Cellular/Molecular

Dual-Phenotype GABA/Glutamate Neurons in Adult Preoptic Area: Sexual Dimorphism and Function

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It is generally assumed that the inhibitory neurotransmitter GABA and the stimulatory neurotransmitter glutamate are released from different neurons in adults. However, this tenet has made it difficult to explain how the same afferent signals can cause opposite changes in GABA and glutamate release. Such reciprocal release is a central mechanism in the neural control of many physiological processes including activation of gonadotropin-releasing hormone (GnRH) neurons, the neural signal for ovulation. Activation of GnRH neurons requires simultaneous suppression of GABA and stimulation of glutamate release, each of which occurs in response to a daily photoperiodic signal, but only in the presence of estradiol (E_2). In rodents, E_2 and photoperiodic signals converge in the anteroventral periventricular nucleus (AVPV), but it is unclear how these signals differentially regulate GABA and glutamate secretion. We now report that nearly all neurons in the AVPV of female rats express both vesicular glutamate transporter 2 (VGLUT2), a marker of hypothalamic glutamatergic neurons, as well as glutamic acid decarboxylase and vesicular GABA transporter (VGAT), markers of GABAergic neurons. These dual-phenotype neurons are the main targets of E_2 in the region and are more than twice as numerous in females as in males. Moreover, dual-phenotype synaptic terminals contact GnRH neurons, and at the time of the surge, VGAT-containing vesicles decrease and VGLUT2-containing vesicles increase in these terminals. Thus, we propose a new model for ovulation that includes dual-phenotype GABA/glutamate neurons as central transducers of hormonal and neural signals to GnRH neurons.

Key words: VGAT; VGLUT; LHRH; GnRH; estradiol; ovulation

Introduction

Ovulation is triggered through estradiol (E_2)-dependent activation of gonadotropin-releasing hormone (GnRH) neurons in the preoptic area (POA) (Levine et al., 1991). Evidence from rodent models indicates that this activation is indirect and mediated primarily by the anteroventral periventricular nucleus (AVPV; a region of the POA). The AVPV is a sexually dimorphic region (Simerly, 1998) with abundant estrogen receptors (ERs) (Simerly et al., 1990; Shughrue et al., 1997). Lesions of the AVPV (Wiegand et al., 1980; Wiegand and Terasawa, 1982; Ronnekleiv and Kelly, 1986; Petersen et al., 1989) or microimplants of antiestrogen (Petersen and Barraclough, 1989) placed into the region block luteinizing hormone (LH) surge release. Furthermore, the AVPV provides the majority of ERα-containing cells that innervate the rostral POA (rPOA), where most GnRH neurons participating in the LH surge reside (Simonian et al., 1999). Finally, the AVPV receives inputs from numerous brain regions that convey sensory and autonomic signals relevant to reproduction (Simerly, 1998). Thus, this nucleus is a critical region for integrating hormonal and environmental signals and communicating them to GnRH neurons.

The AVPV cells responsible for communicating signals to GnRH neurons have not been identified; however, it seems likely for several reasons that they are GABAergic and glutamatergic neurons. First, glutamatergic (Eyigor et al., 2004) and GABAergic (Flugge et al., 1985) neurons in the AVPV contain ER. Furthermore, GABAergic neurons in the AVPV, but not those surrounding GnRH neurons in the rPOA, exhibit changes in GAD67 gene expression that parallel GABA release on the day of LH surge release (Curran-Rauhut and Petersen, 2002). Consistent with these findings, GABA and glutamate terminals provide most of the synaptic input to GnRH neurons (Herbison, 1998; Kiss et al., 2003; Lin et al., 2003; Petersen et al., 2003; Eyigor et al., 2004; Han et al., 2004), and receptors for GABA and glutamate are among the few types found on GnRH neurons (Petersen et al., 2003). Moreover, agonists or antagonists to these receptors disrupt E_2-dependent LH surge release (Herbison and Dyer, 1991; Donoso et al., 1994; Brann and Mahesh, 1995). Other neurotransmitters also regulate the LH surge, but many of these act by regulating GABA and glutamate release (Hartman et al., 1990; Brann and Mahesh, 1995; Bhat et al., 1998; Herbison, 1998; Gore, 2001). Taken together, these data suggest that GABAergic and glutamatergic neurons of the AVPV are likely targets of E_2 and central to the generation of GnRH and LH surge release.

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Although under some conditions GABA may be stimulatory to GnRH neurons (Ondo, 1974; DeFazio et al., 2002), the preponderance of evidence indicates that in postpubertal animals, GABA inhibits them (Herbison, 1998; Han et al., 2002, 2004). In contrast, activation of glutamate receptors consistently stimulates GnRH neurons and LH surge release (Brann and Mahesh, 1995; Gore, 2001; Kuehl-Kovarik et al., 2002). Consistent with these findings, induction of LH surge release requires suppression of GABA and stimulation of glutamate release into the POA (Petersen et al., 2003), changes that occur at the onset of the afternoon surge (Jarry et al., 1995; Mitsushima et al., 2002). These changes are triggered, at least in part, by neurons of the suprachiasmatic nucleus (SCN) that communicate photoperiodic signals to ER-containing cells in the AVPV (de la Iglesia et al., 1995; Watson et al., 1995).

It is not clear how a photoperiodic signal in the presence of E_2 simultaneously activates glutamate release and inhibits GABA release from AVPV neurons, but two possibilities seem most likely. First, it is possible that GABAergic and glutamatergic neurons are separate cells, and the same afferent signal has opposite effects on the two populations. This could be accomplished if only one population contained ER and E_2 changed the responsiveness of that cell type to the photoperiodic signal. An alternative model is that GABA and glutamate are released from the same neurons and that these dual-phenotype neurons have ER. In this model, reciprocal release might be accomplished by photoperiodic signals and autofeedback mechanisms that are regulated by E_2.

A previous obstacle to differentiating between these models was that no reliable method of detecting glutamatergic neurons existed. This obstacle was recently overcome with the characterization of vesicular glutamate transporters (VGLUTs) as specific markers of glutamatergic neurons (Hisano, 2003). Using these markers and markers of GABA neurons, we now report that most of the neurons in the AVPV of females are both GABAergic and glutamatergic under physiological conditions. In addition, we show that these dual-phenotype neurons are the major targets of E_2 in the AVPV and that the incidence of these neurons differs between sexes. Finally, we demonstrate that during the time period in which GnRH and LH surge release begins, E_2 decreases markers of GABAergic vesicles and increases markers of glutamatergic vesicles in terminals contacting GnRH neurons.

