine region.

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.09.001.

REFERENCES

[1] Kurrasch, D.M., Cheung, C.C., Lee, F.Y., Tran, P.V., Hata, K., Ingraham, H.A., 2007. The neonatal ventromedial hypothalamic transcriptome reveals novel markers with spatially distinct patterning. Journal of Neuroscience 27(50): 13624–13634.

[2] Ziegler, D.R., Cullinan, W.E., Herman, J.P., 2002. Distribution of vesicular glutamate transporter mRNAs in rat hypothalamus. Journal of Comparative Neurology 448(3):217–229.

[3] Fremeau Jr., R.T., Troyer, M.D., Palmer, I., Nygaard, G.O., Tran, C.H., Reimer, R.J., et al., 2001. The expression of vesicular glutamate transporters defines two classes of excitatory synapse. Neuron 31(2):247–260.

[4] Fu, L.Y., 2008. van den Pol AN. Agouti-related peptide and MC3/4 receptor agonists both inhibit excitatory hypothalamic ventromedial neuronal activity. Journal of Neuroscience 28(21):5433–5449.

[5] Sterrns, S.M., Shepherd, G.M., Friedman, J.M., 2005. Topographic mapping of VMH+ → arcuate nucleus microcircuits and their reorganization by fasting. Nature Neuroscience 8(10):1356–1363.

[6] Kim, K.W., Zhao, L., Donato Jr., J., Kohno, D., Xu, Y., Elia, C.F., et al., 2011. Steroidogenic factor 1 directs programs regulating diet-induced thermogenesis and leptin action in the ventral medial hypothalamic nucleus. Proceedings of the National Academy of Sciences of the United States of America 108(26):10673–10678.

[7] Tran, P.V., Akana, S.F., Malkovska, I., Dallman, M.F., Parada, L.F., Ingraham, H.A., 2006. Diminished hypothalamic bdnf expression and impaired VMH function are associated with reduced SF-1 gene dosage. Journal of Comparative Neurology 498(5):637–648.

[8] Majdic, G., Young, M., Gomez-Sanchez, E., Anderson, P., Szczepaniak, L.S., Dris, R.L., et al., 2002. Knockout mice lacking steroidogenic factor 1 are a novel genetic model of hypothalamic obesity. Endocrinology 143(2):607–614.

[9] Correa, S.M., Newstrom, D.W., Warne, J.P., Flandin, P., Cheung, C.C., Lin-Moore, A.T., et al., 2015. An estrogen-responsive module in the ventromedial hypothalamus selectively drives sex-specific activity in females. Cell Reports 10(1):62–74.

[10] Xu, Y., Hill, J.W., Fukuda, M., Gautron, L., Sohn, J.W., Kim, K.W., et al., 2010. P3Kδ signaling in the ventromedial hypothalamic nucleus is required for normal energy homeostasis. Cell Metabolism 12(1):88–95.

[11] Dhillon, H., Sigman, J.M., Ye, C., Lee, C.E., McGovern, R.A., Tang, V., et al., 2006. Leptin directly activates SF1 neurons in the VMH, and this action by leptin is required for normal body-weight homeostasis. Neuron 49(2):191–203.

[12] Bingham, N.C., Anderson, K.K., Reuter, A.L., Stallings, N.R., Parker, K.L., 2008. Selective loss of leptin receptors in the ventromedial hypothalamic nucleus results in increased adiposity and a metabolic syndrome. Endocrinology 149(5):2138–2148.

[13] Chiappini, F., Catalano, K.J., Lee, J., Peroni, O.D., Lynch, J., Dhanneshwar, A.S., et al., 2014. Ventromedial hypothalamic–specific Ptpn1 deletion exacerbates diet-induced obesity in female mice. Journal of Clinical Investigation 124(9):3781–3792.

[14] Klockner, T., Hess, S., Belgardt, B.F., Paeger, L., Hagen, L.A., Husch, A., et al., 2011. High-fat feeding promotes obesity via insulin receptor/P3K-dependent inhibition of SF-1 VMH neurons. Nature Neuroscience 14(7):911–918.

[15] Tong, Q., Ye, C., McCrimmon, R.J., Dhillon, H., Choi, B., Kramer, M.D., et al., 2007. Synaptic glutamate release by ventromedial hypothalamic neurons is part of the neurocircuitry that prevents hypoglycemia. Cell Metabolism 5(5):383–393.

[16] Motta, S.C., Goto, M., Gouveia, F.V., Baldo, M.V., Canteras, N.S., Swanson, L.W., 2009. Dissecting the brain’s fear system reveals the hypothalamus is critical for responding in subordinate conspecific intruders. Proceedings of the National Academy of Sciences of the United States of America 106(12):4870–4875.

[17] Silva, B.A., Mattucci, C., Krzywowsky, P., Murane, E., Illarionova, A., Grinevich, V., et al., 2013. Independent hypothalamic circuits for social and predator fear. Nature Neuroscience 16(12):1731–1733.

[18] Zhao, L., Kim, K.W., Ikeda, Y., Anderson, K.K., Beck, L., Chase, S., et al., 2008. Central nervous system-specific knockout of steroidogenic factor 1 results in increased anxiety-like behavior. Molecular Endocrinology 22(6):1403–1415.

[19] Kruk, M.R., Van der Poel, A.M., Meelis, W., Hermans, J., Mostert, P.G., Mos, J., et al., 1983. Discriminant analysis of the localization of aggression-inducing electrode placements in the hypothalamus of male rats. Brain Research 260(1):61–79.

