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Caloric restriction selectively reduces the GABAergic phenotype of mouse hypothalamic proopiomelanocortin neurons

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Key points

- Hypothalamic proopiomelanocortin (POMC) neurons release peptide products that potently inhibit food intake and reduce body weight.
- These neurons also release the amino acid transmitter GABA, which can inhibit downstream neurons.
- Although the release of peptide transmitters from POMC neurons is regulated by energy state, whether similar regulation of GABA release might occur had not been examined.
- The present results show that the GABAergic phenotype of POMC neurons is decreased selectively by caloric deficit and not altered by high-fat diet or stress.
- The fact the GABAergic phenotype of POMC neurons is sensitive to energy state suggests a dynamic physiological role for this transmitter and highlights the importance of determining the functional consequence of GABA released from POMC neurons in terms of the regulation of normal energy balance.

Abstract In addition to peptide transmitters, hypothalamic neurons, including proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons, also release amino acid transmitters that can alter energy balance regulation. While recent studies show that the GABAergic nature of AgRP neurons is increased by caloric restriction, whether the GABAergic phenotype of POMC neurons is also regulated in an energy-state-dependent manner has not been previously examined. The present studies used fluorescence *in situ* hybridization to detect *Gad1* and *Gad2* mRNA in POMC neurons, as these encode the glutamate decarboxylase enzymes GAD67 and GAD65, respectively. The results show that both short-term fasting and chronic caloric restriction significantly reduce the percentage of POMC neurons expressing *Gad1* mRNA in both male and female mice, with less of an effect on *Gad2* expression. Neither acute nor chronic intermittent restraint stress altered *Gad1* expression in POMC neurons. Maintenance on a high-fat diet also did not affect the portion POMC neurons expressing *Gad1*, suggesting that the GABAergic phenotype of POMC neurons is particularly sensitive to energy deficit. Because changes in *Gad1* expression have been previously shown to correlate with altered terminal GABA release, fasting is likely to cause a decrease in GABA release from POMC neurons. Altogether, the present results show that the GABAergic nature of POMC neurons can be dynamically regulated by energy state in a manner opposite to that in AgRP neurons and suggest the importance of considering the functional role of GABA release in addition to the peptide transmitters from POMC neurons.

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Abbreviations AgRP, agouti-related peptide; AS, acute stress; CFR, chronic food restriction; CS, chronic stress; GAD, glutamate decarboxylase; HFD, high-fat diet; POMC, proopiomelanocortin.

Introduction

Many hypothalamic peptide transmitters clearly play important roles in energy balance regulation, including proopiomelanocortin (POMC) and agouti-related peptide (AgRP). Neurons expressing these peptides are thought to act in opposition to one another, mainly because AgRP and the POMC prohormone-derived peptide α-melanocyte stimulating hormone bind to the same melanocortin receptors to stimulate or suppress feeding, respectively. However, recent studies implicate amino acid transmitters from these neurons as important regulators of energy balance. Disruption of synaptic GABA release from AgRP neurons (Tong *et al.* 2008; Wu *et al.* 2009; Atasoy *et al.* 2012), in addition to a number of other hypothalamic neuron types including RIP-Cre neurons (Kong *et al.* 2012) and neurons expressing the PDX-1 transcription factor (Kim *et al.* 2015), reduces food intake and body weight. This suggests an orexigenic role for GABA released from various hypothalamic neurons under normal conditions. Although POMC neurons are also largely GABAergic (Hentges *et al.* 2009; Jarvie & Hentges, 2012; Wittmann *et al.* 2013), the extent to which disruption of GABA release from POMC neurons affects energy balance has not been examined. Only one study thus far has looked at disruption of amino acid transmitter production in POMC neurons and that study focused on the subset of POMC neurons that are glutamatergic (Dennison *et al.* 2016).

Loss of function studies have been pivotal for establishing the necessity of amino acid transmitter release for normal energy balance regulation, but such studies do not address whether transmitter release from these neurons is dynamically regulated in an energy-balance-dependent manner. One study did examine GABA release from AgRP neurons in response to caloric restriction and found that *Gad1* mRNA and GABA release was increased under this condition, which correlated well with increased GABA release from these cells (Dicken *et al.* 2015). This is consistent with the expected general increase in AgRP neuron activity when the animal is in a state of hunger (Hahn *et al.* 1998). The correlation between *Gad1* expression and terminal GABA release is not surprising given that the amount of GABA released is largely dictated by the amount of GABA in the terminal available for vesicular packaging (Mathews & Diamond, 2003; Apostolides & Trussell, 2013; Ishibashi *et al.* 2013; Wang *et al.* 2013; Dicken *et al.* 2015), which is primarily regulated by the activity of the GABA synthetic enzyme glutamate decarboxylase (67 kDa isoform) (GAD67; encoded by *Gad1* mRNA; Lau & Murthy, 2012). Although both GAD67 and glutamate decarboxylase (65 kDa isoform) (GAD65; encoded by *Gad2* mRNA) can produce GABA from the decarboxylation of glutamate, GAD67 is responsible for over 90% of brain GABA levels and is indispensable for most synaptic GABA transmission (Asada *et al.* 1997; Lau & Murthy, 2012). Several previous studies have shown that *Gad1* mRNA expression is more readily altered by various conditions than *Gad2* mRNA (Rimvall & Martin, 1992, 1994; McCarthy, 1995; Bowers *et al.* 1998; Mason *et al.* 2001). The finding that *Gad1* is especially sensitive to changes in physiological state was also found for hypothalamic AgRP neurons where *Gad1* mRNA expression was increased in response to fasting, without altering the percentage of AgRP neurons expressing *Gad2* (Dicken *et al.* 2015).

The present study was designed to determine whether *Gad1* mRNA expression in POMC neurons would be altered by caloric restriction in a manner similar to that observed in AgRP neurons, or rather if fasting would cause a decrease in *Gad1* mRNA in POMC neurons, consistent with the decreased activity of POMC neurons expected under a fasted condition (Mizuno *et al.* 1998; Singru *et al.* 2007). Using dual fluorescence *in situ* hybridization for *Gad* and *Pomc*, the results show that short-term fasting and prolonged caloric restriction selectively decreases the GABAergic nature of POMC neurons.

Methods

Ethical approval

All animal use procedures were approved by the Institutional Animal Care and Use Committee at Colorado State University. The work met United States Public Health guidelines for the ethical use of laboratory animals and was consistent with the policies and regulations for animal experimentation described by *The Journal of Physiology*.

