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Age-dependent changes in amino acid phenotype and the role of glutamate release from hypothalamic proopiomelanocortin neurons

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Abstract

Hypothalamic proopiomelanocortin (POMC) neurons are important regulators of energy balance. Recent studies indicate that in addition to their peptides, POMC neurons can release either the amino acid (AA) transmitter GABA or glutamate. A small subset of POMC neurons appears to have a dual AA phenotype based on co-expression of mRNA for the vesicular glutamate transporter (*vGlut2*) and the GABA synthetic enzyme *Gad67*. To determine whether the colocalization of GABAergic and glutamatergic markers may be indicative of a switch in AA transmitter phenotype, fluorescent *in situ* hybridization was used to detect *vGlut2* and *Gad* mRNA in POMC neurons during early postnatal development. The percentage of POMC neurons expressing *vGlut2* mRNA in POMC neurons progressively decreased from ~40% at day 1 to less than 10% by 8 weeks of age, whereas *Gad67* was only expressed in ~10% of POMC neurons at day 1 and increased until ~45% of POMC neurons coexpressed *Gad67* at 8 weeks of age. To determine whether the expression of *vGlut2* may play a role in energy balance regulation, genetic deletion of *vGlut2* in POMC neurons was accomplished using Cre-lox technology. Male, but not female, mice lacking *vGlut2* in POMC neurons were unable to maintain energy balance to the same extent as control mice when fed a high-fat diet. Altogether, the results indicate that POMC neurons are largely glutamatergic early in life and that the release of glutamate from these cells is involved in sex- and diet-specific regulation of energy balance.

Keywords

POMC; GABA; *in situ* hybridization; plasticity; *vGlut2* knockout; *Gad*; AB_300798; AB_5145000; AB_840257; AB_2307336; AB_2314332

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Conflict of Interest

The authors do not have any conflicts of interests related to this work.

Role of Authors

All authors contributed to the design and analysis of experiments. Performance of experiments: CSD, CMK and MSD. Drafting of manuscript: CSD and STH. Figure preparation: CSD, MSD and STH. All authors critically revised and approved final manuscript.

Introduction

Hypothalamic proopiomelanocortin (POMC) neurons are necessary for the regulation of energy balance and exert many actions on food intake and energy expenditure through the release of their peptide transmitters (Mercer et al., 2013). However, recent work has shown that POMC neurons also release the amino acid transmitters (AA) GABA and glutamate (Dicken et al., 2012; Hentges et al., 2004; Hentges et al., 2009). While the role of GABA and glutamate release from POMC cells is not yet clear, AA transmitter release from other hypothalamic neurons is known to play a key role in regulating energy balance (Kong et al., 2012; Tong et al., 2008; Wu et al., 2009). The majority of the GABAergic and glutamatergic POMC neurons represent two distinct cell populations (Jarvie and Hentges, 2012; Wittmann et al., 2013), although a small subset of POMC neurons express a dual GABAergic and glutamatergic phenotype (Jarvie and Hentges, 2012). A dual AA neurotransmitter phenotype has also been reported for cells in the hippocampus (Zander et al., 2010), basal ganglia (Shabel et al., 2014), ventral tegmental area (Root et al., 2014), preoptic area (Ottem et al., 2004), and presynaptic terminals from the supramammillary nucleus (Boulland et al., 2009). Mounting evidence suggests both GABAergic and glutamatergic markers in an individual cell may indicate a switch between phenotypes. Indeed, some neurons are capable of shifting the expression and distribution of phenotypes in response to ageing or environmental changes and it has been suggested that expression of multiple neurotransmitters and plasticity of neurotransmitter phenotype is a cellular adaptation that can be advantageous in a changing environment (Demarque and Spitzer, 2010).

Early postnatal life has been shown to be a malleable period in development for neurotransmitters. During this time period multiple brain regions, including the hypothalamus, show considerable expression of glutamatergic markers that then declines as rodents reach adulthood (Borgius et al., 2010; Boulland et al., 2004; Nakamura et al., 2005). Studies focusing on specific populations of neurons have shown that neurons in the ventral tegmental area (Berube-Carriere et al., 2009; Mendez et al., 2008), hippocampus (Boulland et al., 2004), and visual cortex (Berry et al., 2012) transiently express glutamatergic markers in early development. Expression of glutamatergic markers in these neurons declines as rodents reach adulthood. In line with these aforementioned studies, Gillespie and colleagues (Gillespie et al., 2005) show glutamate release from neurons in the medial nucleus of the trapezoid body during the first 10 days of postnatal development; subsequently, these neurons become increasingly inhibitory as animals reach adulthood. Although neurons expressing a dual AA transmitter phenotype early in development appear to differentiate into either inhibitory or excitatory neurons, evidence suggests that activity-dependent re-expression of a dual phenotype is possible in mature neurons (Gomez-Lira et al., 2005).

While the specific function of upregulated glutamatergic signaling in postnatal development is likely to be dependent on brain region, evidence suggests glutamate plays a role in synapse formation (Berry et al., 2012; Berube-Carriere et al., 2009; He et al., 2012) and organization of neuronal circuits (Noh et al., 2010). Axonal projections from hypothalamic POMC neurons are formed during the second week of postnatal life (Bouret et al., 2004a), a timeframe coinciding with the increased plasticity of AA transmitters during development. Previous work has shown the distribution of GABAergic and glutamatergic POMC neurons in

adult mice (Jarvie and Hentges, 2012), yet it is not known if the AA phenotype of these neurons exhibits plasticity during early postnatal development.