Materials and Methods

Animals. Adult Sprague Dawley rats (Zivic Miller, Zelienople, PA) were maintained according to *NIH Guidelines for the Care and Use of Laboratory Animals*, and the Institutional Animal Care and Use Committee of the University of Massachusetts approved all treatment protocols. Animals were housed in a temperature- and light-controlled room (14/10 hr light/dark cycle; lights on at 5:00 A.M.) with food and water provided *ad libitum*.

Dual-label in situ *hybridization histochemical studies.* All dual-label *in situ* hybridization histochemical (ISHH) studies used orchidectomized (ORX) male and/or ovariectomized (OVX) female rats. Gonadectomies were performed under isofluorane anesthesia, and animals were killed 1 week later with CO_2. Brains were frozen in powdered dry ice at the time of sacrifice, then wrapped in Parafilm and stored at −80°C in sealed tubes. We obtained serial 12 μm coronal cryosections through the POA region that contains the AVPV, the medial preoptic nucleus (MPO), and the periventricular POA (PePO) (−0.00 to −0.26 from bregma) (Swanson, 1998), as well as through regions containing the hippocampal formation (−2.45 to −4.60 from bregma) and cortex (−2.6 from bregma).

Three dual-label ISHH studies were performed. First, we colocalized mRNAs for glutamic acid decarboxylase (GAD; marker of GABA neurons) and VGLUT2 (marker of hypothalamic glutamatergic neurons) (Ziegler et al., 2002) in the AVPV and in the medial preoptic area (MPO) of OVX rats ($n = 5$). In separate studies, we verified that vesicular GABA transporter (VGAT) mRNA and GAD mRNA were colocalized in all neurons of the AVPV (E. Ottem and S. Petersen, unpublished observations). As a control study, we also colocalized GAD and VGLUT1 mRNAs in the hippocampal formation and cortex. In these regions, VGLUT1 predominates and GABAergic neurons are separate from glutamatergic neurons.

Second, we examined sex differences in the incidence of cells containing both VGLUT2 and GAD mRNAs in the AVPV ($n = 5$ OVX and 5 ORX rats). The rationale for this study was that if the dual-phenotype GABA/glutamate neurons in AVPV are important for LH surge release, there might be sex differences in the incidence of such neurons because only females exhibit the surge in response to E_2.

Third, anti-estrogen microimplants placed into the AVPV block LH surge release (Petersen and Barraclough, 1989), but the affected neurons are unknown; therefore, we next used dual-label ISHH to examine whether the dual-phenotype GABA/glutamate neurons in the AVPV of females were targets of E_2. ERα mRNA is the most abundant isoform in the POA (Shughrue et al., 1997), and in this region, ERβ expression is generally in the same cells as ERα expression (Shughrue et al., 1998). Similarly, preliminary studies showed that GAD-containing neurons were more abundant than VGLUT2-containing neurons, and all VGLUT2 mRNA was found in GAD-containing cells in the AVPV. Therefore, to maximize detection, we used cRNA probes for ERα and GAD mRNAs. We performed these studies in OVX rats ($n = 5$) because endogenous ovarian steroids suppress ER expression (Zhou et al., 1995; Greco et al., 2001).

The cDNA transcription template for probes to VGLUT2 mRNA was a 705 bp fragment corresponding to bases 3197–3901 of the rat VGLUT2 cDNA (GenBank accession number AF271235). It was prepared as described previously (Ottem et al., 2002) using reverse transcription-PCR with a forward primer sequence of 5′-GGAACTCACACCACAAAGC-3′ and a reverse primer sequence of 5′- CAGCAAGGGTTATGGTCACA-3′. The cDNA template for VGLUT1 (predominant form found in the hippocampus and cortex) was a 555 bp fragment corresponding to bases 24–768 of the rat VGLUT1 cDNA (GenBank accession number U07609). It was prepared using a forward primer sequence of 5′-ATAGGAACCGCAAAAGGCTG-3′ and reverse primer sequence of 5′-GGGGGATTGGCAGGGGAC-3′. Fragments were cloned into a TOPO-TA vector (Invitrogen, Carlsbad, CA) and sequenced to verify identity. The cDNA template for ERα was an 880 bp fragment subclone of the full-length clone (Koike et al., 1987) (provided by M. Muramatsu, University of Tokyo, Tokyo, Japan). We used standard *in vitro* transcription to prepare ^{35}S-labeled cRNA probes using each of these cDNA templates (Ottem et al., 2002).

To maximize detection of GABAergic cells, we used a mixture of three digoxigenin-labeled cRNA probes for GAD mRNAs as described previously (Hays et al., 2002). The cDNA templates for GAD65 probes corresponded to bases 315–944 and 944–1769 of the full-length clone (Erlander et al., 1991), and the template for the GAD67 probe to bases 232–767 of the full-length clone (Erlander et al., 1991) (both provided by A. Tobin, University of California–Los Angeles, Los Angeles, CA). Digoxigenin-labeled probes for GAD65 and GAD67 mRNA were prepared and used in dual-label ISHH studies as described previously (Hays et al., 2002). Sections were thawed, fixed, and prehybridized before applying a mixture of ^{35}S-labeled cRNA probes for VGLUT1, VGLUT2, or ERα (1×10^6 cpm), and 0.5 μl each of the digoxigenin-labeled GAD65 (0.5:l each of two probes) and GAD67 (0.5 μl) probes in 0.5 μl of hybridization buffer. To verify specificity, we hybridized representative sections to ^{35}S-labeled sense strand probes in buffer with or without digoxigenin-labeled cRNA probes for GAD mRNAs.

Sections were hybridized at 52°C overnight under glass coverslips, washed, and processed for immunocytochemical (ICC) detection of digoxigenin-labeled probes for GAD mRNAs. We then used standard emulsion autoradiographical procedures (NTB3 emulsion; Eastman Kodak, Rochester, NY) to visualize radiolabeled probes for VGLUT1 or VGLUT2 mRNAs (5 d exposure for each) or ERα (7 d exposure).

To analyze dual-label ISHH studies, we used BioQuant Windows (R & M Biometrics, Nashville, TN) interfaced to a Leitz Laborlux microscope

through a 3CCD color video camera (Hitachi Denshi America, Woodbury, NY). Four to six sections from the region of interest in each animal were analyzed to determine the mean number of neurons containing GAD mRNA, as well as the mean number containing VGLUT2 or ERα mRNA. We also determined the mean percentage of GAD mRNA-positive neurons that contained VGLUT2 or ERα mRNAs and the mean percentage of VGLUT2 or ERα mRNA-containing neurons that also contained GAD mRNA. Grand means for each group were obtained from means of individual animals. In studies comparing males and females, data were analyzed using unpaired two-tailed *t* tests.

