Vasoactive Intestinal Peptide Excites GnRH Neurons in Male and Female Mice

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A variety of external and internal factors modulate the activity of GnRH neurons to control fertility in mammals. A direct, vasoactive intestinal peptide (VIP)-mediated input to GnRH neurons originating from the suprachiasmatic nucleus is thought to relay circadian information within this network. In the present study, we examined the effects of VIP on GnRH neuron activity in male and female mice at different stages of the estrous cycle. We carried out cell-attached recordings in slices from GnRH-green fluorescent protein mice and calcium imaging in slices from a mouse line expressing the genetically encoded calcium indicator GCaMP3 selectively in GnRH neurons. We show that 50%–80% of GnRH neurons increase their firing rate in response to bath-applied VIP (1nM–1000nM) in both male and female mice and that this is accompanied by a robust increase in intracellular calcium concentrations. This effect is mediated directly at the GnRH neuron likely through activation of high-affinity VIP receptors. Because suprachiasmatic nucleus-derived timing cues trigger the preovulatory surge only on the afternoon of proestrus in female mice, we examined the effects of VIP during the estrous cycle at different times of day. VIP responsiveness in GnRH neurons did not vary significantly in diestrous and proestrous mice before or around the time of the expected preovulatory surge. These results indicate that the majority of GnRH neurons in male and female mice express functional VIP receptors and that the effects of VIP on GnRH neurons do not alter across the estrous cycle. (Endocrinology 157: 3621–3630, 2016)

Fertility is governed by a dispersed neuronal network that ultimately controls the activity of the GnRH neurons (1). In female rodents, generation of the preovulatory GnRH-LH surge is dependent on both the midcycle rise in circulating concentrations of estrogen (2, 3) and on daily timing signals arising from the central circadian clock (4, 5). Although estrogen receptor (ER)α-expressing neurons in the rostral periventricular area of the third ventricle (RP3V) relay estrogen signals to the GnRH neurons (6–9), circadian cues are thought to be conveyed via projections from suprachiasmatic nucleus (SCN) (10–12).

Anatomical tracing and functional studies have revealed that SCN neurons may control GnRH neuron activity through both indirect and direct pathways. The indirect route involves SCN projections to ERα-expressing neurons in the RP3V (13–15), including kisspeptin neurons (16, 17), whereas the direct pathway involves SCN projections to the GnRH neurons (18). SCN projections to RP3V kisspeptin neurons use vasopressin (16, 17), a neuropeptide synthesized by dorsal SCN neurons (19) that stimulates LH release and kisspeptin neuron activity (20–22). Direct SCN projections to GnRH neurons, on the other hand, originate from ventral SCN neurons that synthesize vasoactive intestinal peptide (VIP) (23, 24).

Evidence suggests that VIP signaling might play a role in the generation and/or timing of the GnRH/LH surge in females (25, 26). In vivo, female rodent GnRH neurons express the vasoactive intestinal peptide receptor (VPAC)
2 (27) and an early report indicated that central administration of VIP can increase LH release in ovariectomized (OVX) rats (28), although other effects of VIP on LH release have since been reported (29–32). Importantly, however, disrupting endogenous VIP signaling decreases the amplitude of the LH surge (33–35). In good agreement with these observations, in vitro studies have indicated that VIP stimulates GnRH secretion (36–38) and activates GnRH neuron action potential firing in brain slices (39). Interestingly, the latter study reported that VIP actions on GnRH neurons were dependent on the time of day and on estradiol administration to OVX mice, raising the possibility that VIP signaling in GnRH neurons might be modulated by the fluctuations in circulating estrogen seen during the estrus cycle. However, this hypothesis has never been examined in ovari-intact mice.

In addition to the extensively studied modulatory role of SCN VIP neurons upon GnRH neuron activity in females, evidence suggests that SCN neurons project to GnRH neurons in males. VIP-immunoreactive (ir) appositions onto GnRH neurons are also found in male rodents, albeit to a lesser extent than in females (40). However, whether these represent bone fide VIP synapses on GnRH neurons has not been determined and, indeed, the functional role of VIP within the GnRH neuronal network of males is unknown.