Animals

All mice were housed at a controlled temperature (22–24°C) with a 12 h light–dark cycle and given tap water and standard rodent chow (Teklad 2018) *ad libitum* except as noted. All mice were congenic to the C57BL/6 background strain and tissue was collected at 8–10 weeks of age unless otherwise noted. Wild-type mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) or bred in-house. POMC-Cre transgenic mice (Xu *et al.* 2005) were originally obtained from Dr Greg Barsh (Stanford University). For overnight fasting studies, food was removed from the cages between 14.00 and

16.00 h with lights-out at 18.00 h, and mice were killed the following morning for tissue collection. For chronic food restriction studies, experimental mice received ∼70% of their baseline chow intake, adjusted as needed, so that mice would be at 80% of their starting weight 2 weeks after the start of food restriction. In the high-fat diet studies, mice were provided high-fat chow (60% kcal from fat, Teklad D.06414, Madison, WI, USA) or low fat control diet (10% kcal from fat, Teklad D.08806, Envigo, Indianapolis, IN, USA) *ad libitum* for 20 weeks. At the end of the diet period, glucose tolerance was assessed after a 6 h fast beginning at 08.00 h. Glucose was delivered (2 g kg^{-1}, I.P.) at 14.00 h. Samples were analysed using a TRUEtrack blood glucose monitoring system (Trividia Health, Fort Lauderdale, FL, USA) at 0, 15, 30, 60, 90 and 120 min post-injection. A quality control test with the provided Check Strip was performed on the device before each test day. For each sample time, the mouse was restrained in a tailveiner for mice (TV-150, Braintree Scientific, Braintree, MA, USA) for the collection of the blood drop from a tail vein nick. For stress experiments, acute restraint stress was induced by immobilizing mice once for 60 min in a plastic restraint tube just prior to killing and tissue collection. Chronic restraint stress used the same approach, but mice were immobilized for 120 min daily at random times for 12 days and killed on the 13th day.

In situ hybridization

Fluorescence *in situ* hybridization for *Pomc* and *Gad1* or *Gad2* mRNA was carried out as previously described (Dennison *et al.* 2016). In brief, mice were deeply anaesthetized with 200 mg kg^{-1} sodium pentobarbital and transcardially perfused with 10% sucrose in water followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). Brains were removed and postfixed overnight in 4% paraformaldehyde and coronal sections (50 μm) containing the arcuate nucleus were prepared. Endogenous peroxidase activity was quenched by placing sections in 6% H_2O_2 for 15 min. Tissue was then exposed to proteinase K (10 μg ml^{-1}, 15 min) followed by glycine (2 mg ml^{-1} in PBS containing 0.1% Tween 20; PBT) and subsequently postfixed in PBT containing 4% paraformaldehyde and 0.2% gluteraldehyde (20 min). Tissue was dehydrated through ascending concentrations of ethanol and placed into hybridization solution (66% (v/v) deionized formamide, 13% (w/v) 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 h at 60°C. Probes were denatured (85°C, 5 min) and then added to the hybridization solution. The *Pomc* probe (750 pg ml^{-1}, corresponding to bases 532–1000 of GenBank sequence NM_008895.3) and the *Gad1* probe (150 pg ml^{-1}, corresponding to bases 749–1527 of NM_00877.4) were added simultaneously as both probes hybridized at 70°C (18 h). For *Pomc*/*Gad2* dual *in situ* hybridization experiments, the *Pomc* probe was hybridized first and then the tissue was placed into fresh hybridization buffer containing the *Gad2* probe (two probes mixed together that recognize bases 537–1207 and 1201–2032 of NM_008078.2 each at 150 pg μl^{-1}) and hybridized for 18 h at 52°C. The probe sequences have been previously characterized and were prepared as described in an earlier publication (Jarvie & Hentges, 2012). The fluorescein isothiocyanate-labelled *Pomc* probe and the digoxigenin-labelled *Gad* probes were detected as previously described (Dennison *et al.* 2016) such that the *Pomc* probe was labelled with a secondary antibody conjugated to Alexa Fluor 488 and the *Gad* probes were labelled with a secondary antibody conjugated to Alexa Fluor 555. Tissue sections were mounted onto glass slides and coverslipped with Aqua Poly/Mount (Polysciences, Warrington, PA, USA).

Image collection and analysis

Images were collected on a Zeiss 510 Meta confocal microscope with each fluorophore imaged sequentially to avoid crossover between channels. For each tissue section, one or two stacks of images (8 images, 3 μm apart in the *z*-plane) were collected in each channel. For each animal, eight *z*-stack images were collected and analysed from at least four separate slices (1 image from each side of the arcuate nucleus). For analysis, each stack of images was pared down to five sequential images that omitted images taken at the surface of the tissue slice. All imaging parameters were kept constant for a given experiment and each run of the *in situ* hybridization experiment contained tissue from both control and experimental animals. Cell counts were made using a modification of the 3D counting method described by Williams & Rakic (1988) to reduce oversampling. Only *Pomc*-expressing cells completely contained within a 300 μm × 300 μm × 2 μm bounding box on the *x*–*y*–*z* planes were counted and analysed for colocalization with the *Gad* probe. *Pomc*-expressing cells were detected using masks created automatically in NIH ImageJ software to identify Alexa Fluor 488-labelled cells. Each *Pomc* cell was judged to be positive or negative for the *Gad* signal by a rater blinded to the experimental conditions. Average fluorescence intensity had to be greater than 10% above background for a cell to be considered positive for either signal, and for *Gad* mRNA the signal had to be constrained within the somatic region of the *Pomc*-expressing cell for the *Pomc* cell to be considered colabelled. Ten per cent above background was chosen as the threshold because it is the lowest fluorescence level at which a cell could easily be distinguished from the background signal. Choosing this threshold helped to remove individual bias when manually