The purpose of this study was to determine if the distribution of glutamatergic and GABAergic POMC neurons changes during postnatal development relative to adulthood and to determine if altering AA phenotype of POMC neurons would affect energy balance regulation. Fluorescent *in situ* hybridization was used to detect mRNA for the vesicular glutamate transporter-2 (vGlut2) as an indicator of glutamatergic neurons. Putative GABAergic neurons were identified based on the presence of mRNA for the GABA synthetic enzymes glutamate decarboxylase 1 or 2 (referred to as Gad67 and Gad65, respectively). The results indicate that plasticity in the AA transmitter phenotype occurs in hypothalamic POMC neurons during postnatal development from day 1 to 8 weeks of age. Additionally, embryonic deletion of *vGlut2* from POMC neurons resulted in a sex-specific increase in body weight when mice were maintained on a high fat diet. Thus, it appears that glutamate release from POMC neurons contributes to the ability of POMC neurons to regulate energy balance.

MATERIALS AND METHODS

Animals

Male and female mice (aged 1 day to 8 weeks, as indicated) expressing enhanced green fluorescent protein (eGFP; (Cowley et al., 2001)) driven by the POMC promoter and wild-type mice maintained on the C57BL/6J background were used for all *in situ* hybridization experiments. Deletion of the vesicular glutamate transporter type 2 (*vGlut2*) in POMC cells was accomplished by crossing *vGlut2*^{fllox+/-} mice (The Jackson Laboratory stock number 012898) with *vGlut2*^{fllox+/-};POMC-Cre (Xu et al., 2005) double transgenic mice. Standard PCR genotyping was used to detect the floxed alleles and Cre transgene. Animals were housed under controlled temperatures (22–24°C) and a 12-hour light/dark cycle. Mice were given standard rodent chow (except where noted in high-fat diet experiments) and tap water *ad libitum*. All of the animal protocols were approved by the Institutional Animal Care and Use Committee at Colorado State University and were in accordance with the United States Public Health Service guidelines for animal use.

Food intake and bodyweight studies

Upon weaning (3 weeks of age), mice lacking *vGlut2* in POMC-Cre cells and control litter mates were individually housed and given *ad libitum* access to water and standard rodent chow (Teklad 2018; 18% of kcal from fat) or high-fat rodent chow (Teklad 06414; 60% of kcal from fat). Bodyweight and food intake measurements were collected weekly.

Tissue preparation

Mice were anesthetized and perfused transcardially with 10% sucrose in water prior to 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight at 4°C in 4% paraformaldehyde. Coronal sections (50 µm) containing the arcuate nucleus were prepared on a vibratome, collected in cold diethylpyrocarbonate (DEPC)-treated PBS, and processed

for *in situ* hybridization as previously described (Jarvie and Hentges, 2012) and outlined below.

Antibody characterization

All antibodies used in this study are listed in table 1. Antibodies used to detect digoxigenin (DIG) labeled probes (RRID:AB_5145000) and fluorescein (FITC) labeled probes (RRID:AB_840257) for *in situ* hybridization produced a pattern that was unique to each gene target and matched work previously described (Jarvie and Hentges, 2012). Detection of GFP using the chicken anti-GFP antibody (RRID:AB_300798) is consistent with labeling pattern that has been previously described (Jarvie and Hentges, 2012). Western blot analysis has shown that the chicken anti-GFP antibody recognizes a single band around 27–30 kDa, but no band is detected in a WT control (technical information provided by the manufacturer). Negative controls included omitting primary and secondary antibodies as was done previously for these antibodies (Jarvie and Hentges, 2012).

Fluorescent *in situ* hybridization

For fluorescent *in situ* hybridization, sections were prepared from mice of various ages and processed in parallel. Sections were placed in 6% H₂O₂ for 15 minutes to quench endogenous peroxidase activity. Tissue was incubated for 15 minutes in proteinase K (10µg/ml) diluted in PBS containing 0.1% Tween 20 (PBT). Proteinase K was deactivated by exposing tissue to 2mg/ml glycine in PBT for 10 minutes. Following two 5-minute washes in PBT, tissue was postfixed for 20 minutes in solution containing 4% paraformaldehyde and 0.2% glutaraldehyde. Tissue was washed in PBT, then dehydrated in ascending concentrations of ethanol diluted in DEPC-treated water (50, 70, 95, and 100%), and briefly rehydrated in PBT. Sections were transferred to vials and prehybridized in hybridization solution (66% deionized formamide 13% dextran sulfate, 260 mM NaCl, 1.3× Denhardt's solution, 13 mM Tris-HCL [pH 8.0], 1.3 mM EDTA [pH 8.0]) for 1 hour at 60°C. Probes were denatured at 85°C for 5 minutes and then added, along with 0.5mg/ml tRNA and 10 mM DTT, to the hybridization buffer.