ICC studies. We performed three ICC studies with laser confocal analysis. First, we examined whether GABAergic and glutamatergic vesicles were colocalized in the rPOA, a major projection field of ER-containing neurons in the AVPV (Simonian et al., 1999) and a region that contains abundant GnRH neurons (OVX rats; $n = 5$). Second, we tested whether rPOA terminals that contained both GABAergic and glutamatergic vesicles had synaptic specializations as indicated by the presence of synaptophysin (SYN) (Wiedenmann and Franke, 1985) (OVX rats; $n = 5$). Finally, we asked whether levels of GABAergic and glutamatergic vesicles in the dual-phenotype terminals contacting GnRH neurons changed during the day of LH surge release in response to E_2. We examined OVX animals with and without E_2 at 9:00 A.M. when LH release is suppressed, at 12:00 P.M. (around the so-called "critical period" just before LH surge begins), and at 3:30 P.M. during the LH surge in our animal model (Petersen and Barraclough, 1989).

For each study, animals were killed with an overdose of pentobarbital and perfused transcardially with 50 ml of 0.9% saline, followed by 200 ml of 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.2. Brains were postfixed in the same solution at 4°C for 1 hr and placed in a solution of 30% sucrose in 0.1 M PB at 4°C. Forty-micrometer serial cryosections were obtained through the rPOA region (+0.45 to −3.25 from bregma) (Swanson, 1998). Sections were stored in cryoprotectant (30% sucrose, 0.1% polyvinyl pyrolidone-40, in ethylene glycol, and 0.1 M PB) (Watson et al., 1986).

Free-floating sections were washed in phosphate-buffered saline (PBS), pH 7.4, then incubated in blocking buffer (10% normal donkey serum, 0.2% Triton X-100, and 0.02% sodium azide in PBS) at room temperature for 1 hr. To detect GABAergic vesicles, we used antibody to VGAT (McIntire et al., 1997), rabbit polyclonal anti-VGAT (1:1000; Chemicon, Temecula, CA). We incubated sections overnight in blocking buffer containing anti-VGAT, as well as guinea pig polyclonal anti-VGLUT2 (final dilution, 1:5000; Chemicon). To verify that these vesicles were synaptic structures, we also included mouse anti-SYN (1:5000; Chemicon) in the buffer. In studies to examine VGAT and VGLUT2 contacts on GnRH neurons, mouse monoclonal anti-GnRH (1:100; Acris GmbH, Hiddenhausen, Germany) was substituted for anti-SYN.

After incubation in primary antisera, sections were washed in PBS and incubated at room temperature for 1 hr in a mixture of secondary antibodies (diluted 1:150 in PBS containing 0.2% Triton X-100 and 0.02% sodium azide) obtained from Jackson ImmunoResearch (West Grove, PA). We detected immunoreactive (IR) VGAT with Cy5-conjugated donkey anti-rabbit IgG, IRVGLUT2 with Texas Red-conjugated donkey anti-guinea pig IgG, IRSYN with fluorescein (FITC)-conjugated donkey anti-rabbit IgG, and GnRH with FITC-conjugated donkey anti-mouse IgG. After incubation with secondary antibodies, sections were washed in PBS, mounted on glass slides, and allowed to air dry before applying coverslips with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA).

To verify specificity of primary antibodies, we used standard preabsorption protocols, as well as procedures to rule out cross-reactivity between primary and secondary antibodies (Smith et al., 2000). Our findings verified results of previous studies that have extensively characterized the specificity and reactivity of anti-VGLUT2 (Lin et al., 2003; Todd et al., 2003), anti-VGAT (McIntire et al., 1997; Holderith et al., 2003), and anti-SYN (Wiedenmann and Franke, 1985; Masliah et al., 2001).

ICC results were analyzed using a Zeiss LSM 510 META confocal microscope. For image scans, the pinhole diameter was optimized to 1.11 Airy disk, and image size was set at 512 × 512 pixels. All VGAT and VGLUT2 were found in punctate structures; therefore, confocal images were obtained using a high-power objective (63× oil immersion objective; 1.4 numerical aperture) and 2.5× digital zoom. To determine whether IRVGLUT2, IRVGAT, and IRSYN were colocalized, we performed scans at each wavelength independently to eliminate "bleed-through" between individual channels.

To determine whether IRVGAT- and IRVGLUT2-containing terminals contacted GnRH neurons, individual IRGnRH neurons were visualized at 40× and 63× magnification under epifluorescent (mercury vapor) illumination. Next, the illumination was switched to confocal mode (laser), and optical sectioning of a selected GnRH neuron was performed using a 63× oil immersion objective and 3× digital zoom. For each GnRH neuron, we obtained stacks of 20–35 optical sections (Z-thickness, 0.45 μm), depending on the orientation of the individual neuron. Each Z-series was composed of scans through three separate channels (488, 543, and 643 nm excitation). Images from single scans and compiled Z-series were captured and saved. Composite images, as well as individual images from each channel, were converted to TIFF format, saved, and subsequently analyzed using BioQuant Windows Image Analysis software described below. We also used Zeiss LSM 5 Image Browser software that allows virtual rotation of cells to verify that terminals actually contact neurons in multiple fields of view.

Based on previous work showing preferential localization of NMDA-type glutamate receptors in medial subppopulations of GnRH neurons, in each animal we examined 50 neurons in both the medial and lateral subdivisions as defined previously (Ottem et al., 2002). We determined the mean number of terminals that were in contact or closely apposed to each GnRH cell body and that contained IRVGAT only, IRVGLUT2 only, or both IRVGAT and IRVGLUT2. Grand means for each group were obtained and analyzed using two-way ANOVA to determine whether the number of terminals in contact with GnRH neurons changed because of E_2 or time of day. Medial and lateral populations were analyzed separately, and interactions between main effects were probed with Bonferroni's *t* tests.

To determine whether the levels of VGLUT2- and VGAT-containing vesicles changed in contacts with both proteins, we measured the area of each reaction product in terminals contacting perikarya of medial GnRH neurons ($n = 50$/animal) in animals treated with E_2 or oil and killed at 12:00 P.M. or 3:30 P.M. Using Bioquant Windows software, we set a threshold that digitally highlighted pixels corresponding to red signal from IRVGLUT2 or blue signal from IRVGAT and then determined the mean number of highlighted pixels corresponding to each color. Grand means of individual animals in a treatment group were analyzed using two-sample *t* tests to determine whether the mean level of IRVGAT or IRVGLUT2 in dual-phenotype terminals contacting medial GnRH changed between 12:00 P.M. and 3:30 P.M.

