Expression of GABAergic and Glutamatergic Phenotypic Markers in Hypothalamic Proopiomelanocortin Neurons

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Abstract

Hypothalamic proopiomelanocortin (POMC) neurons have traditionally been defined by their peptide transmitters, which are important regulators of energy balance and reward. Recent work shows that POMC neurons can also release the amino acid transmitters γ-aminobutyric acid (GABA) and glutamate, although studying GABAergic and glutamatergic populations of POMC neurons has been hindered by the difficulty in reliably identifying amino acid (AA) transmitter phenotypes. In the present study, fluorescent in situ hybridization and immunohistochemistry were used to identify POMC neurons and to detect the presence of mRNA for the transporters responsible for packaging either GABA (vesicular GABA transporter [vGAT]) or glutamate (vesicular glutamate transporter [vGLUT]) into vesicles, as well as the enzymes responsible for GABA synthesis, glutamic acid decarboxylase (GAD)65 and GAD67. Approximately 7% of POMC neurons expressed *vGlut2* and the highest percentage of *vGlut2*-positive POMC cells were located in the rostral arcuate nucleus. Despite the reports of GABA release from POMC neurons, *vGat* was not detected in POMC neurons, although *Gad65* and *Gad67* were present in ~40% of POMC neurons. Approximately half of the *vGlut2*-expressing POMC cells also expressed *Gad65*. Markers of neurotransmitter phenotype were better detected by using in situ hybridization techniques rather than transgenic expression of fluorophores under the control of the *vGat* or *Gad67* promoters. It is now clear that the expression of markers of AA phenotype provides a useful means to identify distinct subpopulations of POMC neurons. Additionally, the method described will be useful to explore the possibility that plasticity of AA phenotype is an important aspect of POMC neuron function.

INDEXING TERMS

GAD67; GAD65; vGLUT2; in situ hybridization

Proopiomelanocortin (POMC) neurons in the arcuate nucleus of the hypothalamus regulate aspects of feeding behavior and reward, making them an important target for eating disorders and addiction therapies (Coll, 2007; Roth-Deri et al., 2008). These neurons have traditionally been defined by their expression of the *Pomc* gene and the presence of the

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peptides that it encodes, including α-melanocyte–stimulating hormone (α-MSH) and the endogenous opioid β-endorphin. There is growing evidence, however, that subpopulations of POMC neurons are also capable of releasing the amino acids (AA) glutamate and γ-aminobutyric acid (GABA) (Collin et al., 2003; Hentges et al., 2004, 2009), although a comprehensive study to characterize these subpopulations has not previously been undertaken.

The possibility that POMC neurons may release glutamate as a cotransmitter was originally suggested when the vesicular glutamate transporter vGLUT2 was detected in POMC neurons (Collin et al., 2003), which appeared to lack the inhibitory transmitter GABA (Ovesjo et al., 2001). However, another study indicated that POMC neurons release GABA, express mRNA for the GABA synthetic enzyme glutamic acid decarboxylase (GAD)67, and display immunoreactivity for GABA and the vesicular GABA transporter, vGAT (Hentges et al., 2004) indicating a GABAergic phenotype. Subsequently, it was shown that one portion of murine POMC neurons can release GABA and another portion can release glutamate when the cells are maintained in primary culture (Hentges et al., 2009).

To determine the roles of GABAergic and glutamatergic POMC neurons, it is necessary to be able to reliably identify these subpopulations. Traditionally, protein markers indicative of AA phenotype are used to identify GABAergic and glutamatergic cells because GABA and glutamate are not as amenable to detection as enzymes and transporters. To date, there are conflicting reports of AA marker expression in POMC neurons. The discrepancies may arise from the use of a variety of detection methods and reliance on transgenically expressed reporters. The present study was undertaken to identify markers that can be used to reliably detect GABAergic and glutamatergic subpopulations of POMC neurons, which will allow for detailed studies of these POMC neuron subpopulations. Furthermore, the development of in vivo approaches to address the function of AA transmitters in POMC neurons requires a thorough understanding of the markers expressed in these cells. By using fluorescent in situ hybridization for AA phenotype markers, the present results show that ~40% of POMC neurons express *Gad65* and −*67* mRNA, ~7% express *vGlut2* mRNA, and a small population of POMC neurons expresses mRNA for both *Gad65* and *vGlut2*. The highest percentage of *vGlut2*-expressing POMC cells occurs in the rostral arcuate nucleus. The data also indicate that *vGat* is not a reliable marker of the GABAergic nature of POMC neurons.

MATERIALS AND METHODS

Animals

A total of 26 8–12-week-old male mice were used for all experiments. GAD67-green fluorescent protein (GFP) knock-in mice (Tamamaki et al., 2003; originally supplied by Dr. Yuchio Yanagawa, Gunma University, Kiryu, Japan), mice expressing enhanced green fluorescent protein (EGFP) in vGAT neurons (Gong et al., 2003; supplied by the Mutant Mouse Regional Resource Center; Tg(Slc32a1-EGFP) EN128Gsat/Mmcd), and mice expressing either EGFP (Cowley et al., 2001; Jackson Laboratory [Bar Harbor, ME] stock #009593) or discosoma red (DsRed; Hentges et al., 2009; supplied by Dr. Malcolm J. Low, University of Michigan, Ann Arbor, MI) in POMC neurons were maintained on the C57BL/6J genetic background (Jackson Laboratory). POMC-DsRed mice were crossed with

GAD67-GFP mice to create transgenic mice with double-labeled POMC and GAD67-expressing cells as previously described (Hentges et al., 2009). All mice were given standard chow and tap water ad libitum and housed under controlled temperatures (22–24°C) at a constant 12-hour light/dark cycle. All 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.

Tissue preparation

For tissue collection, mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with a 10% sucrose solution, followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Brains were postfixed overnight at 4°C in 4% paraformaldehyde solution in PBS. Then 50-μm sections containing the arcuate nucleus were cut on a Vibratome, collected in cold PBS, and either mounted on slides or processed for in situ hybridization.

Probes for fluorescent in situ hybridization

Antisense and sense RNA probes were synthesized by using T3, SP6, or T7 RNA polymerase in the presence of dNTPs containing digoxigenin (DIG) or fluorescein isothiocyanate (FITC)-labeled UTPs (Roche Applied Sciences, Indianapolis, IN). Template DNA was digested for 30 minutes with DNAse I, and probes were ethanol-precipitated by using lithium chloride and glycogen. Then 70% ethanol was used to wash the RNA before it was resuspended in diethylpyrocarbonate (DEPC)-treated water, and probes were stored at −80°C. All of the probe sequences were made from mouse cDNA and have 100% homology with the appropriate mRNA unless otherwise noted. Specific probe information can be found in Table 1.

The plasmid containing the *Pomc* probe was a gift from Dr. Malcolm J. Low (University of Michigan, Ann Arbor, MI). Plasmids containing *vGlut2* and *vGlut1* were kindly provided by Klas Kullander (Uppsala University, Uppsala, Sweden). Two *Gad65* probes corresponding to different sections of the *Gad65* gene were run simultaneously to maximize the sensitivity of detection of the mRNA. Plasmids containing these probes were provided by Sandra L. Petersen (University of Massachusetts, Amherst, MA). These sequences were originally made from rats, but have a 96 and 97% homology with mouse cDNA. The *vGat* probe containing plasmid was kindly provided by Tomomi Shimogori (Riken Institute, Saitama, Japan). To detect *Gad67*, a probe corresponding to a portion of human *Gad67* cDNA was inserted into pBluescriptII SK(+/−). The plasmid containing the full *Gad67* cDNA was provided by Macdonald J. Christie (Brain and Mind Research Institute, University of Sidney, Australia). This sequence has 92% homology with mouse cDNA. Sense probes were used to establish specificity of antisense probes.