Table 1. Summary of groups and cell counts

	Males			Females		
Group	N^a	Cells per animal[b]	P (t)[c]	N^a	Cells per animal[b]	P (t)[c]
Fasted						
Control, *Gad1*	7	231 ± 31 (155, 306)		11	300 ± 24 (247, 353)	
Gad1	8	200 ± 29 (131, 270)	0.49 (0.72)	15	243 ± 22 (197, 290)	0.09 (1.75)
Control, *Gad2*	5	234 ± 28 (156, 312)		6	283 ± 46 (164, 401)	
Gad2	6	248 ± 22 (191, 304)	0.71 (0.38)	10	224 ± 27 (164, 285)	0.26 (1.17)
Chronic food restricted						
Control, *Gad1*	4	304 ± 40 (178, 430)		4	365 ± 23 (291, 439)	
Gad1	4	261 ± 21 (195, 327)	0.38 (0.96)	4	324 ± 32 (222, 426)	0.34 (1.04)
Control, *Gad2*	4	271 ± 15 (223, 318)		4	379 ± 30 (283, 474)	
Gad2	4	291 ± 37 (172, 410)	0.63 (0.5)	4	320 ± 34 (212, 427)	0.24 (1.3)
High-fat diet						
Control, *Gad1*	5	175 ± 23 (111, 239)		5	188 ± 13 (152, 224)	
Gad1	5	215 ± 20 (160, 270)	0.23 (1.31)	4	227 ± 20 (164, 290)	0.13 (1.7)
Control, *Gad2*	5	281 ± 34 (186, 377)		5	157 ± 23 (92, 222)	
Gad2	5	265 ± 21 (205, 324)	0.69 (0.41)	5	173 ± 29 (92, 255)	0.68 (0.43)
Acute stress						
Control, *Gad1*	4	261 ± 5 (246, 276)		4	298 ± 37 (180, 416)	
Gad1	3	263 ± 11 (216, 310)	0.87 (0.18)	4	314 ± 32 (214, 415)	0.75 (0.33)
Control, *Gad2*	4	299 ± 16 (247, 350)		4	385 ± 23 (311, 458)	
Gad2	4	268 ± 21 (202, 333)	0.28 (1.17)	3	323 ± 29 (199, 446)	0.15 (1.69)
Chronic stress						
Control, *Gad1*	3	277 ± 43 (90, 464)		4	244 ± 38 (123, 364)	
Gad1	5	316 ± 12 (283, 350)	0.31 (1.11)	5	258 ± 21 (199, 316)	0.75 (0.34)
Control, *Gad2*	3	282 ± 9 (242, 312)		4	275 ± 15 (226, 323)	
Gad2	5	266 ± 18 (215, 317)	0.56 (0.62)	5	291 ± 22 (230, 353)	0.57 (0.59)

[a]Number of animals in the group. [b]Number of *Pomc*-expressing cells counted from each animal in a treatment group presented as mean ± SEM (95% CI). [c]Unpaired Student's t test (equal variance) for the number of cells counted between each control and experimental group.

counting cells, as each of the investigators could only count cells as positive if 10% above background, even if they might be inclined to deem a fainter signal as a positive cell. For each *Pomc* cell identified as expressing the *Gad* label, the fluorescence intensity was collected and reported as a percentage above background. Reporting intensity values as a percentage above background helped account for section-to-section variation or experiment-to-experiment variation in fluorescence and allowed us to normalize the data and thus compare intensities between animals The percentage of POMC neurons expressing *Gad* mRNA in any experimental condition is expressed relative to the tissue from control animals processed in parallel to account for possible variability in intensity from one *in situ* hybridization run to another. The number of POMC neurons counted per animal was very consistent (mean ± SEM = 263 ± 5.4 cells per image set per animal) and there was no difference in the number of POMC cells per image set between any treatment group and its matched control group (Table 1).

Statistics

Data were analysed using one-sample, two-tailed Student's t test or two-way ANOVA except when testing for changes in relative intensity of the *Pomc* signal per cell, where Mann–Whitney analyses were performed. All statistics were calculated in Microsoft Excel or Prism 6.0 (GraphPad Software, La Jolla, CA, USA). Differences between groups were considered significant if $P < 0.05$. Data are presented as the mean ± SEM.

Results

Fasting reduces *Gad* expression in POMC neurons

To determine if caloric deficit can alter the GABAergic phenotype of POMC neurons, dual *in situ* hybridization studies were performed to detect *Gad1* or *Gad2* mRNA along with *Pomc* mRNA in tissue sections from mice either *ad lib* fed or with restricted food access. An overnight fast reduced the percentage of *Pomc*-positive cells expressing

Gad1 in male (to 68 ± 4% of control; $t(13) = 4.5$, $P = 0.0006$) and female (to 79 ± 4% of control; $t(24) = 4.6$, $P = 0.0002$) mice (Fig. 1*A*–*C*). Fasting also modestly reduced the fraction of POMC cells expressing *Gad2* in female (to 87 ± 4% of control; $t(14) = 2.6$, $P = 0.02$), but not male (fasted, 90 ± 7% of control, $P = 0.31$) mice (Fig. 1*D*).

Although analyses were largely restricted to assessing the presence or absence of *Gad* signal in *Pomc*-expressing cells as the *in situ* hybridization method used is not strictly quantitative, the median intensity of *Pomc* signal per cell was decreased by fasting in males (control, 258, $n = 806$; fasted, 222, $n = 1153$) and females (control, 293, $n = 1419$; fasted, 254, $n = 1614$) and the distribution of *Pomc* intensities in the control and fasted groups was significantly different for both males (Mann–Whitney $U = 403832$, $P < 0.0001$, two-tailed; Fig. 2*A*) and females ($U = 1014785$, $P < 0.0001$, two-tailed; Fig. 2*B*) consistent with previous reports showing that fasting decreases *Pomc* message and POMC peptide levels (Mizuno *et al.* 1998; Perello *et al.* 2007). Despite the decrease in the overall intensity of the *Pomc* signal after fasting, the number of POMC cells counted was not different between the control and fasted groups (Table 1). Consistent with the reduced number of POMC cells expressing *Gad1* mRNA after caloric restriction, overnight fast caused a decrease in the median intensity of *Gad1* signal per *Pomc*-positive cell compared to control in male (control intensity, 142,