Results

AVPV GABA neurons in females have VGLUT2 mRNA

Results of dual-label ISHH studies showed that in the AVPV of females, most neurons that contained GAD mRNA (as well as VGAT mRNA; data not shown) also contained VGLUT2 mRNA (Fig. 1*A*). Likewise, virtually all VGLUT2 mRNA-containing neurons also contained GAD mRNA, indicating that few cells are solely GABAergic or solely glutamatergic in the AVPV of females. The absence of either signal in sense-strand control assays verified the specificity of the probes (data not shown). In contrast to the AVPV, the MPO (Fig. 2*C*) and PePO (data not shown) had only scattered neurons that expressed both GAD and VGLUT2 mRNAs (Fig. 2*C*). As expected in control studies, we found that VGLUT1 mRNA was found in cells separate from those containing GAD mRNA in the cortex (Fig. 1*C*) and hippocampus (Fig. 1*D*).

Sex differences in dual-phenotype AVPV neurons

Photomicrographs of studies examining colocalization of GAD and VGLUT2 mRNAs in gonadectomized males and females are shown in Figure 2, *A* and *B*, and Table 1 summarizes the data.

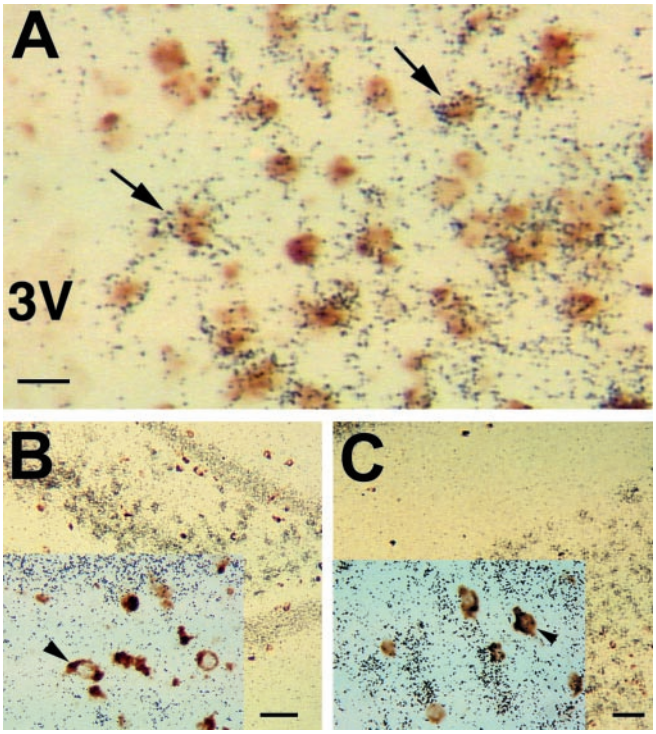

Figure 1. Most GABAergic neurons are also glutamatergic in the AVPV of female rats. *A*, VGLUT2 mRNA (marker of hypothalamic glutamate neurons detected with ^{35}S-labeled cRNA probes; black grains) and GAD mRNA (marker of GABA neurons detected with digoxigenin-labeled cRNA probes; brown stain) are extensively colocalized in the AVPV nucleus (arrows indicate examples of colocalization). VGLUT1 (marker of extrahypothalamic glutamatergic neurons; black grains) and GAD (brown stain) mRNAs are not found in the same cells in either the CA3 region of the hippocampus (*B*) or in the sommatosensory cortex (*C*). Scale bars, 5 μm. 3V, Third ventricle.

Most cells in the AVPV of females contained both GAD and VGLUT2 mRNAs, and females had 2.5 times as many of these dual-phenotype neurons as males. In addition, females had nearly twice as many GAD mRNA-positive cells and also more VGLUT2 mRNA-positive cells than males, consistent with previous evidence that the AVPV of females is significantly larger than that of males (Simerly, 1998). Although the AVPV of males had dual-phenotype neurons, there were more cells that contained only VGLUT2 mRNA or only GAD mRNA than cells that contained both. No such sex differences were seen in the MPO (Fig. 2*C*,*D*).

Most ERα mRNA in the AVPV is in GAD mRNA-positive cells

ERα mRNA was present in 94.5 ± 0.8% of GAD mRNA-containing neurons of the AVPV, and 95.1 ± 0.7% of ERα-positive cells contained GAD mRNA (Fig. 3). Thus, in this brain region, nearly all GABAergic neurons contained ERα and nearly all ERα expression was in GABAergic neurons. This is a significantly higher percentage of ER-containing GABA cells than detected when the POA is considered as a whole (Flugge et al., 1985). This is consistent with our observation that relatively few GAD-containing neurons in the MPO or other regions of the POA contained ER (data not shown). These findings show that dual-phenotype GABA/glutamate neurons are the major targets of E_2 in the AVPV of females and therefore are probably crucial for the signal that activates GnRH neurons and LH surge release.

VGLUT2 and VGAT in synaptic contacts on GnRH cells

The strongest ER-containing projections to GnRH neurons comes from the AVPV region (Simonian et al., 1999). In view of our finding that dual-phenotype GABA/glutamate neurons contain most, if not all, ERα expression in the AVPV, it seemed likely that dual-phenotype terminals from this nucleus comprise the projections. Consistent with this idea, we observed numerous examples of structures containing IRVGLUT2 and IRVGAT in the rPOA, where GnRH neurons reside (Fig. 4). All vesicular transporter immunoreactivity in these structures was colocalized with SYN and, as in a previous report, confined to typical punctate structures characteristic of synaptic vesicles (Lin et al., 2003) (Fig. 5).

We observed contacts with both IRVGLUT2 and IRVGAT on all GnRH neurons in the medial subpopulation of neurons in tissues collected at 12:00 P.M. and 3:30 P.M., but only in E_2-treated animals (Fig. 6). Only 18.4 ± 1.0% of GnRH neurons in lateral subgroups had dual-phenotype contacts. This is an important finding because previous work demonstrates that most medial, but not lateral, subpopulations of GnRH neurons contain NMDA-type glutamate receptors (Ottem et al., 2002), the receptor subtype primarily involved in LH surge release (Brann and Mahesh, 1991). Moreover, we previously showed that a majority of GnRH neurons express the β-3 $GABA_A$ receptor subunit (Petersen et al., 1993), with all medial neurons positive for the subunit (S. McCrone and S. Petersen, unpublished observations).