Digoxigenin (DIG)-labeled and fluorescein isothiocyanate (FITC)-labeled RNA probes were made and used as described by Jarvie and Hentges (2012). Probes for *Pomc*, *vGlut2*, and glutamate decarboxylase 1 (*Gad67*) hybridize at 70°C, therefore combinations of these probes were hybridized simultaneously for 18–20 hours. The glutamate decarboxylase 2 (*Gad65*) probe hybridizes at 52°C and required sequential hybridization for dual *in situ*. For detection of *Gad67* and *Gad65*, the *Gad67* probe was hybridized first at 70°C and then the tissue was incubated for 20 hours at 52°C in new hybridization buffer with *Gad65*. The *Pomc* probe was used at 750 pg/µl and corresponds to bases 532–1,000 of Genbank sequence NM_008895.3. The *Gad67* probe was used at 150 pg/µl and corresponds to NM_00877.4 bases 749–1,527. The *vGlut2* probe recognizes bases 1,854–2,440 of NM_080853.3 and was used at 1 ng/µl. *Gad65* was detected using 2 probes mixed together that recognize bases 537–1,207 and 1,201–2032 of NM_008078.2 and each was used at a final concentration of 150 pg/µl.

Following hybridization, three of the six 30-minute stringency washes at 60°C were in solution containing 50% formamide and 5X SSC followed by three washes in 50% formamide and 2× SSC. Tissue was then digested for 30 minutes at 37°C with RNAse A (20 µg/ml in 0.5 M NaCl, 10 mM Tris-HCL [pH 8.0], 1 mM EDTA) and subsequently placed in three 15-minute TNT (0.1 M Tris-HCL [pH 7.5], 0.15 M NaCl, 0.05% Tween-20) washes. Sections were blocked for 1 hour in TNB (TNT plus 0.5% Blocking Reagent provided in the TSA kit; Perkin Elmer, Oak Brook IL) and then incubated overnight at 4°C in either sheep anti-DIG (1:1,000; Roche Applied Sciences) or sheep anti-FITC (1:1,000; Roche Applied Sciences) antibodies, both conjugated to horseradish peroxidase. The DIG-labeled probes were detected using a TSA PLUS Biotin Kit (Perkin Elmer) and then incubated in 1% H₂O₂ to quench any remaining peroxidase activity. FITC-labeled probes were subsequently detected using a TSA PLUS DNP (HRP) system (Perkin Elmer). Tissue was exposed to three 15-minute TNT washes and then incubated for 30 minutes in a 1:50 dilution of either the Biotin Amplification Reagent or the DNP Amplification Reagent. Tissue was then washed in TNT and DIG-labeled probes were visualized with Streptavidin conjugated to Alexa Fluor 555 (30 minutes in 1:1,000; Invitrogen, Eugene, OR). FITC-labeled probes were visualized with 1:400 rabbit anti-DNP-KLH conjugated to Alexa Fluor 488 (1 hour; Invitrogen, Eugene, OR), both in TNT. Tissue was mounted and cover slipped with Aqua Poly/Mount (Polysciences, Inc., Warrington).

Immunodetection of GFP

Immunodetection of GFP in transgenic POMC-eGFP mice was used for detection of *vGlut2* and *Gad67* in POMC neurons because GFP fluorescence is quenched during *in situ* hybridization, but the antigenicity of GFP is maintained (Jarvie and Hentges, 2012). The POMC-eGFP transgene has been shown to faithfully label POMC neurons in the arcuate nucleus of adult mice (Cowely et al., 2001) as young mice as early as postnatal day 9 (Padilla et al., 2010). Following detection of FITC-labeled probes, tissue was incubated for 2 hours in chicken anti-GFP antibody (1:2,000, Abcam, Boston MA), washed in TNT, and then placed in donkey anti-chicken secondary antibody conjugated to Alexa Fluor 647 for 1 hour (1:1,000, Jackson ImmunoResearch, West Grove, PA).

Imaging

Images were collected on a Zeiss 510 Meta confocal microscope. Green fluorescence (Alexa fluor 488) was imaged using 488 nm excitation and emission was detected using a 505/530 nm bandpass filter. Red fluorescence (Alexa Fluor 555) was imaged using 543 nm excitation and a 560/615 nm bandpass emission filter and far-red fluorescence (Alexa Fluor 647) was imaged using 633 nm excitation and a 650 nm longpass emission filter. Images were taken sequentially at each wavelength to avoid crossover between channels. Images were taken in the z-plane every 3 µm for a total of 18–21 µm from all of the tissue along the rostral-caudal axis containing POMC neurons (~Bregma –1.2 mm to ~Bregma –2.3 mm). Images used for figures were adjusted for brightness and contrast using Photoshop (Adobe Systems, San Jose, CA).

Cell counts and analysis

Cell counts were made using a modification of the 3D counting method described by Williams and Rakic (1988) to limit oversampling. Z-stacks totaling 12 μm were constructed from sequential 3 μm -thick sections of tissue after omitting the upper and lower images collected and the stacks were analyzed for cell number, colocalization, and intensity above background using ImageJ software. Only cells with a clear nucleus and completely contained in a $300 \times 300 \times 12\text{-}\mu\text{m}$ counting box on the x-y-z plane were counted. All N values are reported as number of animals and a minimum of 4 images from throughout the rostral/caudal extent of the arcuate nucleus was analyzed per animal. All visible POMC neurons in each section were counted (mean neurons for all images analyzed/animal = 170 ± 6.61 , $\text{SD} = 46.27$). *Pomc*-FITC labeled cells and POMC-eGFP cells were counted and analyzed for colocalization with *vGlut2*, *Gad67*, or *Gad65*. Presence of *vGlut2*, *Gad67*, or *Gad65* signal had to be in the same z-plane as POMC cells and be contained within POMC cells to be considered colocalized. Colocalization is reported as a percentage of POMC cells to account for variation in the number of cells counted per animal. To determine whether changes in *vGlut2* or *Gad67* label intensity were uniform or unique to POMC neurons, two circular ROIs were drawn in a region of the arcuate nucleus containing POMC cells and a region in the arcuate devoid of POMC cells and intensity of signal within the ROIs was determined. Since the distribution of *vGlut2* and *Gad67* is different across the arcuate nucleus, ROIs for *Gad67* were drawn in the dorsal-medial arcuate nucleus and *vGlut2* ROIs were drawn more towards the dorsal-lateral arcuate nucleus. Intensities are reported relative to background to account for differences in labeling intensity and background between experiments and mice of various ages were represented in each *in situ* hybridization run, with tissue from 8-week-old animals serving as a reference for between assay reproducibility.