Antibody characterization

The patterns revealed by antibody detection of DIG and FITC for in situ hybridization were unique to each gene target, and matched previous reports. The chicken anti-GFP antibody recognizes a single band on western blot of around 27–30 kDa, whereas no band was apparent in a wild-type control (technical information provided by the manufacturer). The

antibody also selectively labeled GFP-positive cells in lamina II of the spinal cord of transgenic mice (technical information provided by the manufacturer). Control tissue from a wild-type mouse lacking GFP revealed an absence of staining with the antibody in the present study (data not shown). Specific antibody information is provided in Table 2.

Fluorescent in situ hybridization

Brains were typically divided into three series, and each series was processed for different mRNA. The protocol for in situ hybridization was adapted from previous investigations (Leupen et al., 2003; de Souza et al., 2005; Watakabe et al., 2010). Free-floating sections were incubated for 15 minutes in 6% H_2O_2 to quench endogenous peroxidases, washed two times for 5 minutes in PBS containing 0.1% Tween 20 (PBT), digested for 15 minutes with proteinase K (10 μg/ml), and then incubated for 10 minutes in 2 mg/ml glycine. All solutions were made up in PBT. After two additional washes for 10 minutes in PBT, sections were postfixed for 20 minutes in a 4% paraformaldehyde/0.2% gluteraldehyde solution, washed, and then dehydrated in ascending ethanol dilutions made with DEPC-treated water (50, 70, 95, and 100%). Tissue was prehybridized for 1 hour at 60°C in hybridization buffer (66% [v/v] deionized formamide, 13% [w/v] dextran sulfate, 260 mM NaCl, 1.3X Denhardt's solution, 13 mM Tris-HCl, pH 8.0, 1.3 mM EDTA, pH 8.0). Probes were denatured at 85°C for 5 minutes before being added to the hybridization buffer, along with tRNA and dithiothreitol (DTT) for a final concentration of 0.5 mg/ml tRNA and 10 mM DTT. Tissue was hybridized for 18–20 hours. The appropriate concentration and hybridization temperature for each probe was empirically determined based on characteristic staining with each probe and a lack of staining in areas of the brain previously reported to be devoid of that mRNA.

After six 30-minute stringency washes at 60°C, three in 50% formamide/5X SSC, and the subsequent three in 50% formamide/2X SSC, sections underwent a 30-minute digestion with RNAse A (20 μg/ml in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 37°C before being washed three times for 15 minutes in TNT (0.1 M Tris-HCl, pH 7.5, 0.15, M NaCl, 0.05% Tween-20) and blocked for 1 hour in TNB (TNT plus 0.5% Blocking Reagent provided in the TSA kit; Perkin Elmer, Oak Brook, IL). Tissue was then incubated overnight at 4°C in either a horseradish peroxidase-conjugated sheep anti-FITC antibody (1:1,000; Roche Applied Sciences) or sheep anti-DIG conjugated to alkaline phosphatase (AP; 1:1,000; Roche Applied Sciences). FITC was visualized by using a TSA PLUS Biotin Kit (Perkin Elmer). After three 15-minute TNT washes, the sections were incubated for 30 minutes in a 1:50 dilution of the Biotin Amplification Reagent working solution (prepared following the manufacturer's instructions), and then washed again before incubation for 30 minutes in 1:600 streptavidin conjugated to Alexa-Fluor 488 (Invitrogen, Carlsbad, CA). The DIG-labeled probes were detected by using an HNPP Fluorescent Detection Kit (Roche).

Briefly, sections were equilibrated for 10 minutes in detection buffer (100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, pH 8.0) before incubation for 1 hour in HNPP/Fast Red TR mix prepared according to the manufacturer's instructions. Each well of tissue was ordered, mounted in PBS with 100 mM EDTA, and coverslipped by using cc/mount (Sigma, St.

Louis, MO). Mounting the tissue quickly was critical, because the HNPP signal diffuses with time. Slides were stored frozen in order to prolong the HNPP signal (Watakabe et al., 2010).

Dual fluorescent in situ hybridization and immunodetection of GFP

Dual fluorescent in situ hybridization was carried out as described above with the following modifications. When using probes that hybridize at the same temperature (*Pomc* and *vGlut2*, *vGlut1*, or *vGat*), the probes were added simultaneously and hybridized for 18–20 hours at 70°C. However, the *Gad65* and *Gad67* probes hybridize at a different temperature (52 and 60°C, respectively), so a sequential hybridization was performed. The probe that hybridized at the higher temperature was hybridized first for 20 hours before the tissue was transferred to new hybridization buffer containing the second probe, whereupon the tissue was hybridized for an additional 20 hours at the lower temperature (see Table 3 for specific combinations of probes). Antigenicity of POMC peptides and DsRed did not survive the hybridization conditions; thus GFP transgenic animals were used for triple detection (Table 3). Although GFP fluorescence was quenched by the in situ hybridization procedure, the antigenicity was maintained. Therefore, after stringency washes, tissue was incubated at 4°C in a cocktail of primary antibodies made up in TNB. This included the anti-FITC POD and anti-DIG AP antibodies, as well as a chicken-anti-GFP antibody at 4°C (1:2,000; Abcam, Boston, MA) to detect GFP when using the transgenic animals. GFP was visualized first by incubating tissue for 2 hours at room temperature in donkey anti-chicken antibody conjugated to DyLight 649 (1:400; Jackson ImmunoResearch, West Grove, PA). FITC was detected next by using the TSA Biotin Kit, and DIG-labeled probes were always visualized last by using the HNPP Fluorescent Detection Kit as described above.

Imaging

Images were taken with a Zeiss 510-Meta laser-scanning confocal microscope. Green fluorescence (Alexa-Fluor 488) was imaged by using a 488/543-nm bandpass filter, and emission was detected by using a 505/530-nm bandpass filter. HNPP was imaged by using a 488/543-nm bandpass excitation filter and a 585/615-nm bandpass emission filter, and antibody-conjugated DyLight 649 was imaged by using a 514/633-nm bandpass excitation filter and a 650-nm longpass emission filter. Images at each wavelength were taken sequentially to avoid crossover between channels. Z-stack images were taken every 3 μm, for a total of 15 μms, from all of the tissue containing POMC neurons within a series of tissue (typically five to six tissue sections representing the rostral-caudal extent of the arcuate nucleus). Images were collected beginning with the most rostral slice containing POMC neurons (~Bregma −1.2 mm) through the most caudal slice containing POMC neurons (~Bregma −2.3). Images to be used for figures were pseudo-colored by using the LSM Image Browser software (Carl Zeiss, Thornwood, NY), and then adjusted for brightness and contrast by using Photoshop (Adobe Systems, San Jose, CA).

Cell counts and analysis

A modification of the 3D counting method (Williams and Rakic, 1988) was employed to estimate cell numbers. A "counting box" was defined as a 300 × 300-μm box on the x–y

plane, with a z-axis depth of 15 μm. Only cells completely within the counting box or with a clear nucleus were counted, as this provided enough of the cell body to look for colabeling. LSM Image Browser software was used to move through the z-stacks and to identify and mark cells. Cells that contained multiple labels (e.g., green and red fluorescence) in more than one section in the z-stack were defined as colabeled cells, or if a clear nucleus was apparent in both colors. Marked images were then exported into Adobe Photoshop CS4, in which the markings were counted manually by using the "count tool." Although the counting method employed may result in some oversampling, applying corrections to accommodate for this does not alter the percentage of the cells that coexpress the various markers. Given that the number and density of POMC neurons change significantly throughout the rostral–caudal extent of the arcuate nucleus, reporting coexpression as a percentage of POMC cells is more representative than providing absolute numbers. The number of POMC cells counted is provided in Table 4, however, to illustrate the sample size.