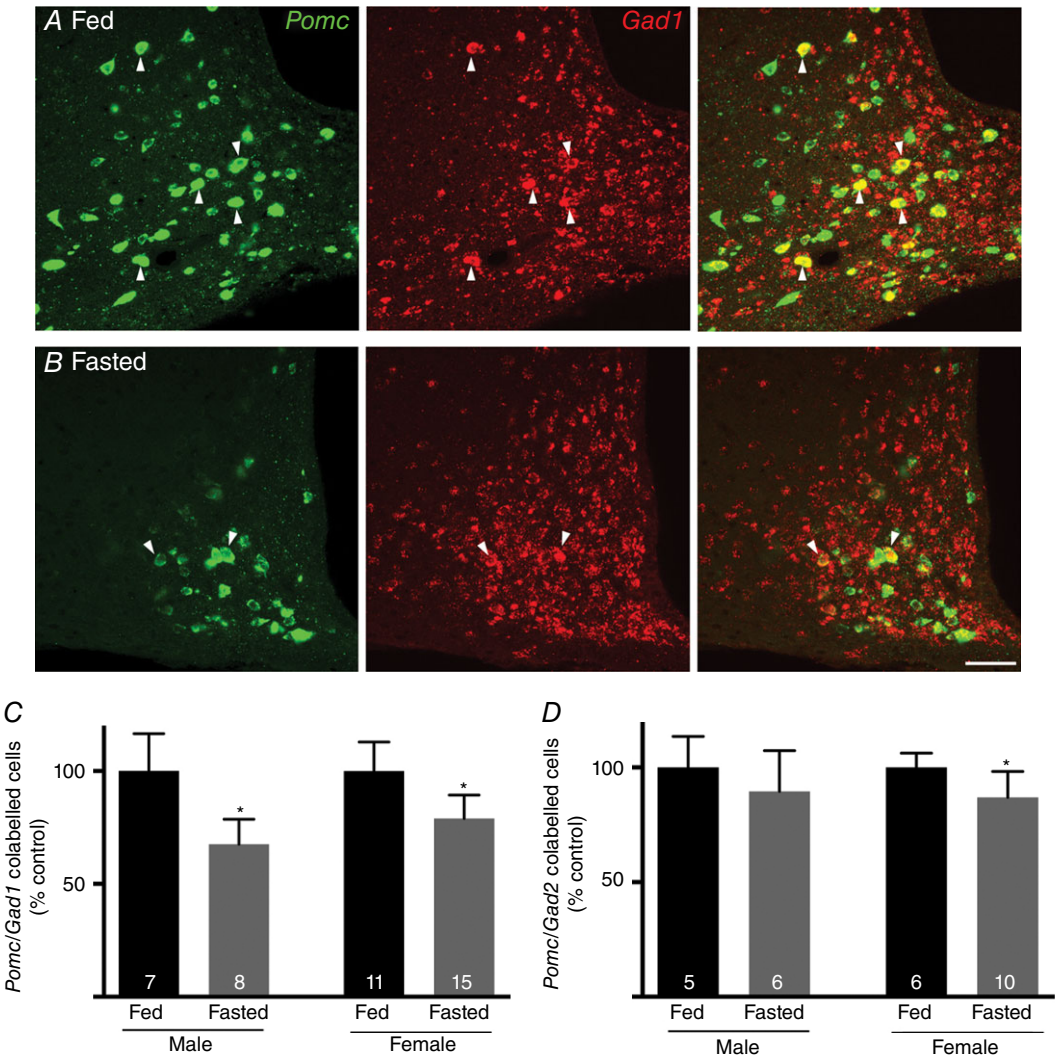

Figure 1. Fasting decreases the GABAergic phenotype of POMC neurons
A and *B*, representative images showing *Pomc* mRNA (green) and *Gad1* mRNA (red) in sections from an *ad lib* fed mouse (*A*) or a mouse that had been fasted overnight (*B*). A merge of the left and centre images is shown in the far right image. White arrowheads indicate some of the double-labelled cells. The scale bar in the lower right image is 50 μm. *C* and *D*, bar graphs showing the percentage of *Pomc*-positive cells that express *Gad1* (*C*) or *Gad2* mRNA (*D*) normalized to control in fed (black bars) and fasted mice (grey bars). $^*P < 0.05$ compared to matched control group. The number of animals included in each group is noted at the bottom of the bars.

$n = 571$; fasted intensity, 104, $n = 368$; Mann–Whitney $U = 71584$, $P < 0.0001$, two-tailed) and female (control, 144, $n = 560$; fasted, 121, $n = 480$; $U = 114123$, $P < 0.0001$ two-tailed) mice (Fig. 2*C* and *D*). There was no difference in *Gad2* intensity in POMC cells from fed (73, $n = 335$) and fasted (73, $n = 404$) male mice ($U = 66{,}542$, $P = 0.7$), whereas females showed a small difference in *Gad2* intensity (control, 160, $n = 676$; fasted, 177, $n = 788$; $U = 237{,}080$, $P = 0.003$).

Limiting food access to ∼70% of normal for 2 weeks (chronic food restriction; CFR) also decreased the portion of POMC neurons expressing *Gad1* (Fig. 3*A–C*) in male (to 61 ± 3% of control; $t(6) = 5.0$, $P = 0.002$) and female (CFR, 56 ± 4% of control; $t(6) = 6.8$, $P = 0.0005$) mice. CFR also caused a modest decrease in the percentage of POMC neurons expressing *Gad2* (Fig. 3*D*) in male mice (CFR, 78 ± 6% of control; $t(6) = 2.8$, $P = 0.03$) but not in females (CFR, 83 ± 10 of control, $P = 0.23$).

High-fat diet does not alter the proportion of GABAergic POMC neurons

To determine if fat consumption and caloric excess would have the opposite effect on the number of POMC cells expressing *Gad*, mice were maintained on a high-fat diet (HFD) for 20 weeks. Mice on the HFD gained significantly more weight than those on the low fat control diet (by 2-way ANOVA, male weight curves $F(1,160) = 121.3$, $P < 0.0001$; females $F(1,160) = 30.1$, $P < 0.0001$, Fig. 4*A*) and showed reduced glucose clearance rates (by 2-way ANOVA male glucose curves, $F(1,48) = 36.2$, $P < 0.0001$; females, $F(1,48) = 8.1$, $P = 0.007$, Fig. 4*B*). Despite the increased weight and decreased glucose tolerance, maintenance on HFD did not alter the percentage of POMC cells expressing *Gad1* or *Gad2* in male (*Gad1*, HFD 95 ± 4% of control, $P = 0.78$; *Gad2*, HFD 106 ± 3% of control, $P = 0.26$) or female (*Gad1*, HFD 101 ± 4% of control, $P = 0.95$; *Gad2*, HFD 116 ± 5% of control, $P = 0.09$) mice (Fig. 4*C* and *D*). Thus, *Gad* in POMC neurons might be selectively sensitive to caloric deficit.