Evoked transmitter release

To determine whether the Cre-mediated excision of *vGlut2* effectively prevented glutamate release from POMC-Cre neurons, an optogenetic/electrophysiologic approach was used. Channelrhodopsin2 was expressed in POMC-Cre neurons by injecting AAV2/9.EF1.dflox.hChR2(H134R)-mCherry.WPRE.hGH (obtained from the Penn Vector Core at the University of Pennsylvania School of Medicine, Philadelphia, PA) into the arcuate nucleus of POMC-Cre transgenic mice as previously described (Dicken et al., 2012). Brief (2 ms) pulses of blue light were used to evoke transmitter release from ChR2-expressing POMC-Cre neurons. Voltage-clamp (-60 mV holding potential) recordings were made in unlabeled neurons near the ChR2-mCherry neurons as previously described (Dicken et al., 2012). Pharmacologic blockade of AMPA receptors (6,7-dinitroquinoxaline-2,3(1*H*, 4*H*), 10 μM ; Sigma-Aldrich) or GABA_A receptors (bicuculline methiodide, 10 μM ; Tocris) was used to determine whether the postsynaptic current was mediated by glutamate or GABA, respectively.

Statistics

For *in situ* hybridization and food intake data, statistical significance was determined using two-tailed Student's t-tests or ANOVA (Bonferroni's post hoc analysis) with or without

repeated measures (RMANOVA). No significant differences were found between sexes in any of the *in situ* hybridization experiments and thus, data from both sexes have been combined in the final analyses. Bodyweight data were analyzed using repeated measures ANOVA (RMANOVA) and Sidak's posthoc tests. Results were considered significant if $p < 0.05$. Data are presented as mean $\pm$ SEM in the text and figures and standard deviation (SD) is also reported in the text.

RESULTS

Expression of *vGlut2* in POMC neurons during postnatal development

Dual fluorescent *in situ* hybridization was used to evaluate the expression of *vGlut2* in *Pomc*-containing neurons in mice at the following ages: p1, 1 week, 3 weeks, 5 weeks, and 8 weeks. The relative number of *Pomc* cells expressing *vGlut2* was significantly different across all age groups (p1=40.29 $\pm$ 3.18, n=7, SD=8.43; 1 wk=35.29 $\pm$ 3.44, n=4, SD=6.88; 3 wk=25.62 $\pm$ 3.36, n=12, SD=11.96; 5 wk=17.98 $\pm$ 2.47, n=11, SD=8.2; 8wk=8.19 $\pm$ 1.98, n=12, SD=6.87; ANOVA $p < 0.0001$, $F_{4,41}=17.11$, Fig. 1). Compared to 8-week-old mice, the percentage of *Pomc* neurons expressing *vGlut2* was significantly higher at p1, 1 week, 3 weeks, and 5 weeks of age (p1 vs. 8 wk, $t(41)=7.49$, $p < 0.0001$; 1 wk vs. 8 wk, $t(41)=5.21$, $p < 0.0001$; 3 wk vs. 8 wk, $t(41)=4.31$, $p = 0.0004$; 5 wk vs. 8 wk, $t(41)=4.31$, $p = 0.03$).

Overall, the intensity of the *vGlut2* signal in the arcuate nucleus appeared to be higher in younger animals relative to older mice (Fig. 1A–C center images). To determine if *vGlut2* labeling intensity is relatively high throughout the arcuate or is high specifically in POMC neurons during early postnatal development, the intensity of *vGlut2* signal in a region lacking *Pomc* neurons and in a region containing *Pomc* neurons was evaluated at each age group (Fig. 2). Age had a significant effect on the intensity of *vGlut2* signal in areas of the arcuate nucleus without *Pomc* neurons (Fig. 2 A–C, dashed circle and D; intensity above background at 8 wk=20.20 $\pm$ 3.14, n=12, SD=10.98; at p1=131.6 $\pm$ 26.9, n=8, SD=76.02, $p < 0.0001$, $t(41)=6.73$ and with *Pomc* neurons (Fig. 2 A–C, solid circle & D, $p < 0.0001$, $t(41)=7.71$; intensity above background at 8 wk=22.74 $\pm$ 2.83, n=12, SD=9.83; at p1=103.4 $\pm$ 12.84, n=8, SD=36.32). While signal intensity may not faithfully represent absolute mRNA levels or reflect changes in protein, the reduced intensity with age correlates well with the noted decline in the percentage of POMC neurons expressing *vGlut2* mRNA as mice age and suggests that a general age-related decrease in *vGlut2* signal occurs in the arcuate nucleus.