RESULTS

Specificity of probes

Seven different probes were used to detect markers of GABAergic and glutamatergic phenotypes in POMC neurons (Table 1). The most effective order of probe binding and the optimal visualization methods were empirically determined and are outlined in Table 2. Probe concentrations and detection protocols were optimized to ensure specificity and are roughly equal intensity of signal for each of the detected mRNAs. Probe specificity was indicated by the lack of signal in regions of the brain previously shown to lack the mRNA of interest, and by the lack of sense probe binding (Fig. 1).

Glutamatergic POMC neurons

Signal for *vGlut2* was present throughout the brain as previously described (Fremeau et al., 2001), including strong expression in the ventromedial hypothalamus (VMH; Fig. 1G) and various nuclei throughout the thalamus (data not shown). *vGlut2*-positive cells detected within the arcuate nucleus appeared fainter than those in the VMH (Fig. 2A, lower and upper portion of images, respectively), suggesting that these cells may express lower levels of the mRNA. *vGlut2*-positive cells not expressing POMC-EGFP were scattered throughout the arcuate nucleus. Overall, the density of *vGlut2*-expressing cells was much lower in the arcuate nucleus compared with the VMH (Figs. 1G, 2). The retrochiasmatic arcuate and most rostral regions of the arcuate nucleus contained a higher percentage of POMC cells expressing *vGlut2* than more caudal areas (Fig. 2B,C; $30.7 \pm 12.2\%$, $P < 0.01$ by one-way ANOVA, $n = 7$) such that the majority of v*Glut2*-positive POMC neurons occurred within ~ 100 μm of the most rostral POMC neurons (~Bregma −1.2–1.3 mm). Within the rostral arcuate nucleus, the *vGlut2*-expressing POMC neurons appeared to be evenly distributed in the dorsal-ventral and medial-lateral planes. Of the total POMC-EGFP neurons present in the arcuate, $7.4 \pm 1.2\%$ ($n = 7$) were found to express *vGlut2* (Table 4).

No *vGlut1* expression was detected in POMC neurons or in any other cells in the arcuate nucleus, although labeling was detected in the cortex (Fig. 1K), the lateral and basolateral

amygdaloid nuclei, and to a lesser extent in various thalamic nuclei, as has been previously reported (Fremeau et al., 2001; Poulin et al., 2008).

GABAergic POMC neurons

Strong *Gad65* and *Gad67* expression was apparent throughout the brain, including the arcuate nucleus, reticular thalamic nucleus, and several amygdaloid complexes, as previously described (Esclapez et al., 1993; Ovesjo et al., 2001; Poulin et al., 2008). *Gad* expression was not detected in areas that have previously been shown to lack GABAergic neurons, including the VMH (Fig. 1E,I; Esclapez et al., 1993). Of POMC-EGFP neurons located in the arcuate nucleus, $44.7 \pm 2.8\%$ expressed *Gad65* mRNA (Fig. 3, $n = 7$), and 38 $\pm 1\%$ of POMC neurons expressed *Gad67* (Fig. 3B, $n = 3$). *Gad*-positive cells without POMC expression were observed, consistent with the many other GABAergic cell types present within the arcuate nucleus. The percentage of *Gad65* or *–67*-positive POMC neurons was fairly uniform throughout the rostral two-thirds of the arcuate nucleus, whereas fewer POMC cells in the caudal portion of the arcuate nucleus expressed *Gad65* or *–67* (Fig. 3C,D, $P < 0.01$ by one-way ANOVA). The *Gad65* probe was used to label putative GABAergic neurons in the majority of later double- and triple-labeling experiments because this probe gave more robust and distinct labeling than the *Gad67* probe.

The expression of *vGat* in POMC neurons was examined because this vesicular transporter is also considered an indicator of the GABAergic phenotype (McIntire et al., 1997). In situ hybridization revealed *vGat* mRNA in a very low percentage of *Pomc*-containing neurons even at saturating probe concentrations (3.7 and 3.9% in the two brains counted; Fig. 4A). The lack of detectable *vGat* mRNA in POMC neurons is not likely to reflect low efficiency of the probe labeling because the signal is readily detected in many brain regions (Fig. 1A) and in non-POMC cells within the arcuate nucleus (Fig. 4A). In situ hybridization for *Pomc* was also carried out in tissue from mice expressing EGFP under the control of the *vGat* promoter. Immunodetection of EGFP revealed that less than 1% of *Pomc* neurons in the transgenic mice ($0.4 \pm 0.3\%$, $n = 4$) expressed vGAT–EGFP (Fig. 4B). The number of cells in the arcuate nucleus labeled with EGFP in the vGAT–EGFP transgenic mouse appeared to be less than the number of cells with detectable – mRNA (Fig. 4A,B, center panels), suggesting that the vGAT–EGFP transgene does not label all cells that produce *vGat* mRNA. All of the cells expressing the vGAT–EGFP label also expressed mRNA for *Gad67*, although there were many cells with *Gad67* expression that were not vGAT–EGFP positive (Fig. 4C), suggesting that vGAT is not as strongly expressed in the arcuate nucleus as *Gad67*. The near lack of vGAT–EGFP label in POMC neurons is consistent with the lack of detectable mRNA for *vGat* in POMC neurons, further suggesting that POMC cells either lack vGAT or express the mRNA for vGAT at very low levels.

Dual-phenotype POMC neurons

To determine the relative number and distribution of POMC neurons with GABAergic and glutamatergic markers, dual fluorescent in situ hybridization was performed to simultaneously detect *vGlut2* and *Gad65* in tissue from POMC–EGFP transgenic mice. Immunodetection of EGFP was used to identify the POMC cells. A clear distinction between glutamatergic and GABAergic cell populations was observed in many areas

throughout the brain. Within the arcuate nucleus, most cells clearly expressed either *vGlut2* or *Gad65*, although coexpression was occasionally observed (Fig. 5). Of the POMC–EGFP neurons, $3.6 \pm 0.6\%$ ($n = 7$) expressed both *vGlut2* and *Gad65* mRNA, suggesting that some POMC neurons may be capable of releasing both inhibitory and excitatory AA transmitters.

GAD67–GFP transgenic mouse

Earlier studies indicated that POMC neurons might be GABAergic because a cross between mice expressing red fluorescent protein under the control of the POMC promoter (POMC–DsRed transgenic mouse) and GAD67–GFP transgenic mice revealed that approximately 40% of POMC neurons contained GFP (Hentges et al., 2009). However, later experiments using POMC–DsRed and GAD67–GFP double-transgenic animals showed a steady decline in the occurrence of colocalization between the DsRed and GFP (data not shown). To determine whether the apparent decline in GAD67–GFP expression may be due to loss of transgene expression, fluorescent in situ hybridization for *Pomc* was performed in tissue from GAD67–GFP transgenic mice. By using this method, only $10.5 \pm 1.3\%$ ($n = 3$) of *Pomc* neurons colabeled with GAD67–GFP, considerably less than the $38 \pm 1\%$ of POMC neurons that expressed *Gad67* mRNA (Table 4). Thus, the GAD67–GFP transgene is not expressed at detectable levels in all *Gad67*-positive cells.

