Original article

MOLECULAR METABOLISM

Sex-dependent changes in metabolism and behavior, as well as reduced anxiety after eliminating ventromedial hypothalamus 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 *Sf1-Cre* and a *Vglut2* floxed allele, *Vglut2* was knocked-out in SF-1 VMH neurons ($Vglut2^{Sf1\text{-}Cre}$). Metabolic and neurobehavioral assays were carried out initially on $Vglut2^{fl/fl}$ and $Vglut2^{Sf1\text{-}Cre}$ 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 $Vglut2^{Sf1\text{-}Cre}$ mice were largely unexpected based on prior studies that have perturbed VMH development or VMH glutamate signaling. In our hands, $Vglut2^{Sf1\text{-}Cre}$ 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 $Vglut2^{Sf1\text{-}Cre}$ females. $Vglut2^{Sf1\text{-}Cre}$ 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 $Sf1^{Nestin\text{-}Cre}$ mice that lack a fully formed VMH, both male and female $Vglut2^{Sf1\text{-}Cre}$ 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

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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, Pl3K* (p110α), or *Ptpn1* with the *Sf1-Cre* increases susceptibility to DIO [10–13]. On the other hand, deleting insulin signaling (*Insr*) using the same *Sf1-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 *Sf1-Cre* ($Vglut2^{Sf1\text{-}Cre}$) in a mixed genetic background [15]. Surprisingly, only a modest increase in weight gain is observed in $Vglut2^{Sf1\text{-}Cre}$ 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* ($Sf1^{Nestin\text{-}Cre}$), 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 $Sf1^{-/-}$ knockout, $Sf1^{Nestin\text{-}Cre}$ mice show gross abnormalities in VMH architecture. Hence, it is unclear if the increased anxiety phenotype noted in the $Sf1^{Nestin\text{-}Cre}$ 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 $Vglut2^{Sf1\text{-}Cre}$ allele using *Sf1-Cre* and a *Vglut2* floxed allele ($Vglut2^{fl/fl}$) [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 $Vglut2^{Sf1\text{-}Cre}$ 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

$Vglut2^{+/fl}$ mice in a mixed C57BL/6, Sv129J background were provided by Dr. R.D. Palmiter, (University of Washington) [22]. $Sf1^{+/Cre}$ 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. $Vglut2^{fl/fl}$ 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 $Sf1^{+/Cre}$; $Vglut2^{fl/fl}$ with $Vglut2^{fl/fl}$ mice. We previously generated and characterized the $Sf1^{TauGFP}$ 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 (5058; LabDiet, 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 (DISH) 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 1% HIDS were added to the sections and incubated overnight at 4 °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, pH7.5; 5 M NaCl; 0.05% Tween 20), IF and ISH signals were detected sequentially using the TSA Biotin System (Perkin Elmer) and the HNPP Fast Red Detection Kit (Roche), respectively, according to the manufacturers' instructions. Sections were counter-stained with DAPI (1:1000) for 5 min at room temperature and mounted with aqueous mounting media.

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