Expression of *Gad67* in POMC neurons during postnatal development

Dual fluorescent *in situ* hybridization was used to determine the number of *Pomc* neurons expressing *Gad67* at the following ages: p1, 1 week, 3 weeks, 5 weeks, and 8 weeks. The overall expression of *Gad67* in the arcuate nucleus was similar across all age groups (Fig. 3A, middle column) and age did not have a significant effect on the intensity of *Gad67* signal in POMC ($p = 0.07$, $F_{4,41}=1.49$) and non-POMC cells ($p = 0.93$, $F_{4,41}=0.21$). The relative number of *Pomc* cells expressing *Gad67* increased with age (Fig. 3B, $p < 0.0001$, $F_{4,37}=67.65$). Compared to animals at 8 weeks of age (46.16% $\pm$ 1.49, n=9, SD=5.2), the percentage of *Pomc* neurons expressing *Gad67* was significantly lower at p1 (8.04% $\pm$ 1.57,

n=7, SD=4.18), 1 week (29.15%±2.23, n=3, SD=3.87), and 3 weeks (35.42%±1.52, n=13, SD=5.51) of age (p1 vs. 8 wk, $p<0.0001$, $t(37)=13.59$; 1 wk vs. 8 wk, $p=0.0004$, $t(37)=4.40$; 3 wk vs. 8 wk, $p=0.0093$, $t(37)=3.27$). By the time animals reached 5 weeks of age, the percent of *Pomc* cells expressing *Gad67* was not significantly different from 8-week-old mice (5 wk= 48.26%±1.99, n=10, SD=6.31, 5 wk vs. 8 wk, $p=0.76$, $t(37)=1.33$). To determine whether the decreased *Gad67* expression in POMC neurons during early postnatal development might reflect a shift between *Gad67* and *Gad65* expression, dual fluorescent *in situ* was used to determine the number of *Pomc* neurons colocalized with *Gad65* in 1 week old mice. The percentage of *Pomc* neurons expressing *Gad65* was not significantly different than *Pomc* neurons expressing *Gad67* (Fig. 3C; 32.37%±4.78, n=3, SD=8.29 colocalize *Gad65*, 29.15%±2.23, n=3, SD=3.87 colocalize *Gad67*, $p=0.57$, $t(4)=0.61$) suggesting that *Gad65* and *Gad67* have overlapping expression patterns.

Dual phenotype POMC neurons during postnatal development

To determine if the relatively high number of glutamatergic POMC neurons during early postnatal development represents a population of cells that might express a dual AA transmitter phenotype during early development and later mature into GABAergic POMC cells, dual fluorescent *in situ* hybridization was used to detect *Gad67* and *vGlut2* mRNA in POMC-eGFP neurons. Although eGFP fluorescence is quenched during tissue processing for the *in situ* hybridization procedure, antigenicity of eGFP survives and can be used for immunodetection of POMC-eGFP cells (Jarvie and Hentges, 2012). The majority of POMC cells do not express a dual AA transmitter phenotype in animals aged 1 day, 3 weeks, or 5 weeks (9.7%, 7.6%, and 6.6%, respectively; Fig 4).

Effect of deletion of *vGlut2* in POMC on body weight regulation

To determine whether glutamate release from POMC neurons contributes to energy balance regulation, Cre/lox technology was used to delete *vGlut2* from POMC-Cre expressing neurons. An optogenetic approach was used to demonstrate that deletion of *vGlut2* from POMC neurons prevented glutamate release from these cells. When ChR2 was expressed in POMC-Cre neurons, a brief pulse of blue light evoked transmitter release and caused a postsynaptic current in downstream cells as previously reported (Dicken et al., 2012). In tissue from POMC-Cre;*vGlut2*^{lox/lox} mice, the light evoked currents were mediated exclusively by GABA (9/9 recordings displayed GABA-mediated currents; 0/9 recordings that showed evoked currents displayed glutamate mediated currents). This is in contrast to previous reports showing that 30% of evoked currents from POMC neurons in control tissue are mediated by glutamate (Dicken et al., 2012). Thus, the Cre/lox approach effectively eliminated glutamate release from Cre-expressing neurons as shown previously for other hypothalamic neurons (Tong et al., 2007).

The deletion of *vGlut2* from POMC-Cre neurons did not alter the bodyweight of female mice maintained for 8 weeks after weaning on regular chow (final weight 20.2±0.58g, n=10, SD=1.84 for control, 19.3±0.6g, n=10, SD=1.82 for POMC-*vGlut2*^{-/-}, $p=0.27$, $t(15)=0.18$; RMANOVA for entire weight curve, $p=0.29$, $F_{1,170}=1.14$, n=10; Fig. 5A) or high-fat chow (final weight 21.6±0.4g, n=8, SD=1.16 for control, 21.5±0.9g, n=9, SD=2.68 for POMC-*vGlut2*^{-/-}, $p=0.99$, $t(15)=0.18$; RMANOVA for entire weight curve, $p=0.60$, $F_{1,15}=0.28$,