E_2-induced changes in IRVGAT and IRVGLUT2 levels

In medial, but not lateral, subpopulations of GnRH neurons, the mean number of dual-phenotype terminals increased between morning and afternoon in OVX E_2-treated animals (Fig. 7*A*). There were no dual-phenotype contacts at any time in OVX animals, and the number of IRVGAT only or IRVGLUT2 only did not change during the day in these animals.

Overall, neither the number of IRVGLUT2-containing contacts nor the total number of contacts changed during the day (data not shown). However, the number of contacts with IRVGAT alone was lower at 12:00 P.M. and 3:30 P.M. than during the morning (Fig. 7*B*). We reasoned that these data could be explained if IRVGAT declined in terminals and IRVGLUT2 reached detectable levels, thus causing the appearance of more dual-labeled structures. To test this idea, we examined levels of IRVGAT and IRVGLUT2 reaction product in dual-labeled terminals. Consistent with our hypothesis, IRVGLUT2 increased significantly, whereas levels of IRVGAT decreased between 12:00 P.M. and 3:30 P.M. in E_2-treated, but not oil-treated, animals (Fig. 8). These findings support the idea that dual-phenotype terminals on medial GnRH neurons are responsible for the reciprocal changes in GABA and glutamate that are necessary for LH surge release.

Discussion

Our results provide compelling evidence that in the AVPV of female rats, GABA and glutamate are released from the same neurons. We also made the surprising discovery that this unique population of neurons is sexually dimorphic and the major target of E_2 in the AVPV. Finally, we showed that during the afternoon, IRVGAT declines and IRVGLUT2 rises in dual-phenotype terminals contacting GnRH neurons, but only in animals treated with E_2. The timing of these changes is similar to previously described daily photoperiodic signals necessary for the LH surge (Chappell et al., 2000). Moreover, the direction of the changes is consistent with demonstrated roles of these neurotransmitters in the induction of the surge (Petersen et al., 2003). Together, our findings indicate that dual-phenotype GABA/glutamate neurons of the

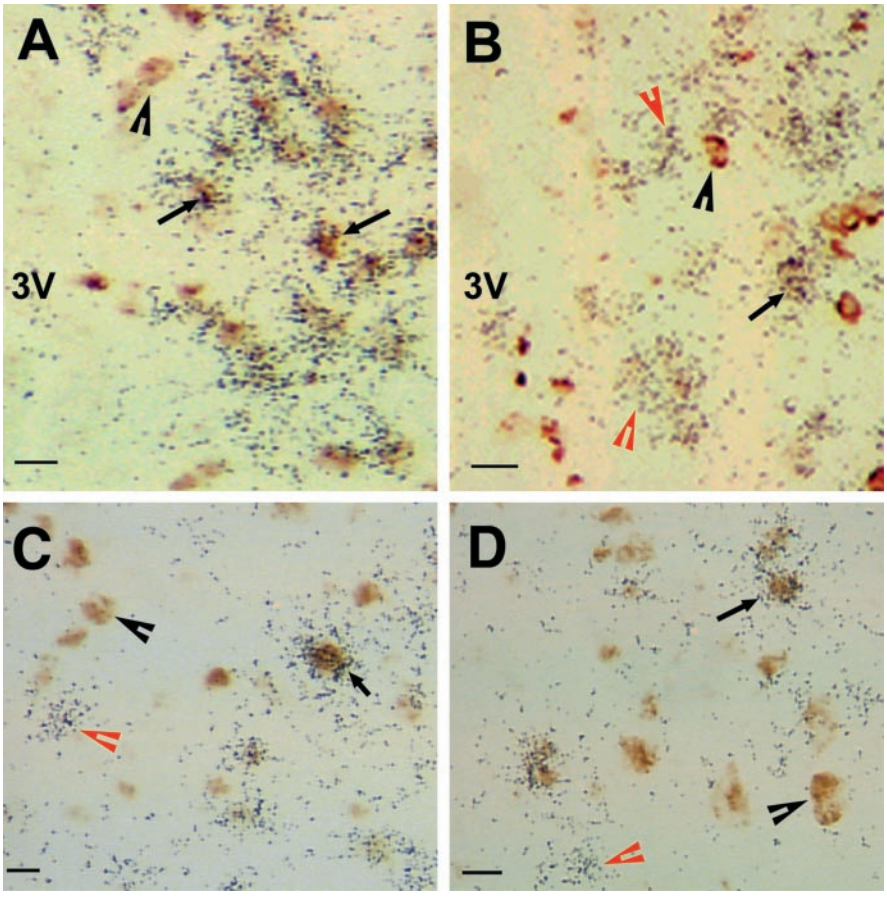

Figure 2. Sex differences in VGLUT2 and GAD mRNA colocalization. In females (*A*), but not males (*B*), most VGLUT2 mRNA (marker of glutamate neurons; black grains) is in GABAergic neurons (brown stain) in the AVPV. However, in the MPO of both females (*C*) and males (*D*), most neurons contain either VGLUT2 or GAD mRNA, not both. The black arrows indicate neurons positive for VGLUT2 and GAD mRNAs. The black arrowheads denote cells positive only for GAD mRNA, and the red arrowheads indicate cells positive only for VGLUT2 mRNA. Scale bars, 5 μm. 3V, Third ventricle.

AVPV integrate hormonal and photoperiodic signals necessary for LH surge release and ovulation.

GABA/glutamate neurons in the female AVPV were densely packed and appeared to be the major neural phenotype of the AVPV. In contrast, few such dual-phenotype neurons were found in the adjacent MPO region, and none was detected in either the hippocampus or cortex. Therefore, it seems likely that these neurons are important for a function unique to the AVPV, namely the induction of LH surge release. In support of this idea, we found that most ERα gene expression in the AVPV was in GABA/glutamate neurons. Thus, these neurons are likely the targets through which AVPV anti-estrogen microimplants block both E_2-dependent LH surge release and changes in GnRH gene expression (Petersen and Barraclough, 1989; Petersen et al., 1989).

We found that the ER-containing GABA/glutamate neurons were 2.5 times more prevalent in the AVPV of females than of males. This is significant because E_2 elicits LH surge release only in females (Gorski, 1979). It is notable that a previous study did not detect any dual-function neurons in the hypothalamus or POA of males (Ziegler et al., 2002), but the method they used was different than the one used in the present studies. Regardless, it is clear from the present data that the dual-phenotype GABA/glutamate neurons are sexually dimorphic populations and likely to be important for female-specific LH surge release.