DISCUSSION

To fully understand all the roles that POMC neurons play in the regulation of energy balance and reward, it is essential to identify the complete transmitter phenotype of these important neurons. Previous studies have suggested that POMC neurons are glutamatergic (Collin et al., 2003; Vong et al., 2011), GABAergic (Hentges et al., 2004), or both (Hentges et al., 2009). However, the difficulty of detecting GABA and glutamate or GAD, vGLUT, and vGAT proteins by using antibody-based detection methods in the hypothalamus has limited comprehensive analysis of POMC neuron phenotype. The present study used fluorescent in situ hybridization to demonstrate that POMC neurons express *Gad65*, *−67*, and/or *vGlut2*, suggesting a striking heterogeneity in AA transmitter phenotypes for POMC neurons. Interestingly, whereas *Gad65* and *−67* were readily detectable in POMC neurons, *vGat* was not, although POMC neurons release GABA from intact terminals (Dicken et al., 2012). The differing expression of phenotypic markers may need to be taken into account when determining transmitter release and designing experiments to alter GABA or glutamate release from POMC neurons. Overall, the in situ hybridization methods used here appear to provide a more sensitive and reliable means of detecting AA phenotype than transgenically expressed protein markers.

Glutamatergic POMC neurons

A small but distinct portion of POMC neurons may release the excitatory AA glutamate, as is indicated by the ~7% of POMC-EGFP neurons found in this study to express *vGlut2*.

vGLUT2 is one of three vesicular transporter proteins (vGLUT1–3) known to package glutamate into vesicles (Fremeau et al., 2001; Herzog et al., 2001; Schafer et al., 2002) and the only vGLUT that appears to be expressed in POMC neurons (Collin et al., 2003; present

data). The presence of a vesicular transporter for glutamate in POMC neurons is consistent with the previous report of glutamate release from POMC neurons in primary cultures (Hentges et al., 2009).

Interestingly, a higher percentage of *vGlut2*-expressing POMC–EGFP cells were present in the retrochiasmatic and rostral arcuate nucleus compared with more central and caudal regions of the nucleus, suggesting a potential anatomical divergence in the function of POMC cells. Specific functions for POMC neurons located in distinct regions of the arcuate nucleus has previously been suggested based on the finding that administration of estradiol and testosterone can increase *Pomc* mRNA preferentially in neurons located in the rostral arcuate nucleus (Chowen-Breed et al., 1989; Cheung and Hammer, 1995). Although glutamate may be released from only a small portion of POMC neurons, recent tract-tracing studies suggest that small populations of POMC neurons project selectively to specific target sites (King and Hentges, 2011). Therefore, the rostrally located glutamatergic POMC neurons may mediate distinct physiologic actions.

GAD expression in POMC neurons

The GAD65 and −67 enzymes synthesize GABA, and their expression is generally indicative of GABA-releasing neurons (Erlander et al., 1991). The expression of both *Gad65* and *Gad67* mRNA in POMC neurons is consistent with the previous reports of GABA release from POMC neurons in primary cultures and hypothalamic slice preparations (Hentges et al., 2004; Atasoy et al., 2008; Hentges et al., 2009). In the present study *Gad* mRNA was detected in ~40% of POMC neurons, which is consistent with a previous report in which isotopic in situ hybridization was used to detect *Gad* (Hentges et al., 2004). Additionally, ~40% of POMC neurons were reported to be GABAergic on the basis of expression of GAD67 promoter-driven GFP expression in POMC neurons (Hentges et al., 2009), although another group found GAD67-GFP in only ~5% of POMC neurons (Yee et al., 2009). The low expression of GAD67-GFP reported by Yee et al. (2009) is consistent with our unpublished observation that GAD67-GFP expression has significantly declined over time, leading to the conclusion that this reporter no longer reliably labels the majority of POMC neurons that express *Gad67.* In situ hybridization appears to be a more reliable means of detection for *Gad65* and −*67* expression in POMC neurons.

Lack of vGAT in POMC neurons

In addition to the synthetic enzymes for GABA, vGAT has been considered a reliable marker for GABAergic neurons and is the only known protein capable of packaging GABA into vesicles for release (McIntire et al., 1997). The data shown here, however, suggest that neither the vGAT-EGFP transgene nor detectable levels of mRNA for *vGat* are present in a significant population of POMC neurons. Although both in situ hybridization and the vGAT-EGFP transgenic animal revealed very little coexpression of – in – neurons, there appeared to be a greater density of cells labeled for the mRNA than for the transgenic reporter (Fig. 4) suggesting that the vGAT-EGFP transgene does not label the entire population of *vGat*-expressing cells that can be detected by using in situ hybridization.

The apparent lack of vGAT in the majority of POMC neurons is consistent with a recent report from Vong et al. (2011) in which the authors used a transgenic reporter to detect vGAT expression. Interestingly, granule cells of the dentate gyrus of the hippocampus exhibit multiple markers of GABAergic cells, but either they do not express *vGat* (Chaudhry et al., 1998; Sperk et al., 2003), or else *vGat* occurs at extremely low levels, but can be upregulated under certain conditions (Lamas et al., 2001; Gómez-Lira et al., 2005). It seems possible that POMC neurons could express *vGat* at a low enough level that the methods used here were not sensitive enough to detect it, and perhaps the upregulation of *vGat* occurs during nonhomeostatic conditions that would allow for increased GABA release. Alternatively, POMC neurons may employ an alternative method of packaging GABA. In one study, an examination of the lateral oliva superior using electron microscopy revealed that ~25% of the GABA-containing terminals did not contain *vGat* (Chaudhry et al., 1998). Additionally, the data from another study showed that a portion of Gad67-immunoreactive cells did not express a vGat promoter-driven fluorescent reporter (Zhao et al., 2011), which agrees with the much denser labeling of *Gad67* than of vGat-EGFP shown in this study.

Although an apparent lack of *vGat* expression could be a function of the detection methods used or indicative of mRNA not localized to the cell body (Giuditta et al, 2002), it is also possible that some cells are capable of releasing GABA independent of *vGat.* Reversal of the plasma membrane GABA transporter, for example, has been shown to underlie evoked GABA release in some conditions (Wu et al., 2007). However, in POMC neurons, pharmacologic inhibition of the GABA reuptake transporters does not affect GABA release from POMC terminals (our unpublished observation). Furthermore, GABA release from POMC terminals appears to be vesicular, based on the onset, amplitude, and duration of currents resulting from evoked GABA release (Hentges et al., 2004, 2009; Atasoy et al., 2008; Dicken et al., 2012). Further studies are needed to determine whether *vGat* levels are too low to detect under basal conditions or whether an alternative mechanism is used to package GABA in POMC neurons.

POMC neurons with Gad and vGlut2

In the present study, ~3.5% of POMC neurons were found to express both *vGlut2* and *Gad65* mRNA, suggesting the possibility of co-release of glutamate and GABA from these neurons. Although GABA and glutamate co-release has not been commonly reported, there is growing evidence that neurons in the adult brain can coexpress the machinery necessary for GABA and glutamate release. For example, the presence of *vGat* and *vGlut* or *Gad* and *vGlut* has been found in the hippocampus (Somogyi et al., 2004; Zander et al., 2010), cerebellum (Zander et al., 2010), and ex (Hioki et al., 2004; Somogyi et al., 2004; Fattorini et al., 2009), in terminals originating from the supramammillary nucleus (Boulland et al., 2009), and in the cat retina (Kao et al., 2004). Transmitter co-release during development has been attributed to the maturation and refinement of synapses (Anderson et al., 2004; Noh et al., 2010), whereas in adulthood it seems more likely that this is an indication of plasticity. For example, neurons in the preoptic area display hormone-dependent changes in the coexpression of vGLUT2 and vGAT (Ottem et al., 2004). It may be that AA transmitter phenotype is dynamically regulated in POMC neurons as well. Regardless of whether the AA phenotype of an individual POMC neuron is constant or plastic, the existence of

excitatory and inhibitory subpopulations of POMC neurons is another indicator of heterogeneity of POMC neurons. It is now becoming clear that POMC neurons are heterogeneous in a variety of ways, and such heterogeneity is probably important to the wide range of functions attributed to these neurons (Sohn and Williams, 2012).