n=8–9; Fig. 5B). The deletion of *vGlut2* from POMC-Cre neurons also did not alter the bodyweight of male mice maintained on regular chow (final weight 24.6 ± 0.7 g, n=10, SD=2.07 for control, 24.9 ± 0.9 g, n=13, SD=3.24 for POMC-*vGlut2*^{-/-}, p=0.83, t(21)=0.22; RMANOVA for entire weight curve, p=0.23, $F_{1,210}=1.45$, N=10–13, Fig 5C). When male mice were maintained on high-fat chow after weaning, the mice were significantly heavier at 12 weeks of age (25.2 ± 0.8 g, n=8, SD=2.3 for control versus 30.2 ± 1.3 g, n=8, SD=1.35 for POMC-*vGlut2*^{-/-}, p=0.007, t(14)=3.15). There was a significant difference in the weight curves between control and POMC-*vGlut2*^{-/-} males maintained on high-fat diet (RMANOVA, p=0.01, $F_{1,14}=8.8$ by genotype and p<0.0001, $F_{9,126}=5.85$ for interaction between age and genotype). Posthoc analysis showed that bodyweights were significantly different between the genotypes beginning at 9 weeks of age (Fig. 5D). The food intake curves were also significantly different between control and POMC-*vGlut2*^{-/-} males maintained on high-fat chow (RMANOVA, p<0.0001, $F_{1,125}=67.95$, n=8) with the POMC-*vGlut2*^{-/-} mice eating significantly more at weeks 9–12. The deletion of *vGlut2* from POMC neurons did not cause any apparent difference in the number of POMC neurons, the number of neurons expressing Pomc-Cre-dependent-ChR2 or the percentage of POMC neurons expressing *Gad67* in the arcuate nucleus of adult mice. Although peptide content in POMC neurons was not assessed, the lack of phenotype on normal chow suggests that the mutant mice do not have extreme alterations in alpha-MSH content. Altogether, it appears that loss of glutamate release from POMC neurons selectively inhibited the ability of male mice to regulate their bodyweight on a high-fat diet as compared to control mice.

DISCUSSION

While the AA transmitter phenotype of hypothalamic POMC neurons has been characterized in adolescent and young adult mice (Jarvie and Hentges, 2012; Wittmann et al., 2013), it was not known if the distribution of GABAergic and glutamatergic POMC neurons is stable from early postnatal development until adulthood. The present study used fluorescent *in situ* hybridization to show that POMC neurons exhibit plasticity in their AA transmitter phenotype during postnatal development and used genetic deletion of *vGlut2* to examine the role of glutamate release from POMC neurons in energy balance regulation.

POMC neuron expression of *vGlut2* during postnatal development

This investigation found that early in postnatal development mice have *vGlut2* expression in a significant portion of POMC neurons and that the expression of *vGlut2* progressively declines as animals approach 8 weeks of age. The relatively high degree of colocalization in young mice may explain why a previous study (Wittmann et al., 2013) reported more *vGlut2* positive POMC neurons than the earlier Jarvie and Hentges (2012) report, although the two studies also used different *in situ* hybridization methods. The pattern of *vGlut2* expression across ages was not unique to POMC neurons since other cells in the arcuate nucleus devoid of POMC had a similar pattern of expression during postnatal development. These findings are in line with previous reports of relatively high *vGlut2* mRNA throughout the hypothalamus during postnatal development (Borgius et al., 2010; Boulland et al., 2004). Consistent with an increase in glutamatergic POMC neurons is the observation of increased expression of metabotropic glutamate receptor mGluR1 and mGluR5 in the hypothalamus

during early postnatal development (van den Pol et al., 1994; van den Pol et al., 1995). Additionally, the activity of these receptors in the hypothalamus is highest at postnatal day 10 (Palmer et al., 1990; Sortino et al., 1990) indicating that hypothalamic neurons are capable of receiving increased glutamatergic signaling during early postnatal development. The relative contribution of POMC-derived glutamate in enhanced hypothalamic glutamate signaling remains to be determined.

Although the significance of the transiently high *vGlut2* expression in the arcuate nucleus during postnatal development is unknown, in other brain regions developmentally regulated increases in glutamatergic signaling is involved in cell proliferation, neuron migration (Haydar et al., 2000; Komuro and Rakic, 1993; Luhmann et al., 2015) and guiding neurite outgrowth (Zheng et al., 1996). Interestingly, the transiently high *vGlut2* mRNA observed in the present study coincides with the development of axon projections from neurons in the arcuate nucleus to other hypothalamic regions, which occurs during the first three weeks of postnatal life in rodents (Bouret et al., 2004a; b). While proper development of these neural circuits depend on neonatal exposure to leptin (Bouret et al., 2004b; Bouyer and Simerly, 2013) and are sensitive to insulin and maternal nutrition (Vogt et al., 2014), some projections from POMC neurons appear to mature independent of leptin and insulin (Bouyer and Simerly, 2013; Vogt et al., 2014). Heterogeneity in the signals driving circuit development is known to occur for subpopulations of neurons within other hypothalamic nuclei (McClellan et al., 2008; Tong et al., 2007), thus it is plausible glutamatergic signaling during early postnatal developmental is important for a subset of projections from arcuate neurons.

POMC neuron expression of *Gad67* during postnatal development

The current study found that *Gad67* expression in POMC neurons progressively increases during early postnatal development. Expression of *Gad67* did not significantly change in the arcuate nucleus overall during postnatal development. Importantly, the relatively low expression of *Gad67* mRNA in POMC neurons at young ages was not compensated for by *Gad65*. Consistent with previous data showing similarity in the number and pattern of POMC neurons expressing *Gad65* or *Gad67* in adult mice (Jarvie and Hentges, 2012), the current findings suggest the distribution of *Gad* expressing POMC neurons is also similar during early postnatal development.