Stress does not affect *Gad* expression in POMC neurons

It is likely that caloric deficit is stressful for mice as suggested by fasting-induced increases in corticosterone (Makimura *et al.* 2003). Therefore, to determine if caloric deficit, or perhaps rather the stress of dieting, was responsible for the decrease in *Gad* colocalization observed

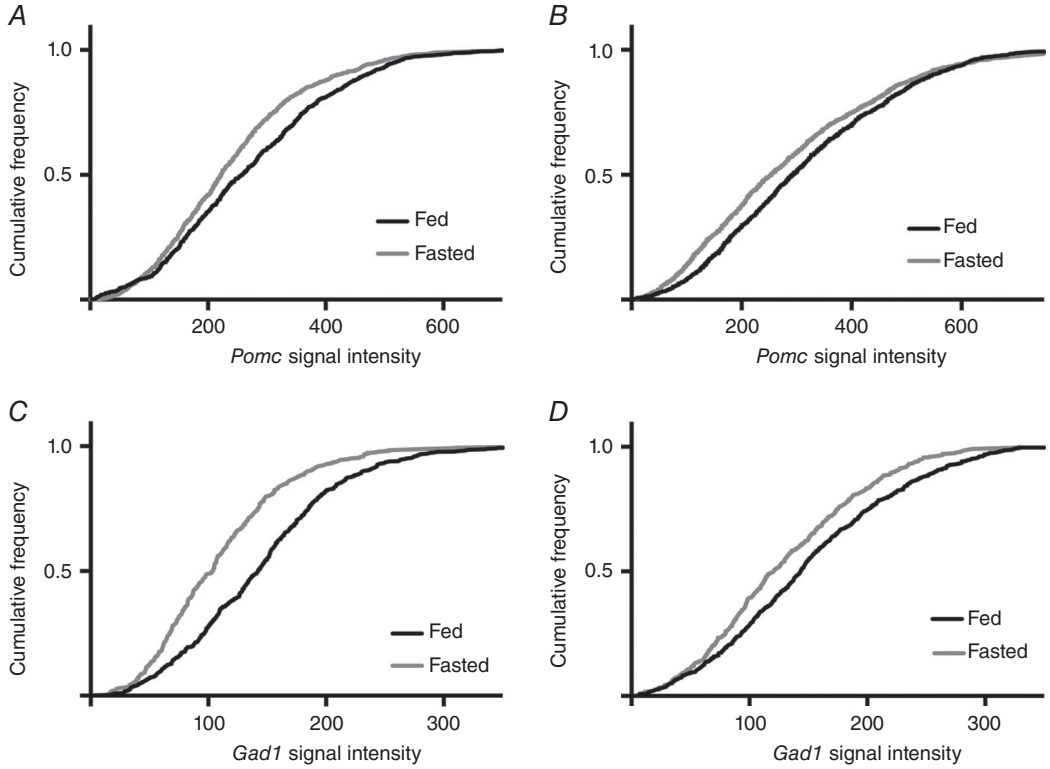

Figure 2. Overnight fasting decreases the intensity of *Pomc* and *Gad1* signals in POMC neurons
A and *B*, cumulative frequency plots showing decreased signal intensity per cell (percentage above background) for *Pomc* in males (*A*) and females (*B*). *C* and *D*, cumulative frequency distribution showing the intensity of *Gad1* signal in *Pomc* neurons is also left-shifted for males (*C*) and females (*D*). The distribution of intensities is significantly different ($P < 0.0001$) between fed and fasted mice for all groups shown based on Mann–Whitney tests.

with fasting and CFR, mice were exposed to restraint stress either one time (acute stress, AS) or once a day for 12 days (chronic stress, CS). AS did not alter the percentage of POMC neurons expressing *Gad1* (males, 96 ± 8% of control, $P = 0.72$; females, 85 ± 9% of control, $P = 0.31$) or *Gad2* (males, 94 ± 5% of control, $P = 0.33$; females, 82 ± 9% of control, $P = 0.36$) in mice of either sex (Fig. 5*A*). Similarly, chronic stress (Fig. 5*B*) also had no effect on the percentage of POMC neurons coexpressing *Gad1* (males, 86 ± 10% of control, $P = 0.37$; females, 94 ± 5% of control, $P = 0.61$) or *Gad2* (males, 96 ± 3% of control, $P = 0.41$; females, 101 ± 3% of control, $P = 0.84$). Therefore, it appears that caloric restriction specifically, rather than the associated stress is responsible for the decrease in *Gad* colocalization in POMC neurons after fasting or CFR. Consistent with previous reports (Baubet *et al.* 1994; Liu *et al.* 2007), daily restraint stress increased the relative intensity of the *Pomc* label in female mice compared to the non-stressed control (intensity: control, 1908, $n = 1885$; stressed group, 2226, $n = 2431$; Mann–Whitney $U = 2036437$, $P < 0.0001$, two-tailed) in the present study. Together, these results suggest that *Gad1* and *Pomc* transcription can be independent of one another and that *Gad1* transcription does not necessarily