Advantages and technical considerations for using in situ hybridization to explore phenotype

Although the use of transgenically expressed markers to label cell types and manipulate cell function has many strengths, transgene expression may not always faithfully reflect native gene expression. The current results indicate that some transgenic markers are detectable only in a fraction of cells that can be identified by using in situ hybridization techniques. Conversely, transgenes can be ectopically expressed and genes may be expressed developmentally in a pattern not observed in the adult. A striking example is the recent demonstration that there is transient, widespread expression of the *Pomc* gene promoter during development, which can lead to a considerably high occurrence of off-target recombination of floxed alleles when a *Pomc*-cre mouse is used (Padilla et al., 2011). Thus, for some purposes, detection via in situ hybridization has clear advantages over transgenic markers.

In situ hybridization, however, can be technically challenging, and several approaches were tested to optimize the current methods. For example, after the dual fluorescent in situ hybridization procedure used here, many antibodies tested did not recognize their target proteins. POMC peptides and Ds-red lost their antigenicity and were not detectable with antibodies after the hybridization process (data not shown). Therefore, most of the studies were carried out in tissue from mice transgenically expressing EGFP because it was possible to perform immunodetection of EGFP even after the dual FISH procedure. Experiments were originally designed by using a biotin-labeled *Pomc* probe and antibody labeling to detect the biotin. This approach proved to be much less sensitive than TSA- or HNPP-based detection methods or immunohistochemistry for POMC-EGFP. Only ~50% of the POMC neurons were detected when immunodetection of the biotin-labeled probe was used, with ~80% of detected *Pomc* neurons expressing *Gad65* mRNA. This highlights the importance of careful controls for in situ hybridization to ensure that maximum sensitivity has been achieved. To ensure that such maximal sensitivity was being achieved in the present study, probe concentrations were increased until background/nonspecific labeling was noted, and then the concentration of probe that gave the maximal signal without background labeling was used in the studies, as reported in Table 1. It should be noted, however, that what was considered excess signal (label in regions not previously reported to express the mRNA) with high probe concentrations could reflect not only nonspecific binding, but also detection of lower levels of message present in some cells (Hrabovszky and Petersen, 2002).

Thus, it could be that the stringent criteria used here to avoid false positives yielded an underestimate of the absolute number of cells expressing markers for GABA and/or glutamate phenotypes. Despite the potential limitations of in situ hybridization, the technique as employed here allowed for the simultaneous detection of markers for GABA and glutamate phenotypes in POMC neurons. Now it is clear that under the same conditions,

in a single section of tissue, some POMC neurons are GABAergic, some are glutamatergic, and some may be capable of releasing both GABA and glutamate based on coexpression of GAD and vGLUT.

CONCLUSIONS

The present results show that POMC neurons can express *vGlut2*, indicating a glutamatergic phenotype, and *Gad65* and *Gad67*, indicating a GABAergic phenotype. Together with previous studies demonstrating release of GABA and glutamate from POMC neurons, there is compelling evidence that this population of peptide-releasing neurons is heterogeneous in its AA phenotype. *Gad* and *vGlut2*, but not *vGat*, will provide reliable markers to further identify and study the distinct subpopulations of POMC neurons and the consequence of AA transmitter release from these neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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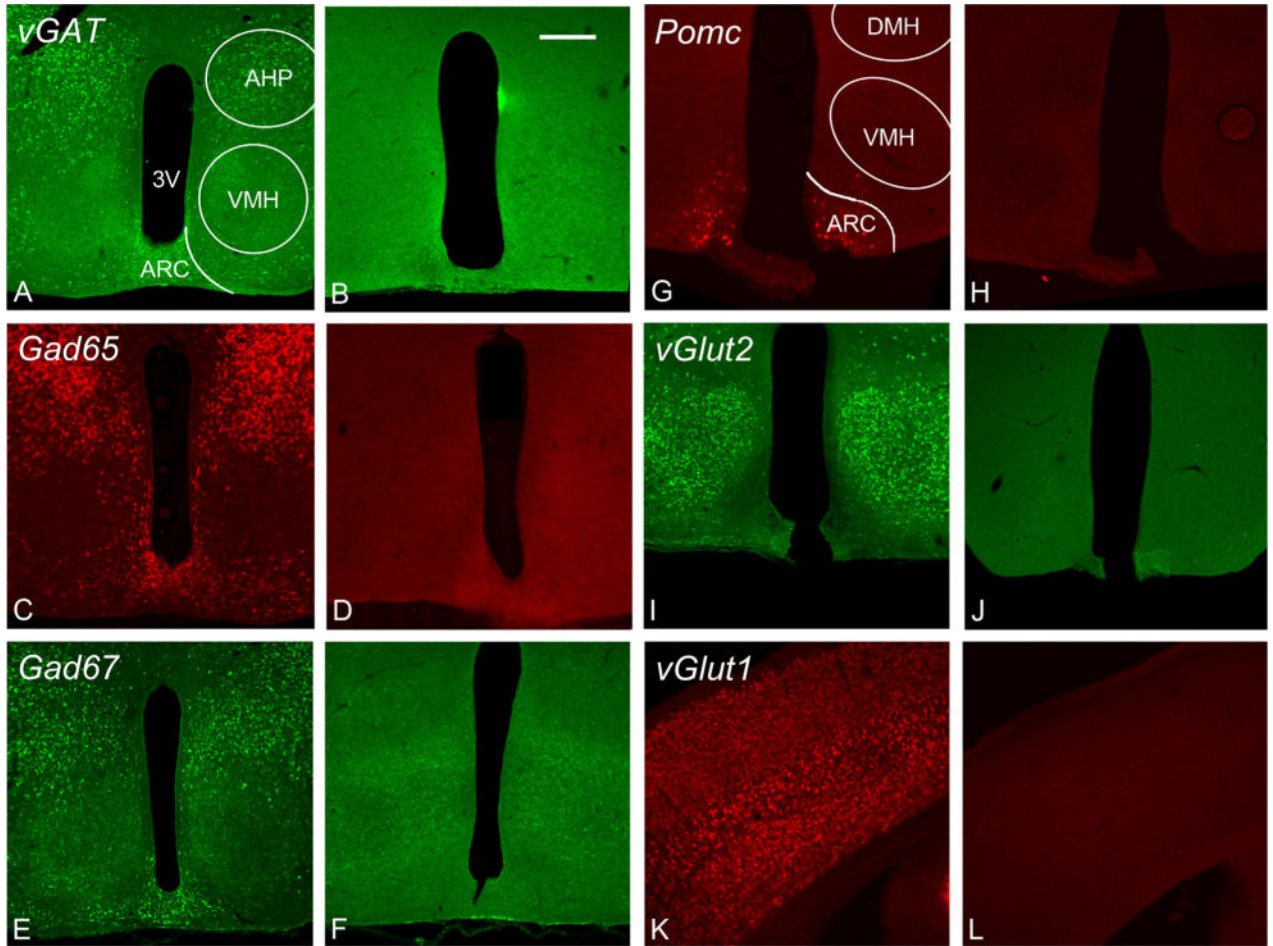
Figure 1.
Specificity of probe binding. **A–L**: Tissue was treated with antisense and sense probes to control for nonspecific labeling of the probe. *vGat* (A), *Gad65* (C), and *Gad67* (E) labeling is shown in the rostral arcuate where the ventromedial hypothalamus (VMH) is apparent. The VMH contains primarily glutamatergic cells, so the lack of GABAergic markers in this nucleus is characteristic. Labeling can be seen in the arcuate nucleus and the anterior hypothalamic area (AHP). *Pomc* expression was present exclusively in the arcuate nucleus with robust labeling in the center of the nucleus (G). The *vGlut2* probe displayed strong labeling in the VMH (I), with scattered cells throughout the arcuate nucleus. *vGlut1* was not present in the arcuate nucleus. An image from cortex with strong *vGlut1* labeling is shown (K). There was no labeling above background with any of the sense probes (B,D,F,H,J,L). AHP, anterior hypothalamic area (posterior); ARC, arcuate nucleus; 3V, third ventricle; DMH, dorsal medial hypothalamus; VMH, ventral medial hypothalamus. (A magenta–green version of this figure is included as a supplementary file for the assistance of color-blind authors.) Scale bar = 200 μm in B (applies to A–L).
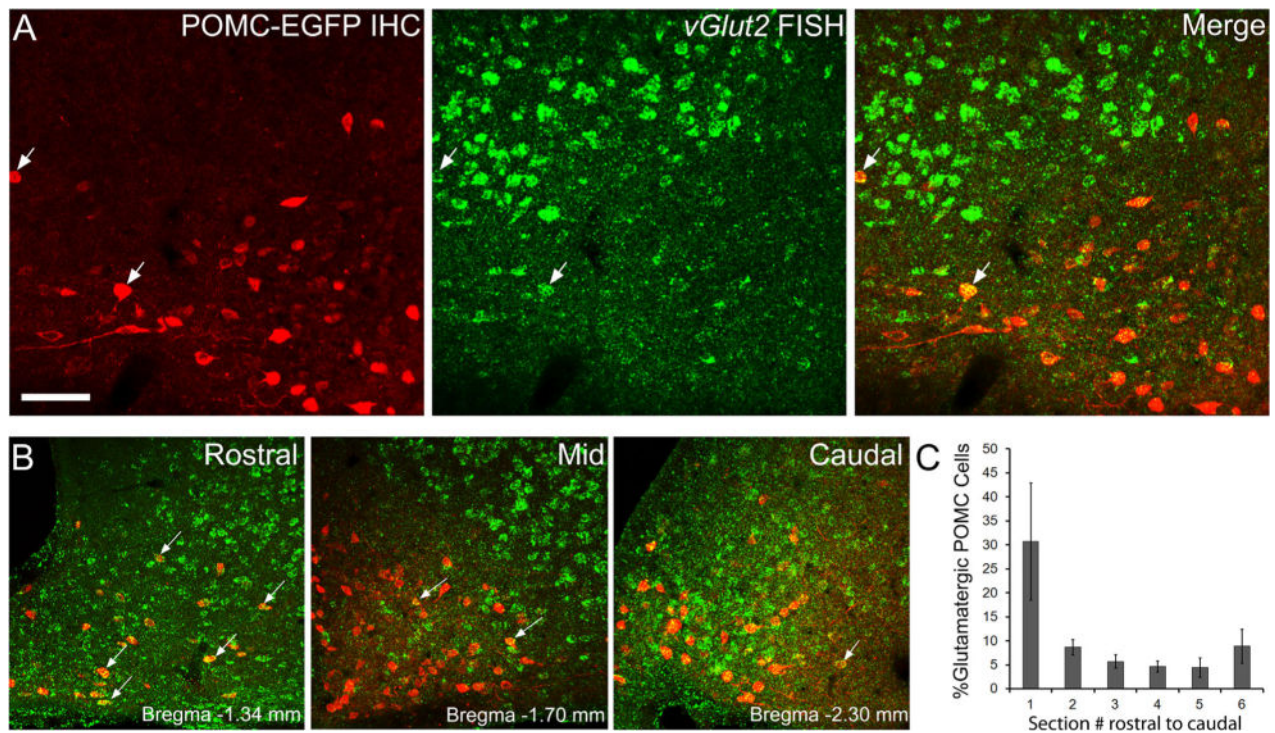
Figure 2.