The functional significance of GABAergic POMC neurons is currently unknown. Given that GABA signaling is important in maintaining energy balance (Kong et al., 2012; Tong et al., 2008; Wu et al., 2009), it is likely that this population of POMC neurons also have a role in mediating energy balance. Nevertheless, body weight does not appear to be affected by a loss of GABA until animals reach adulthood (Kong et al., 2012; Tong et al., 2008); thus, raising the possibility that GABA's effects on feeding circuits occurs later in development. This notion is supported by evidence showing that GABA can influence hypothalamic circuit development after the second week of postnatal life (Di Giorgio et al., 2014; Frahm et al., 2012). Interestingly, the current study found *Gad67* expression in POMC neurons progressively increases until adult levels are reached between the third and fifth week of

postnatal life, which raises the possibility of GABAergic POMC neurons being involved in the later stages of postnatal neural development.

Switch in *vGlut2* and *Gad67* abundance in POMC neurons during postnatal development

Transient, widespread changes in expression of neurotransmitters, particularly AA transmitters, during development may precede neurotransmitter specification (Demarque and Spitzer, 2010). Additionally, expression of multiple neurotransmitters by an individual cell during development is thought to be involved in determining neurotransmitter phenotype (Gomez-Lira et al., 2005; Root et al., 2014) and involved in adapting to changing environments (Demarque and Spitzer, 2010; Gomez-Lira et al., 2005). Given the shift in the distribution of glutamatergic and GABAergic POMC neurons from day 1 to 8 weeks of age found in the present study, it was speculated that *vGlut2*⁺ POMC neurons in young animals might be part of the population of *Gad67*⁺ POMC neurons in adult mice. However, it does not seem likely that glutamatergic POMC neurons in young mice become GABAergic in adulthood since the majority of POMC neurons do not express a dual AA transmitter phenotype at any of the ages examined in the present study. While it is possible that phenotype switching occurs rapidly in POMC neurons and might have been missed in the age groups evaluated, this seems unlikely since changes in neurotransmitter phenotype appear to occur over multiple days and stages of development (Gillespie et al., 2005; Gomez-Lira et al., 2005; Root et al., 2014). Taken together, these data suggest the majority of glutamatergic and GABAergic POMC neurons are non-overlapping subpopulations. In line with this idea is recent evidence showing transcription factors *Ptf1a* and *Atoh1* are capable of controlling specification of GABAergic and glutamatergic cells in the cerebellum, respectively, and producing neurons with distinct AA transmitter phenotypes via mutual suppression of expression (Yamada et al., 2014). Deletion of *vGlut2* did not alter the percentage of POMC neurons expressing GABAergic markers in the arcuate nucleus of adult mice, further suggesting that the glutamatergic and GABAergic populations may develop independent of one another.

Deletion of *vGlut2* and energy balance regulation

In the present study, *vGlut2* was deleted from POMC neurons to determine if glutamate release from these neurons may contribute to energy balance regulation. The finding that male mice lacking *vGlut2* in POMC-Cre neurons gained significantly more weight on a high-fat diet than control mice indicates that glutamate release from POMC-Cre neurons must normally contribute to energy balance regulation in a sex- and diet-specific manner. This study appears to be one of the first to indicate a physiologic role for POMC-neuron-derived AA transmitters. It may be that additional studies examining other parameters of energy balance regulation or looking at later ages may reveal additional consequences of the loss of glutamate release from POMC neurons. It is important to note that the current approach of using a transgenic mouse breeding strategy to remove *vGlut2* has some limitations and caveats that must be considered. First, given that *vGlut2* expression is relatively high in POMC neurons postnatally compared to later in age, it may be that constitutive deletion of *vGlut* from POMC neurons leads to development differences that could potentially underlie the altered regulation of energy balance in the adult animals. Studies that disrupt *vGlut2* expression or activity in POMC neurons of adult mice will be

needed to distinguish potential developmental effects from the role of acute glutamate release from POMC terminals.

A second caveat to the transgenic approach is that the POMC-Cre transgene may be transiently expressed in neurons not fated to become adult POMC neurons. Previous studies show that during development, the POMC gene promoter is expressed in some cells in the arcuate nucleus and the nucleus of the solitary tract that do not express POMC peptides or *Pomc* mRNA in the adult (Padilla et al., 2010; Padilla et al., 2012). Thus, the approach used here could result in the deletion of *vGlut2* in cells other than authentic POMC neurons. However, the overall contribution from non-POMC neurons is likely to be minimal given the relatively small number of neurons that transiently express POMC that are not authentic POMC neurons and the small number of glutamatergic neurons in the adult arcuate nucleus. Deleting *vGlut2* from POMC neurons in adult mice using an inducible approach could be a means to avoid contributions from putative non-POMC neurons. Despite the limitations of the current experiments, the data clearly show that *vGlut2* deletion from neurons that express POMC-Cre during development can alter energy balance regulation and suggest that the physiologic consequence of AA transmitter release from POMC neurons and mechanisms of glutamate actions should be further explored.

Conclusion

It is well documented that hypothalamic POMC neurons exhibit heterogeneity in their neurotransmitter phenotype, receptor expression, and in the regions they innervate. The current results show that POMC neurons also exhibit plasticity in their AA transmitter phenotype during early postnatal development. It is not known if this plasticity represents a sensitive period for AA transmitter specification in POMC neurons, but future studies could help elucidate whether or not environmental perturbations alters AA transmitter phenotype of POMC neurons and the metabolic consequences later in life. It is also possible that the role for glutamate release from POMC neurons early in development is to help establish the circuitry needed for proper energy balance regulation. Future studies that abrogate POMC-neuron derived glutamate release in adulthood will help distinguish between developmental and sustained roles for glutamate release from these important neurons.