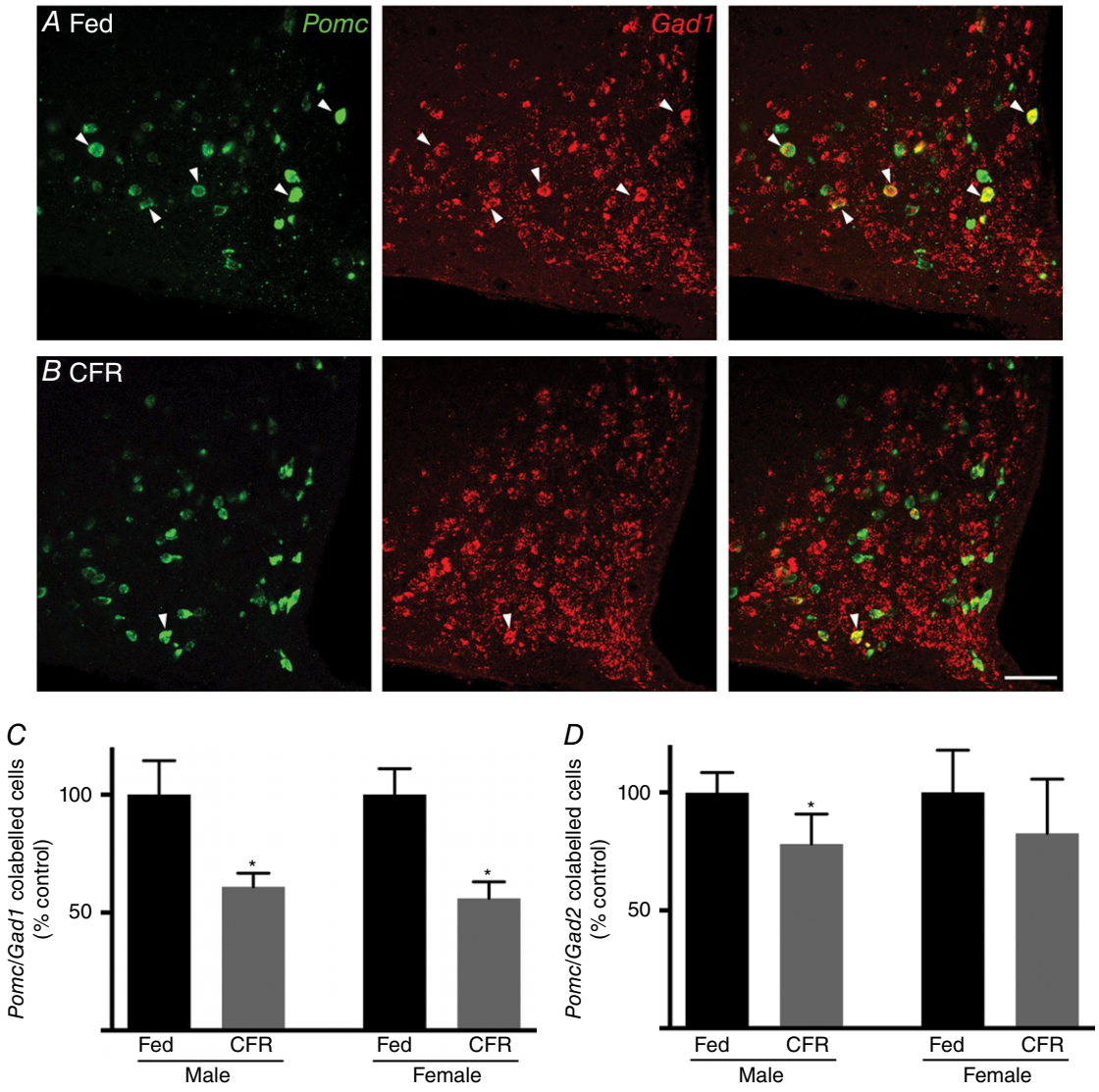

Figure 3. Chronic food restriction decreases *Gad1* colocalization in POMC neurons
A and *B*, representative images showing *Pomc* mRNA (green) and *Gad1* mRNA (red) in sections from an *ad lib* fed mouse (*A*) or a mouse that had been chronically food restricted (CFR, *B*). A merge of the left and centre images is shown in the far right image. White arrowheads indicate some of the double-labelled cells. The scale bar in the lower right image is 50 μm. *C* and *D*, bar graphs showing the percentage of *Pomc* cells expressing *Gad1* (*C*) or *Gad2* mRNA (*D*) normalized to control in fed (black bars) and fasted mice (grey bars). $^*P < 0.05$ compared to matched control group. $n = 4$–5 mice/group.

parallel POMC neuron activation as restraint stress has been shown to increase cFos expression in POMC neurons (Liu *et al.* 2007).

Caloric restriction does not change the overall expression of *Gad1* or *Gad2* in the arcuate nucleus

To determine whether the decrease in *Gad1* signal in POMC neurons after caloric restriction selectively occurs in POMC neurons, or rather reflects overall changes in *Gad1* expression in the arcuate nucleus, the intensity of the *Gad1* signal was determined for a large area within the region that included both POMC and non-POMC neurons. Neither fasting nor CFR affected the overall *Gad1* signal in male (fasting: control, 2.7 ± 0.2-fold over background; fasted, 2.6 ± 0.1; $P = 0.79$; CFR: control, 3.1 ± 0.2-fold over background; CFR, 3.3 ± 0.5; $P = 0.71$) or female (fasting: control, 2.6 ± 0.1-fold over background; fasted, 2.6 ± 0.1; $P = 0.96$; CFR: control, 3 ± 0.3-fold over background; CFR, 2.9 ± 0.1; $P = 0.62$)