vGlut2 mRNA in POMC neurons. **A,B:** Example images of immunohistochemistry for POMC-EGFP (red) and fluorescent in situ hybridization for *vGlut2* using an FITC-labeled probe and TSA-Biotin detection (green). Only a small portion of POMC-EGFP neurons expressed *vGlut2* (A, white arrows). *vGlut2*-positive neurons lacking POMC-EGFP expression (A, right panel, green cells) and POMC-EGFP neurons without *vGlut2* (A, right panel, red cells) are also present. Representative images of rostral (B, left panel), mid (B, center panel), and caudal (B, right panel) areas of the arcuate nucleus in coronal sections show *vGlut2* present in POMC-EGFP neurons (white arrows), particularly in the rostral arcuate. 3V, third ventricle. *C*: Graph depicting the percentage of POMC-EGFP cells expressing *vGlut2* throughout the rostral to caudal extent of the arcuate nucleus (*n* = 7). (A magenta–green version of this figure is included as a supplementary file for the assistance of color-blind authors.) Scale bar = 50 μm in A (applies to A,B).
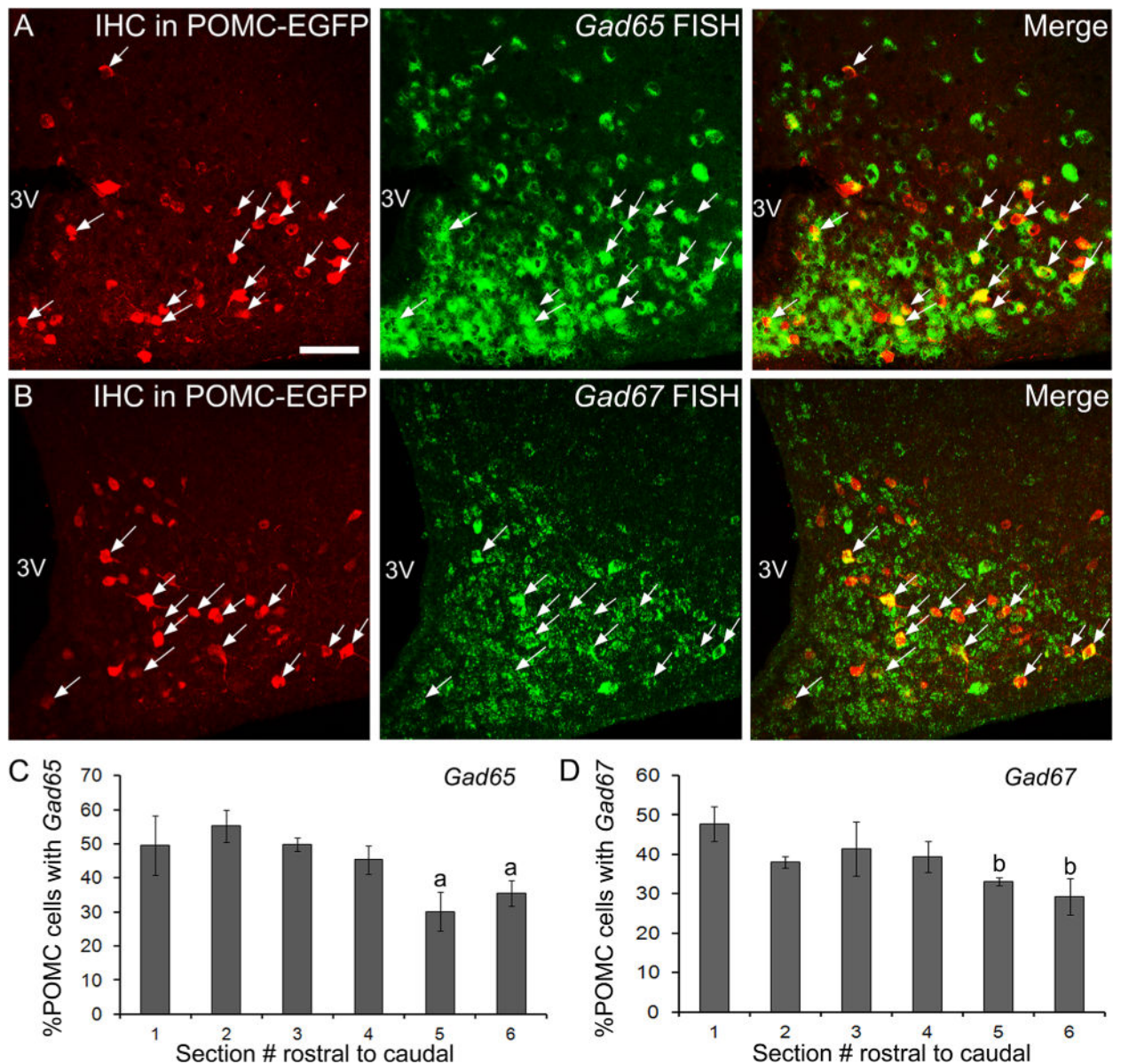
Figure 3.