Other evidence that dual-phenotype neurons play a role in the LH surge comes from our findings that in the projection fields of these neurons, terminals containing both IRVGAT and IRVGLUT2 contacted GnRH neurons. These contacts were observed only in E_2-treated animals and only during the afternoon at the onset of the LH surge in this model. Moreover, dual-phenotype contacts were found on all medial GnRH neurons examined, but on few lateral GnRH neurons. This is a key finding because in rats the majority of medial, but not lateral, GnRH neurons contains functional NMDA receptors (Ottem et al., 2002), a glutamate receptor subtype that regulates GnRH synthesis (Petersen et al., 1991; Liaw and Barraclough, 1993; Gore and Roberts, 1994), and surge release (Brann and Mahesh, 1995). All midline GnRH neurons also contain $GABA_A$ receptors (Petersen et al., 1993; Petersen, unpublished) and $GABA_B$ receptors (Petersen et al., 2003). Consistent with these findings, midline green fluorescent protein-expressing GnRH neurons exhibit altered firing patterns when glutamate and GABA receptors are blocked, whereas lateral GnRH neurons do not (Nunemaker et al., 2002). Interestingly, it is also the medial population of GnRH neurons that is initially activated on the day of LH surge release (Hiatt et al., 1992; Rubin et al., 1995). These findings indicate that dual-phenotype GABA/glutamate neurons are important for GnRH regulation.

In addition to this neuroanatomical evidence for a role of GABA/glutamate terminals in LH surge release, we found physiologically relevant changes in dual-phenotype terminals. During the period preceding LH surge release in E_2-treated animals, IRVGAT decreased and IRVGLUT2 increased in dual-phenotype terminals on medial GnRH neurons. These fluctuations occurred around the time GABA release decreases and glutamate release into the POA increases in this animal model (Jarry et al., 1995). Such changes appear to be necessary for the LH surge because GABA generally inhibits (Herbison, 1998; Han et al., 2004) and glutamate stimulates (Brann and Mahesh, 1995; Spergel et al., 1999; Kuehl-Kovarik et al., 2002) GnRH activity in adult animals. This interpretation is in line with evidence that GABA signaling must be inhibited in order for stimulatory signals to trigger LH surge release (Hartman et al., 1990).

Although we did not show directly that the changes we observed in IRVGAT and IRVGLUT2 are in terminals of AVPV GABA/glutamate neurons, there is reason to presume that this is the case. First, our present work identifies AVPV GABA/glutamate neurons as the E_2-sensitive cells that send strong projections to GnRH neurons (Simonian et al., 1999). Similarly, AVPV GABA/glutamate neurons are most likely the ER-containing targets to which the SCN afferents project (de la Iglesia et al., 1995; Watson et al., 1995) and therefore the neurons that receive the daily signal necessary for the LH surge (Legan and Karsch, 1975). This interpretation is supported by recent work demonstrating that a daily rise in cAMP levels occurs in AVPV neurons (Chappell et al., 2000). Importantly, this rise is necessary for the LH

Table 1. Sex differences in the number of GABA, glutamate, and dual-phenotype neurons in the AVPV (mean ± SEM)

	GAD mRNA	VGLUT2 mRNA	Percentage of GAD-positive cells with VGLUT2 mRNA	Percentage of VGLUT2-positive cells with GAD mRNA
Female	676 ± 10.4	632 ± 13.9	95.5 ± 0.8	98.5 ± 0.3
Male	307 ± 8.0[a]	459 ± 24.5[a]	76.8 ± 1.0[a]	48.6 ± 1.8[a]

[a]Significantly different from corresponding values in females ($p < 0.001$).

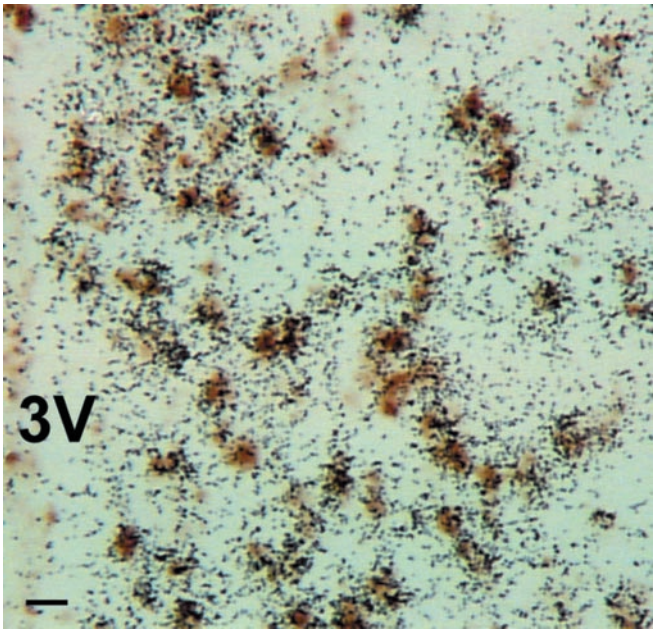

Figure 3. Virtually all ERα mRNA is found in GABAergic cells of female AVPV. A photomicrograph shows results of dual-label ISHH studies using ^{35}S-labeled cRNA probes for ERα mRNA (black silver grains) and digoxigenin-labeled cRNA probes for GAD mRNAs (brown stain). Scale bar, 10 μm. 3V, Third ventricle.

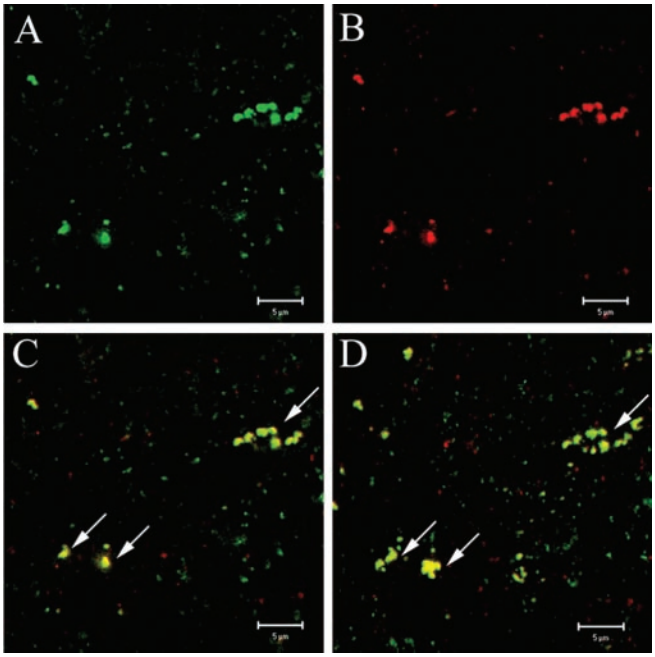

Figure 4. Immunoreactivity for VGAT and VGLUT2 is colocalized in the rPOA projection site of AVPV neurons. A single laser confocal optical section shows clustering of IRVGAT (*A*) and IRVGLUT2 (*B*). Merged images show colocalization of VGAT and VGLUT2 signals (*C*). The projected image was obtained from 10 stacked 1 μm optical sections in the same field as images in *A–C* at 63× with 4× zoom. The arrows indicate colocalization of IRVGAT and IRVGLUT2.