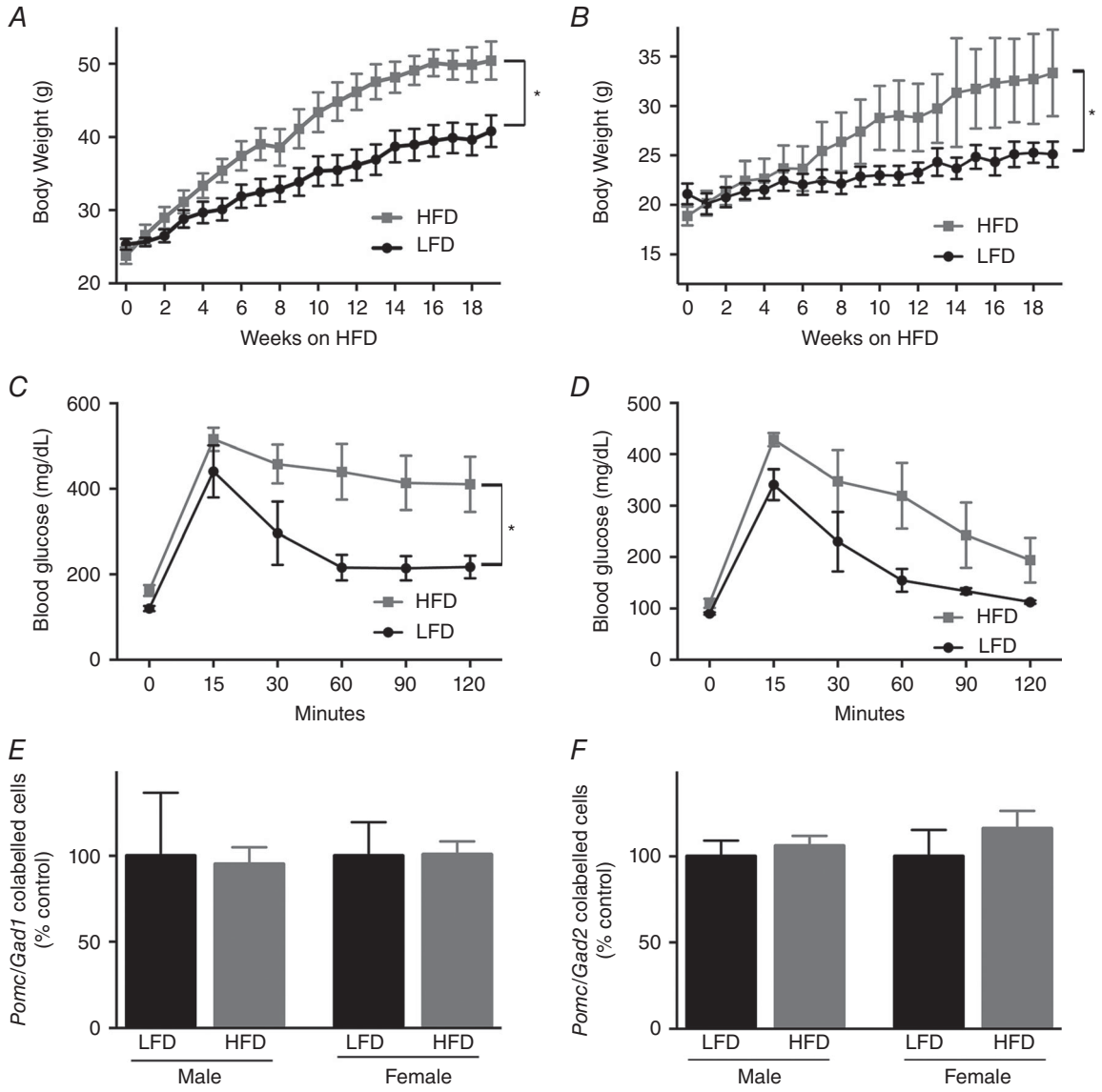

Figure 4. High-fat diet induces weight gain and decreases glucose clearance, but does not alter the fraction of POMC neurons expressing *Gad* mRNA
A and *B*, weight curves for male (*A*) and female (*B*) mice showing that HFD increases body weight over the 19 weeks on the diet. *C* and *D*, glucose clearance was also decreased in both males (*C*) and females (*D*) at the end of the HFD period. *$P < 0.05$. *E* and *F*, bar graphs representing the percentage of *Pomc*-positive cells that express *Gad1* (*E*) or *Gad2* mRNA (*F*) normalized to control in mice fed standard low fat diet (LFD, black bars) or fed a high-fat diet (HFD, grey bars). No significant difference was detected between any control and HFD group. $n = 4$–5 mice/group.

mice (Fig. 6), consistent with the possibility that *Gad1* is selectively decreased in POMC neurons in response to caloric deficit.

Discussion

Despite the well-described roles for POMC neuron-derived peptides in the regulation of energy balance and the growing evidence indicating that GABA from hypothalamic neurons contributes to energy balance, the possibility that energy state can alter GABA release has received little attention. The present results show that energy deficit can inhibit the portion of POMC neurons expressing *Gad*, whereas HFD and stress do not. Together with previous studies correlating *Gad1* expression with terminal GABA release, the results suggest that GABA release from POMC neurons can be dynamically regulated and is selectively sensitive to caloric restriction.

Dynamic regulation of *Gad1* in POMC neurons

The GABAergic phenotype of POMC neurons has been recognized since early electron microscopy studies identified small clear vesicles in axon terminals labelled with antibodies against POMC peptides (reviewed in Mercer *et al.* 2013). More recent work identified the presence of *Gad* mRNA in POMC neurons and showed functional release of GABA from these cells (Hentges *et al.* 2004, 2009; Dicken *et al.* 2012; Jarvie & Hentges, 2012; Wittmann *et al.* 2013). The GABAergic nature of POMC neurons is a bit perplexing given that AgRP neurons are also GABAergic (Wu & Palmiter, 2011), despite the fact that these two populations of neurons generally act in opposition to one another. While it is clear that GABA release from AgRP neurons plays a role in the stimulation of food intake (Wu & Palmiter, 2011), whether GABA release from POMC neurons has a similar or opposing effect is not yet known. It is also not known whether these two cell groups release GABA onto the same or different

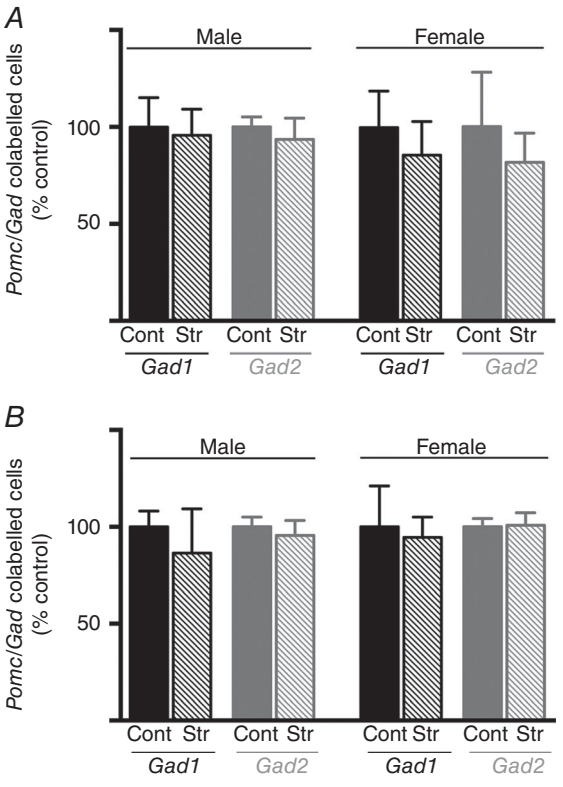

Figure 5. Stress does not alter the fraction of POMC neurons expressing *Gad* mRNA
Bar graphs representing the percentage of *Pomc*-positive cells that express *Gad1* (black bars) or *Gad2* mRNA (grey bars) normalized to control (filled bars) in mice that were exposed to an acute stressor (*A*, hatched bars) or chronic intermittent stress (*B*, hatched bars). No significant difference was detected between any control and HFD group. $n = 3$–5 mice/group.