Gad65 and −*67* mRNA in POMC neurons. **A,B:** Example images of POMC–EGFP detected by immunohistochemistry (A,B, red) and fluorescent in situ hybridization for *Gad65* using a DIG-labeled probe and HNPP detection (A, green) or *Gad67* using an FITC-labeled probe and TSA-Biotin detection (B, green). POMC–EGFP cells with (white arrows, yellow cells in right panels) and without *Gad* label, as well as *Gad*-positive cells lacking POMC–EGFP (green cells in right panels) can be seen. **C,D:** The percentage of POMC cells expressing Gad65 (C, *n* = 7) and Gad67 (D, *n* = 3) throughout the arcuate nucleus is depicted in the bar graphs. Error bars represent the SEM. a, significantly different from sections 1–4; b. significantly different from section 1. 3V, third ventricle. (A magenta–green version of this figure is included as a supplementary file for the assistance of color-blind authors.) Scale bar = 50 μm in A (applies to A,B).
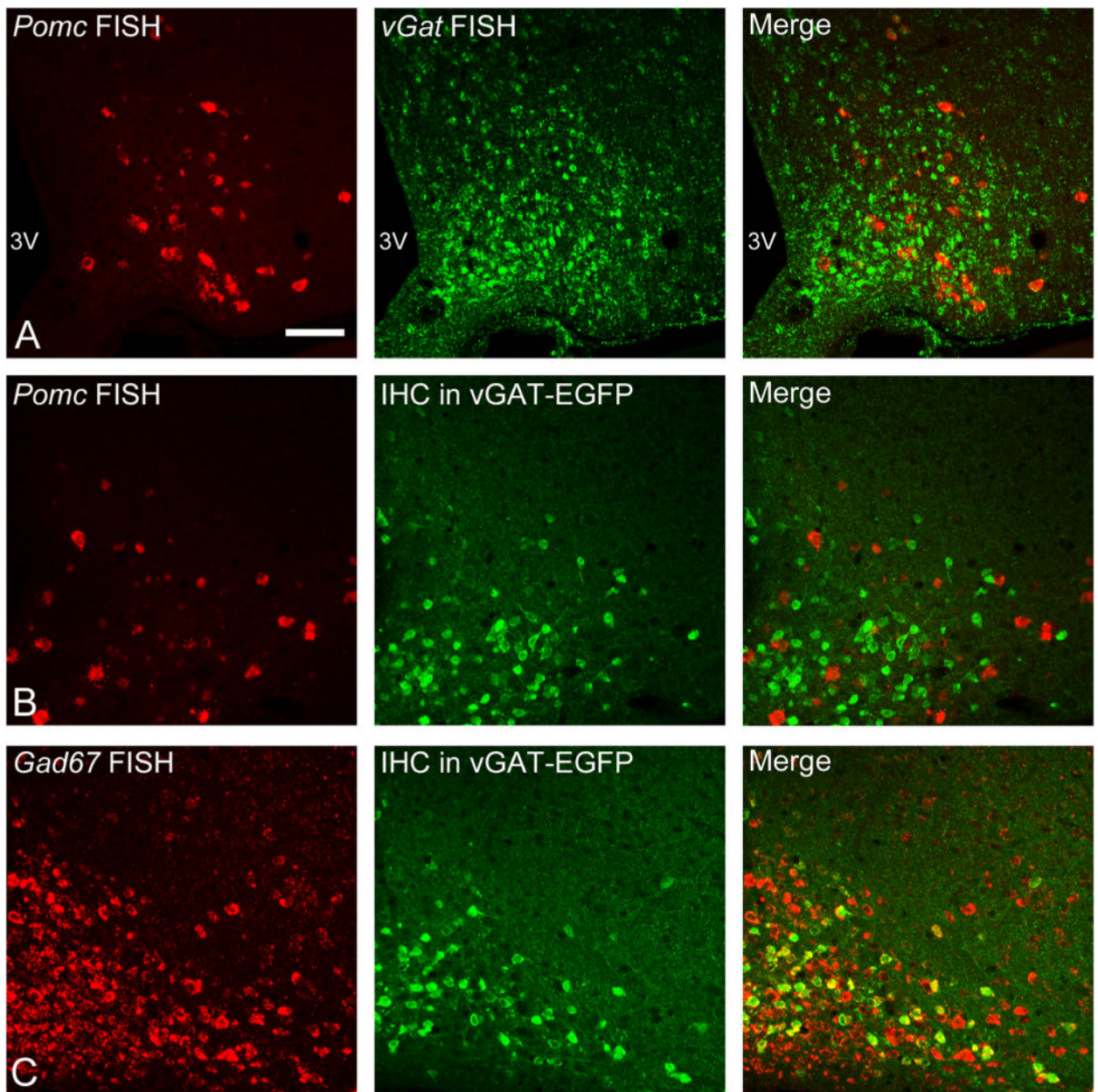
Figure 4.
vGAT mRNA and vGAT–EGFP expression in POMC neurons. **A:** An example image of dual in situ hybridization for *Pomc* using a DIG-labeled probe and HNPP detection (red) and *vGAT* using an FITC-labeled probe and TSA-Biotin detection (green). **B:** In situ hybridization for *Pomc* in tissue from vGAT–EGFP transgenic mice reveals that the transgene is not expressed in a notable number of POMC neurons. **C:** In situ hybridization for *Gad67* in tissue from vGAT–EGFP mice reveals that vGAT–EGFP is expressed only in a portion of *GAD67*-expressing cells (yellow cells in right panel). 3V, third ventricle. (A magenta–green version of this figure is included as a supplementary file for the assistance of color-blind authors.) Scale bar = 50 μm in A (applies to a–C).
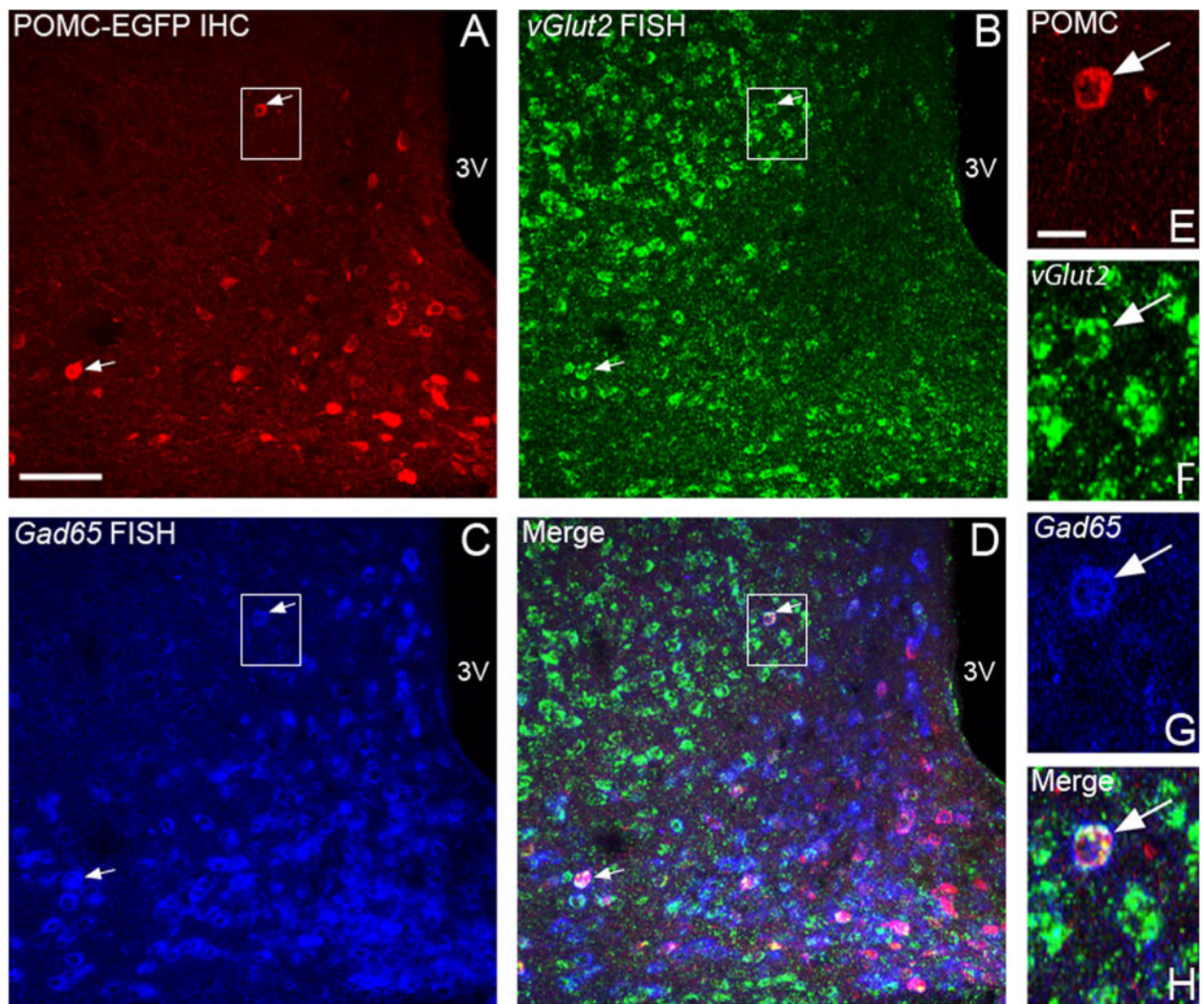
Figure 5.
Simultaneous detection of *vGlut2* and *Gad65* in POMC neurons. **A–C:** POMC-EGFP was detected with a GFP antibody (A, red) after in situ hybridization for both *vGlut2* using an FITC-labeled probe and TSA-Biotin detection (B, green) and *Gad65* using DIG-labeled probes and HNPP detection (C, blue). POMC-EGFP cells expressing both *vGlut2* and *Gad65* are denoted with white arrows. **D:** Cells positive for only *vGlut2*, *Gad65*, or POMC–EGFP are apparent, especially in the merged image. **E–H:** The boxed areas are enlarged to highlight a cell with POMC–EGFP (E), *vGlut2* (F), and *Gad65* (G). 3V, third ventricle. Scale bar = 50 μm in A (applies to A–D); 10 μm in E (applies to E–H).