surge, and it coincides with a rise in GAD67 gene expression in neurons found specifically in the AVPV (Curran-Rauhut and Petersen, 2002). These changes also coincide with the timing of E_2-dependent changes in IRVGAT and IRVGLUT2 we observed, as well as in GABA and glutamate release into the POA observed by others using this animal model (Jarry et al., 1995). This body of work provides convincing evidence that AVPV GABA/glutamate neurons communicate hormonal and photoperiodic information to GnRH neurons and that this information is encoded as a change in the balance between GABA and glutamate release from dual-phenotype terminals.

Without electrophysiological evidence, which would be difficult to obtain in this system, we cannot unambiguously show that VGAT- and VGLUT2-containing vesicles in dual-phenotype terminals are released. However, there is strong indirect evidence to support such a notion. First, the number of synaptic contacts between GnRH neurons and IRVGAT and IRVGLUT2 terminals is similar to the total number of synapses found on GnRH neurons (Witkin et al., 1995). Considering that GABA and glutamate provide most of the input to GnRH neurons (Herbison, 2003), it seems likely that they are released from the VGAT- and VGLUT2-containing vesicles we observed in contact with GnRH neurons. Second, the decline in IRVGAT before the onset of LH surge parallels the E_2-dependent suppression of GABA release into the POA (Jarry et al., 1992, 1995), as well as the decline in GAD67 gene expression in AVPV neurons (Curran-Rauhut and Petersen, 2002). Notably, work examining other brain regions shows that VGAT and GAD67 mRNA levels are correlated with GABA release (Litwak et al., 1990; Drengler and Oltmans, 1993; Falkenberg et al., 1997; Lamas et al., 2001; Ramirez and Gutierrez, 2001; Gutierrez, 2002; Kang et al., 2003). No similar correlates of glutamate release are available; however, it has been suggested that in other brain regions, VGLUT2-containing vesicles are found in terminals that have a high probability of release (Fremeau et al., 2004). Together, these data support the idea that reciprocal changes in IRVGAT and IRVGLUT2 in terminals contacting GnRH neurons are responsible for corresponding changes in GABA and glutamate release required for LH surge release.

The present work identified a population of dual-phenotype GABA/glutamate neurons in the AVPV. Previously, the main evidence for such neurons came from work showing that glutamatergic granule cells of the dentate gyrus contain low levels of GABA (Sandler and Smith, 1991; Bergersen et al., 2003), as well as GAD67 (Sloviter et al., 1996). Seizure activity upregulates these levels (Schwarzer and Sperk, 1995; Lehmann et al., 1996) and triggers simultaneous release of GABA and glutamate (Gutierrez, 2000; Gutierrez and Heinemann, 2001). Importantly, GAD67, but not GAD65, gene expression in these neurons is also upregulated in an activity-dependent manner in the absence of seizures (for review, see Gutierrez, 2003). This finding is similar to our previous observation that changes in GAD67 mRNA levels in AVPV GABA/gluamate neurons also reflect changes in GABA release, whereas levels of GAD65 mRNA do not (Curran-Rauhut and Petersen, 2002). Likewise, changes in levels of VGAT mRNA are linked to GABA release in granule cells (Lamas et al., 2001), and we found that IRVGAT in AVPV projection fields decreased

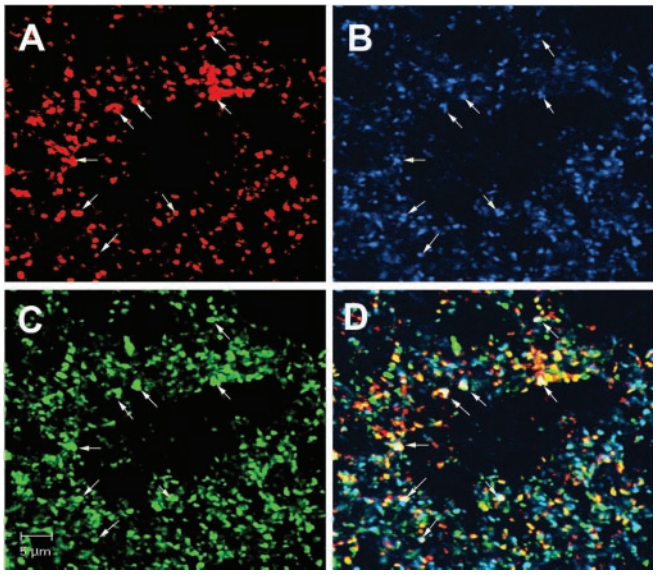

Figure 5. Structures positive for VGLUT2 and VGAT also contain the synaptic marker SYN. Single laser confocal optical sections of triple-label fluorescent ICC for VGLUT2 (*A*), SYN (*B*), and VGAT (*C*) in the rPOA are shown. In the overlay (*D*), virtually all IRVGAT (green) and IRVGLUT2 (red) overlaps with IRSYN (blue) in punctate, terminal-like structures (63× with 2× zoom). The arrows indicate IRVGLUT2, IRrSYN, and IRVGAT contained in triple-labeled structures.