[20] Lin, D., Boyle, M.P., Dollar, P., Lee, H., Lein, E.S., Perona, P., et al., 2011. Functional identification of an aggression locus in the mouse hypothalamus. Nature 470(7333):221–226.
Original article

[21] Yang, C.F., Chiang, M.C., Gray, D.C., Prabhakaran, M., Alvarado, M., Juntti, S.A., et al., 2013. Sexually dimorphic neurons in the ventromedial hypothalamus govern mating in both sexes and aggression in males. Cell 153(4):896–909.

[22] Hnasko, T.S., Chuhma, N., Zhang, H., Goh, G.Y., Sulzer, D., Palmiter, R.D., et al., 2010. Vesicular glutamate transport promotes dopamine storage and glutamate corelease in vivo. Neuron 65(5):643–656.

[23] Moechars, D., Weston, M.C., Leo, S., Callaerts-Vegh, Z., Goris, I., Daneels, G., et al., 2006. Vesicular glutamate transporter VGLUT2 expression levels control quantal size and neuromuscular pain. Journal of Neuroscience 26(46):12055–12066.

[24] Cheung, C.C., Kurrasch, D.M., Liang, J.K., Ingraham, H.A., 2013. Genetic labeling of steroidogenic factor-1 (SF-1) neurons in mice reveals ventromedial nucleus of the hypothalamus (VMH) circuitry beginning at neurogenesis and development of a separate non-SF-1 neuronal cluster in the ventrolateral VMH. Journal of Comparative Neurology 521(6):1268–1288.

[25] Stuber, G.D., Hnasko, T.S., Britt, J.P., Edwards, R.H., Bonci, A., 2010. Dopaminergic terminals in the nucleus accumbens but not the dorsal striatum corelease glutamate. Journal of Neuroscience 30(24):8229–8233.

[26] Montgomery, M.K., Hallahan, N.L., Brown, S.H., Liu, M., Mitchell, T.W., Cooney, G.J., et al., 2013. Mouse strain-dependent variation in obesity and glucose homeostasis in response to high-fat feeding. Diabetologia 56(5):1129–1139.

[27] Collins, S., Martin, T.L., Surwit, R.S., Robidoux, J., 2004. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. Physiology & Behavior 81(2):243–248.

[28] Crawley, J.N., 2008. Behavioral phenotyping strategies for mutant mice. Neuron 57(6):809–818.

[29] Rodgers, R.J., Johnson, N.J., 1995. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacology Biochemistry & Behavior 52(2):297–303.

[30] Habrovszy, E., Kall, I., Turf, G.F., May, K., Wittmann, G., Fekete, C., et al., 2006. Expression of vesicular glutamate transporter-2 in gonadotrope and thyrotrope cells of the rat pituitary. Regulation by estrogen and thyroid hormone status. Endocrinology 147(8):3818–3825.

[31] Borg, M.A., Sherwin, R.S., Borg, W.P., Tamborlane, W.V., Shulman, G.I., 1997. Local ventromedial hypothalamic glucose perfusion blocks counterregulation during systemic hypoglycemia in awake rats. Journal of Clinical Investigation 99(2):361–365.

[32] Garfield, A.S., Shah, B.P., Madara, J.C., Burke, L.K., Patterson, C.M., Flak, J., et al., 2014. A parabrachial-hypothalamic cholecystokinin neurocircuit controls counterregulatory responses to hypoglycemia. Cell Metabolism 20(6):1030–1037.

[33] Xu, Y., Nedungadi, T.P., Zhu, L., Sobhani, N., Irani, B.G., Davis, K.E., et al., 2011. Distinct hypothalamic neurons mediate estrogenic effects on energy homeostasis and reproduction. Cell Metabolism 14(4):453–465.

[34] Musatov, S., Chen, W., Pfaff, D.W., Mobbs, C.V., Yang, X.J., Clegg, D.J., et al., 2007. Silencing of estrogen receptor alpha in the ventromedial nucleus of the hypothalamus leads to metabolic syndrome. Proceedings of the National Academy of Sciences of the United States of America 104(7):2501–2506.

[35] Martinez de Morentin, P.B., Gonzalez-Garcia, I., Martins, L., Lage, R., Fernandez-Mallo, D., Martinez-Sanchez, N., et al., 2014. Estradiol regulates brown adipose tissue thermogenesis via hypothalamic AMPK. Cell Metabolism 20(1):41–53.

[36] Ogawa, S., Eng, V., Taylor, J., Lubahn, D.B., Korach, K.S., Pfaff, D.W., 1998. Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice. Endocrinology 139(12):5070–5081.

[37] Kunwar, P.S., Zelikowsky, M., Remedios, R., Cai, H., Yilmaz, M., Meister, M., et al., 2015. Ventromedial hypothalamic neurons control a defensive emotion state. Elife, 4.

[38] Canteras, N.S., Simerly, R.B., Swanson, L.W., 1994. Organization of projections from the ventromedial nucleus of the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. Journal of Comparative Neurology 348(1):41–79.

[39] Guamaraes, F.S., Carobrez, A.P., De Aquiar, J.C., Graeff, F.G., 1991. Anxiolytic effect in the elevated plus-maze of the NMDA receptor antagonist AP7 microinjected into the dorsal periaqueductal grey. Psychopharmacology (Berlin) 103(1):91–94.

[40] Miguel, T.T., Nunes-de-Souza, R.L., 2008. Anxiogenic-like effects induced by NMDA receptor activation are prevented by inhibition of neuronal nitric oxide synthase in the periaqueductal gray in mice. Brain Research 1240:39–46.

[41] Gross, C.T., Canteras, N.S., 2012. The many paths to fear. Nature Reviews Neuroscience 13(9):651–658.