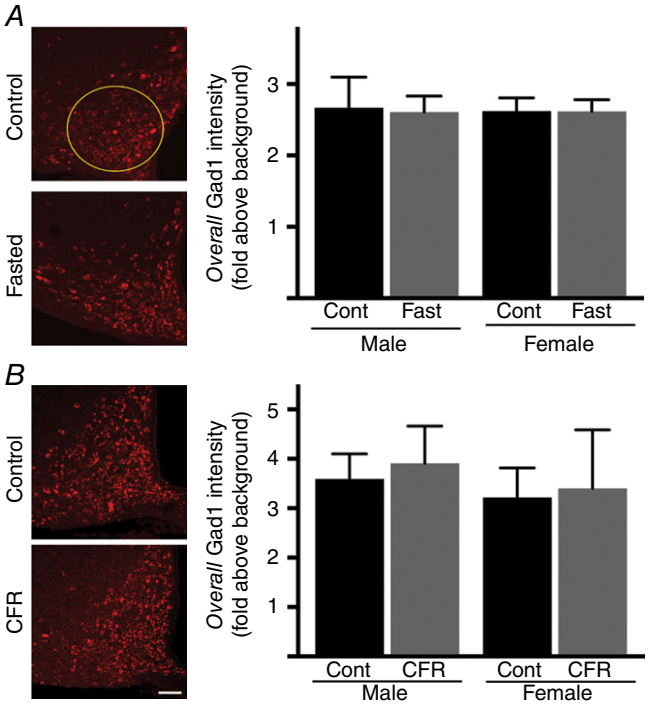

Figure 6. The overall intensity of the *Gad1* signal in the arcuate nucleus is not altered by caloric deficit
Representative images of *Gad1* mRNA detected in the arcuate nucleus in sections from *ad lib* fed (control) mice or a mouse that had been fasted (*A*) or chronically food restricted (CFR, *B*). The yellow circle in the upper panel indicates the large region of interest (ROI) from which average signal intensities were taken. The ROI was placed adjacent to the 3rd ventricle and the ventral border of the slice for each slice analysed. The scale bar in the lower right image is 50 μm. The bar graphs show the percentage of *Pomc*-positive neurons that coexpress *Gad1* in *ad lib* fed mice (black bars) or mice with caloric deficit (grey bars). $n = 4$–5 mice/group.

downstream target neurons. What is clear from the present results is that GABA release from POMC neurons is likely to be regulated in a manner opposite to that in AgRP neurons in response to fasting. A recent study shows that fasting increases *Gad1* expression in AgRP neurons and that this correlates with an increase in vesicular GABA release, counter to the decrease in *Gad1* in POMC neurons found here. The differential regulation of *Gad1* expression in these cell types may explain why a global change in *Gad1* was not detected in the arcuate nucleus overall in response to fasting in the present work or in previous studies (Schwartz *et al.* 1993; Dicken *et al.* 2015).

Recently, Dennison *et al.* (2016) showed that the expression of *Gad1* is limited to very few POMC neurons in the early postnatal period and increases until adult levels of expression are reached at approximately 5 weeks of age. Thus, this period of rapid growth in early life appears to repress the GABAergic phenotype of POMC neurons similar to that found here for conditions of caloric deficit. The decrease in *Gad1* mRNA in POMC neurons in the calorically deficient state correlates with the decrease in POMC neuron activity, *Pomc* mRNA expression and reduced α-melanocyte stimulating hormone release observed after fasting (Mizuno *et al.* 1998; Perello *et al.* 2007; Singru *et al.* 2007). Thus, it may be that *Gad1* expression directly correlates with cellular activity, although present results show that stress can increase *Pomc* without altering *Gad1* signal in POMC neurons. Additionally, *Gad1* transcription is directly sensitive to a number of physiological factors including glucose, which can increase *Gad1* (Pedersen *et al.* 2001), perhaps independently of altered cellular activity.

The present results showing that energy deficit decreases the GABAergic phenotype of POMC neurons would be consistent with a putative anorexigenic role for POMC-neuron-derived GABA. Interestingly, the results in Dennison *et al.* (2016) also suggest a potential anorexigenic role for glutamate released from POMC neurons. It may be that these transmitters exert opposing actions on distinct target cells to similarly effect food intake, but further studies are needed to explore this possibility directly.

Differential sensitivity of *Gad1* and *Gad2* to caloric restriction

The two enzymes that catalyse the conversion of glutamate to GABA are products of two separate genes with distinct transcriptional regulatory elements (Soghomonian & Martin, 1998). Additionally, GAD65 (encoded by the *Gad2* gene) is heavily regulated by post-translational activation by its coenzyme pyridoxal 5′-phosphate, whereas the majority of GAD67 (encoded by the *Gad1* gene) is maintained in the active holoenzyme state (Wei & Wu, 2008), and thus its activity is primarily regulated at the transcriptional level (Pedersen *et al.* 2001). *Gad1* and its protein product are sensitive to a number of physiological and pathological conditions including immune activation, stress, reproductive state, schizophrenia and energy status, whereas *Gad2* expression tends to be more stable (Cashion *et al.* 2004; Dent *et al.* 2007; Zhao *et al.* 2012; Dicken *et al.* 2015; Makinson *et al.* 2015; Cassella *et al.* 2016). Consistent with this trend, the present results show that *Gad1* expression in POMC neurons was more sensitive to caloric deficit compared to *Gad2* expression, which showed smaller and inconsistent changes in colocalization after fasting.

Gad1 expression and implications for GABA release

Gad1 expression is not only more sensitive to physiological challenge compared to *Gad2*, but GAD67 is also responsible for the vast majority of GABA production and release from neurons (Asada *et al.* 1997). Importantly, it appears that the level of cytosolic GABA, dictated primarily by GAD67 activity and not vesicular GABA transporter expression, is the key determinant of vesicular GABA content and release (Lau & Murthy, 2012). Thus, the expression of *Gad1* can be a reliable indicator of terminal GABA release, as was recently shown to be the case in AgRP neurons (Dicken *et al.* 2015). In AgRP neurons, fasting increased the expression of *Gad1* and enhanced the readily-releasable pool of GABA from AgRP neurons, whereas the inhibition of GAD activity decreased GABA release. Therefore, it is reasonable to expect that the decrease in *Gad1* colocalization observed here after fasting would correlate to reduced release of synaptic GABA from POMC neurons and relative disinhibition of target neurons, which would likely affect food intake. Testing the regulation of postsynaptic cells directly will require the development of new methods that would allow the reliable identification of postsynaptic cells directly receiving GABAergic POMC inputs.

Conclusions

Altogether, the present results show that *Gad1* coexpression in POMC neurons is inhibited by caloric deficit, but is not altered by stress or high-fat diet and weight gain. This inhibition is in contrast to the increase in *Gad1* observed in AgRP neurons after fasting (Dicken *et al.* 2015). Unlike the known role for GABA released from AgRP neurons to stimulate food intake, the consequence of GABA released from POMC neurons has not been demonstrated, although glutamate release from POMC neurons does affect normal energy balance regulation (Dennison *et al.* 2016). The fact that *Gad* expression in POMC neurons is dynamically regulated throughout postnatal development and in response to caloric deficit, provides evidence for the importance of determining the role that GABA release from POMC neurons normally

plays in energy balance regulation and suggests that understanding the overall actions of POMC neurons needs to consider not only peptide release, but also amino acid transmitter release from these cells.

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Additional information

Competing interests

The authors have no competing financial interests.

Author contributions

B.C.J. and S.T.H. conceived and designed the experiments. All authors collected and analysed data. S.T.H. and B.C.J. wrote the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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