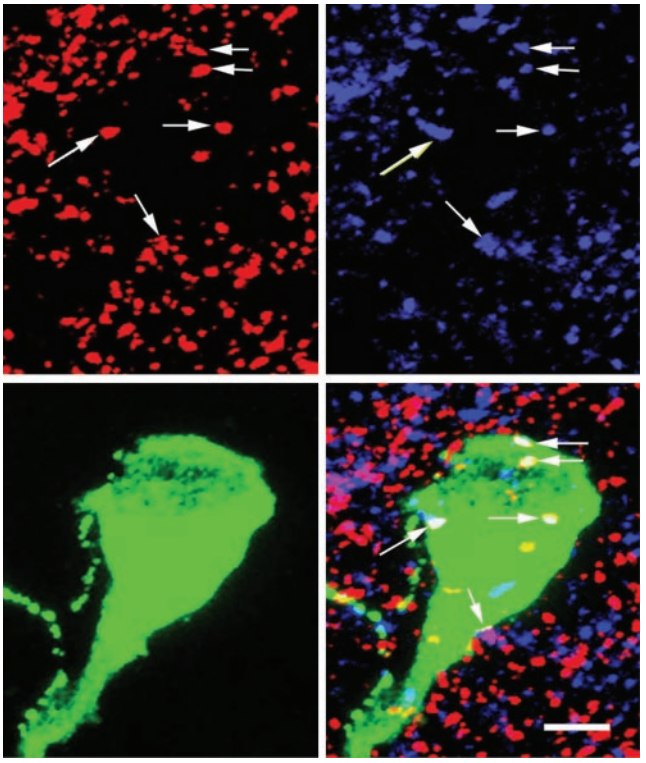

Figure 6. Dual-phenotype GABAergic/glutamatergic terminals contact medial GnRH neurons. A compilation of 30 optical sections from a Z-series (Z-thickness, 0.45 μm) from an OVX, E_2-treated rat killed at 12:00 P.M. shows triple-label ICC for VGLUT2 (*A*), VGAT (*B*), and GnRH (*C*). The overlay (*D*) shows terminal-like structures containing both IRVGAT and IRVGLUT2 in contact with a GnRH neuron. The white arrows indicate terminals with both IRVGAT and IRVGLUT2. Scale bar, 5 μm.

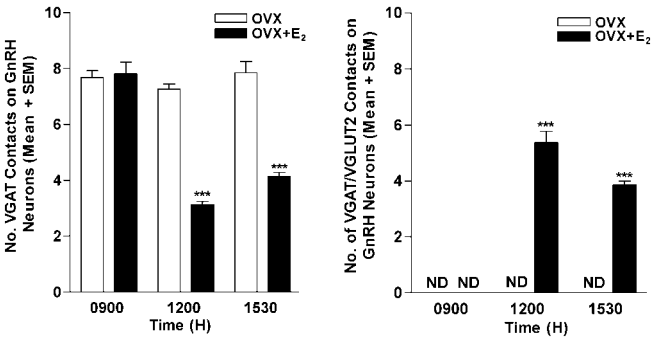

Figure 7. Effects of E_2 on temporal changes in the number of dual-phenotype IRVGAT/IRVGLUT2 or IRVGAT contacts on medial subpopulations of GnRH neurons. Right, Dual-phenotype terminals were observed in OVX rats that received E_2 ($n = 5$/group), but not in those that received vehicle ($n = 5$/group). Dual-phenotype terminals were seen only in animals examined in the afternoon. Left, The number of terminals containing only IRVGAT declined during the period in which LH surge begins in our animal model. ND, Nondetectable. ***Significantly greater than observed at 9:00 A.M. or in OVX animals ($p < 0.001$).

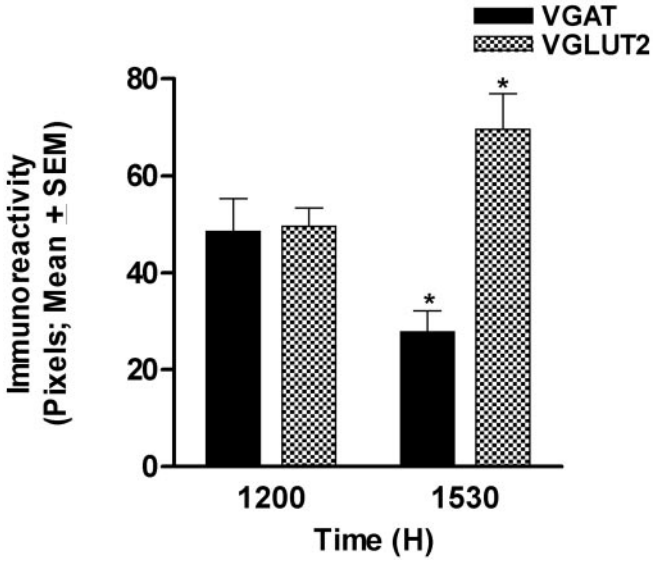

Figure 8. Reciprocal changes in levels of IRVGAT and IRVGLUT2 in dual-phenotype terminals contacting medial GnRH neurons in OVX, E_2-treated animals (no such terminals are seen in OVX animals treated with oil or in animals examined during the morning hours; see Fig. 7). At the time of the LH surge (3:30 P.M. in our animal model), IRVGAT decreases and IRVGLUT2 increases in dual-phenotype terminals. *Significantly different from corresponding values at 12:00 P.M.; $p < 0.05$.

at the time GABA release into the region declines (Jarry et al., 1995). However, one difference is that we found relatively high expression of IRVGAT in terminals, as well as high VGAT mRNA (data not shown) in cell bodies of dual-phenotype GABA/glutamate neurons in AVPV neurons. In contrast, IRVGAT is undetectable (Chaudhry et al., 1998; Sperk et al., 2003) in granule cells, and VGAT mRNA is present only at low levels in adults (Lamas et al., 2001). Regardless of these differences, work from granule cells and our present findings from AVPV neurons provide compelling evidence that dual-phenotype GABA/glutamate neurons are not artifacts, but rather serve important physiological functions.

Based on our findings, we propose that the physiological function of AVPV GABA/glutamate neurons is to integrate hormonal and environmental signals and communicate them to GnRH neurons. Specifically, we propose that in the presence of E_2, a photoperiodic cue to dual-phenotype neurons inhibits GABA release while stimulating glutamate release. These changes allow other neurotransmitters and neuropeptides to elicit GnRH hypersecretion and the preovulatory surge of LH release. The intracellular mechanisms regulating the switch from GABA to gluta-

mate release remain to be determined; however, the fact that glutamate is converted by GAD to GABA suggests one possibility. In prepubertal monkeys, central administration of GAD67 antisense oligonucleotides suppresses GABA and then stimulates glutamate release (Terasawa et al., 1999). In our E_2-treated OVX rat model, GAD67 gene expression in the AVPV declines (Curran-Rauhut and Petersen, 2002) during the same period VGAT decreased and VGLUT2 increased in the present study. These changes also coincide with a decline in GABA and rise in glutamate release into the region (Jarry et al., 1995). Therefore, it is possible that in the presence of E_2, the daily signal to AVPV neurons inhibits GAD67 synthesis, thereby decreasing utilization of glutamate for GABA synthesis and providing more glutamate for release.

Our findings relate directly to the mechanisms through which steroids regulate the neural control of ovulation. However, considering that the balance between GABA and glutamate release regulates myriad functions, it will be important to determine whether dual-phenotype GABA/glutamate neurons are present in other brain regions in which they might regulate sexually dimorphic neural functions and susceptibility to disease.

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