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Proopiomelanocortin (POMC) neurons are heavily studied due the involvement of their peptide transmitters in the regulation of energy balance. Here, the authors show that the amino acid transmitter (AA) phenotype of these neurons changes throughout early postnatal development and that disruption of AA phenotype alters energy balance regulation.

Figure 1. *vGlut2* expression in POMC neurons declines in early life. **A–C)** Representative images of *Pomc* (green) and *vGlut2* (magenta) mRNA in mice at ages 1 week, 3 weeks, and 8 weeks. White arrows denote cells coexpressing *Pomc* and *vGlut2* mRNA. 3V, third ventricle. Scale bar = 50 μ m. Boxed areas in 1 week image are enlarged to show colocalization of *Pomc* and *vGlut2* mRNA. Scale bar = 10 μ m for enlarged image. **D)** The percentage of POMC cells expressing *vGlut2* in the arcuate nucleus across postnatal development. Data are expressed as mean $\pm$ SEM. The numbers in parentheses represent the number of animals in each group.

Significance was determined relative to 8-week-old mice. *** $p < 0.0001$; ** $p = 0.0004$; * $p = 0.03$.

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Figure 2. *vGlut2* expression drops throughout the arcuate nucleus during postnatal development. **A)** Representative image from an animal at postnatal day 1 with analysis regions (ROIs) containing *Pomc* cells (green cells, solid circle) and lacking *Pomc* cells but containing *vGlut2* positive cells (dashed circle). White arrows indicate some cells coexpressing *Pomc* and *vGlut2* mRNA. 3V, third ventricle. Scale bar = 50 μ m. **B)** Intensity of *vGlut2* fluorescence above background in ROIs lacking *Pomc*. *** $p < 0.0001$; * $p = 0.018$. **C)** Intensity of *vGlut2* fluorescence above background in ROIs with *Pomc* neurons. *** $p < 0.0001$; ** $p = 0.006$. Data are expressed as mean $\pm$ SEM. The numbers in parentheses represent the number of animals in each group. Significance was determined relative to 8-week-old mice.

Figure 3.

Gad67 expression in POMC neurons increases with age. **A)** Representative images of *Pomc* (green) and *Gad67* (magenta) mRNA expressing cells in the arcuate nucleus from mice 1 day (top), 3 weeks (middle), and 8 weeks (bottom) of age. White arrows indicate cells coexpressing *Pomc* and *Gad67* mRNA. 3V, third ventricle. Scale bar = 50 μ m. Boxed areas in 8 week images are enlarged to show colocalization of *Pomc* and *Gad67* mRNA. Scale bar = 10 μ m for enlarged image. **B)** The percentage of POMC cells expressing *Gad67* in the arcuate nucleus across postnatal development. Data are expressed as mean $\pm$ SEM. The

numbers in parentheses represent the number of animals in each group. Significance was determined relative to 8-week-old mice. *** $p < 0.0001$; ** $p = 0.0004$; * $p = 0.009$. C) Representative image of *Pomc* (green) and *Gad65* (magenta) mRNA in mice at 1 week. White arrows denote cells coexpressing *Pomc* and *Gad65* mRNA. Scale bar = 50 μm .

Figure 4.

Detection of POMC neurons expressing both *vGlut2* and *Gad67* during postnatal development. Representative images of *vGlut2* (**A**, green) and *Gad67* (**B**, red) mRNA, and immunodetection of POMC-eGFP (**C**, blue) expression in the arcuate nucleus of an animal aged 5 weeks. **D**) Merged image of A–C. White arrows indicate a POMC cell containing *vGlut2* and *Gad67*. Scale bar = 50 μ m. Boxed area in A–D is enlarged in **E–H**.

Figure 5. *vGlut2* deletion in POMC neurons increases weight gain in males on a high-fat diet. **A–D**) Weight curves of control mice (black circles) and mice lacking *vGlut2* in POMC neurons maintained on normal chow (**A&C**) or high-fat diet (**B&D**). All data points are mean $\pm$ SEM. *= $p<0.05$ compared to same age control mice (*vGlut2*^{flox/flox}).

TABLE 1**Antibodies Used in This Study**

Antibody	Immunogen	Company, Cat#, RRID	Conc.
Primary			
Green Fluorescent Protein (GFP) Chicken, polyclonal	Recombinant full-length GFP	Abcam, Cat#ab13970 RRID:AB_300798	1:2,000
Digoxigenin conjugated to POD Sheep, polyclonal	digoxigenin (DIG)	Roche, Cat#11207733910 RRID:AB_5145000	1:1,000
Fluorescein conjugated to POD Sheep, polyclonal	fluorescein	Roche, Cat#11426346910 RRID:AB_840257	1:1,000
Secondary			
Anti-chicken IgY(IgG) (H+L)Alexa647 Donkey, polyclonal	chicken IgY(IgG) (H+L)	Jackson ImmunoResearch, Cat#703605155	1:1,000
Streptavidin Alexa555 Polyclonal	Biotin	Life Technologies Cat#S32355, RRID:AB_2307336	1:1,000
Anti-DNP-KLH Alexa488 conjugate Rabbit, polyclonal	dinitrophenyl (DNP)	Molecular probes, Cat#A11097 RRID:AB_2314332	1:400