TABLE 1

Probe Information[1]

Gene	Probe concentration	Hybridization temperature(°C)	Accession ID no.	Probe position (bp)
Pomc	750 pg/μl	70	NM_008895.3	532–1,007
Gad67	150 pg/μl	60	NM_008077.4	749–1,527
Gad65 (628 bp)	150 pg/μl	52	NM_008078.2	537–1,207
Gad65 (824 bp)	150 pg/μl	52	NM_008078.2	1,201–2,032
vGat	1 ng/μl	70	NM_009508.2	1,321–1,713
vGlut2	1 ng/μl	70	NM_080853.3	1,854–2,440
vGlut1	250 pg/μl	70	NM_182993.2	714–1,320

[1]RNA probes corresponding to each gene were used at the specified concentration, and the hybridization reaction took place at the temperatures listed. The accession ID for the Genbank database is provided for each probe sequence, as well as the corresponding base pairs the probes binds to on the mRNA sequence.

TABLE 2

Antibodies Used in This Study

Antibody	Animal	Antigen	Manufacturer and cat. no.
GFP	Chicken, polyclonal	Recombinant full-length GFP	Abcam Ab13970
Digoxigenin conjugated to alkaline phosphatase	Sheep, polyclonal		Roche Applied Sciences 11093274910
Fluorescein conjugated to POD (horseradish peroxidase)	Sheep, polyclonal		Roche Applied Sciences 11426346910
Donkey anti-chicken IgY (IgG) (H+L) DyLight 649			Jackson ImmunoResearch 703-495-155
Streptavidin, Alexa Fluor 488 conjugate			Invitrogen S-32354

TABLE 3

Summary of Experiments[1]

Mouse phenotype	Hybridized first	Hybridized second	Method of visualization
GAD67–GFP	*Pomc*-DIG	n/a	IHC (dαck-649) + HNPP
vGAT-EGFP	*Pomc*-DIG + *vGat*-FITC	n/a	IHC (dαck-649) + TSA Biotin Plus kit (SA-488) + HNPP
vGAT-EGFP	*Gad67*-FITC	n/a	IHC (dαck-649) + TSA Biotin Plus kit (SA-488)
POMC–EGFP	*vGlut2*-FITC	*Gad65*-DIG	IHC (dαck-649) + TSA Biotin Plus kit (SA-488) + HNPP
POMC–EGFP	*Gad67*-FITC	n/a	IHC (dαck-649) + TSA Biotin Plus kit (SA-488)
Wild type	*vGlut1*-DIG	n/a	HNPP

[1]Probes labeled with either digoxigenin (DIG) or fluorescein (FITC) were hybridized individually, at the same time, or sequentially. The target mRNA is listed alongside the phenotype of the animals used for each experiment, as well as a brief summary of the immunological methods used to detect each marker (GFP, DIG, or FITC). IHC, immunohistochemistry.

TABLE 4

Percentage of *Pomc* Cells With Glutamatergic or GABAergic Markers[1]

	% of POMC neurons	No. of animals	Total POMC cells counted/animal
vGlut2 mRNA	7.36 ± 1.21	7	263 ± 25.50
Gad65 mRNA	44.66 ± 2.76	7	263 ± 25.50
Gad67 mRNA	37.99 ± 1.03	3	407.67 ± 88.10
vGlut2 + Gad65 mRNA	3.55 ± 0.56	7	263 ± 25.50
vGat mRNA	3.77 ± 0.10	2	200 ± 45
vGAT–EGFP	0.42 ± 0.30	4	257 ± 51.10
GAD67–GFP	10.53 ± 1.33	3	208.33 ± 28.42

[1]The percentage of GABAergic and/or glutamatergic markers present in POMC cells with SEM, as well as the label used to detect each marker (mRNA or the fluorescent reporter, EGFP/GFP). The number of animals used in each experiment is provided, as well as the average number of cells counted per animal per experiment with SEM.