Using brain slice electrophysiology and calcium imaging, we have examined here whether GnRH neurons express functional VIP receptors in both male and female mice. To address whether endogenous gonadal steroids may modulate the ability of VIP to excite GnRH neurons in females, we have investigated whether VIP signaling in GnRH neurons is modulated in a diurnal fashion on diestrus and proestrus.

Materials and Methods

Animals and brain slice preparation

Adult (2- to 7-mo-old) male and female GnRH-green fluorescent protein (GFP) (41) and GnRH-Cre+/−/ROSA26-CAG-GCaMP3+/− (42, 43) (GnRH-GCaMP3) mice were group housed under conditions of controlled temperature (22 ± 2°C) and lighting (12:12-h light, 12:12-h dark cycles) with ad libitum access to food and water. All mice used in this study were bred from homozygous GnRH-GFP, GnRH-Cre, or ROSA26-CAG-GCaMP3 parents accordingly. The University of Otago Animal Ethics Committee approved all animal experimental protocols.

Male mice were killed 5–6 hours after the onset of the light phase (zeitgeber time [ZT]5–ZT6) and recordings performed between ZT6 and ZT11. In female mice, estrous cycle stage was determined by daily vaginal smear each morning, and mice exhibiting at least 2 regular 4- or 5-day estrous cycles were studied either on diestrus (appearance of leukocytes) or proestrus (nu-
Intracellular calcium imaging

Slices obtained from GnRH-GCaMP3 mice were obtained as described above. Slices were placed under an upright microscope (Olympus), constantly perfused (1.0–1.5 mL/min) with warm (30°C) aCSF and allowed to stabilize for more than 15 minutes. Variations in intracellular calcium concentration ([Ca2+]i) were estimated by measuring GCaMP3 fluorescence changes in GnRH neurons. Slices were illuminated through a 40X immersion objective, using the xenon arc light source (300 W; filtered by a GFP filter cube, excitation 470–490 nm; Chroma) and shutter of a DG-4 (Sutter Instruments). Epifluorescence (495 nm long pass and emission 500–520 nm) was collected using a Hamamatsu ORCA-ER digital CCD camera. Light source, shutter and camera were controlled and synchronized with the μ-manager 1.4 software (47). A focal plane including at least one fluorescent soma was chosen and acquisitions (100-ms light exposure at 2 Hz for 10–20 min) were started. VIP was applied in the bath for 1–2 minutes after a 3- to 5-minute control period. At the end of experiments, the responsiveness of fluorescent cells in the slice was tested by 20mM KCl.

For analysis, time series of images were processed in ImageJ (48). Regions of interest including individual, in-focus fluorescent somata were selected (2.4 ± 0.3 per slice, range 1–5). Average fluorescence intensity within each region of interest was measured in each frame. Fluorescence intensity data were analyzed using scripts written in R (http://www.r-project.org/) or using pClamp. Relative fluorescence changes were calculated using the following formula: ΔF/F = (Ft – F)/F × 100, where F is the baseline fluorescence intensity calculated as the mean fluorescence intensity over a 2-minute period preceding drug applications and Ft is the fluorescence measured at any time point. For statistical comparisons, Ft was averaged over 2-minute periods in control and in the presence of VIP. VIP was considered to have an effect on [Ca2+]i if GCaMP3 fluorescence increased over the mean basal fluorescence by more than twice the SD of basal fluorescence.

Statistics

Statistical analyses were performed in Prism 6 (GraphPad Software). Values given in the text and illustrated in figures are mean ± SEM. Nonparametric statistics were applied, using number of neurons as the sample size. Differences were considered significant for \( P < .05 \).

Drugs

DL-2-amino-5-phosphonopentanoic acid sodium salt (AP5) (catalog number 3693), 6-cyano-7-nitroquinoxaline-2,3-dione disodium (CNQX) (catalog number 1045), 6-imino-3-(4-ethoxyphenyl)-1(6H)-pyridazinethiobutanoic acid hydrobromide (gabazine) (catalog number 1262), VIP (catalog number 1911), and VIP(6–28) (catalog number 1905) were purchased from Tocris Bioscience. Tetrodotoxin citrate (catalog number T-550) was purchased from Alomone Labs. All drugs were dissolved in water to a stock concentration, kept at 20°C until use and diluted to the appropriate concentration in aCSF.

Results

VIP increases electrical activity and intracellular calcium in male GnRH neurons

We first examined whether VIP could modulate GnRH neuron electrical activity in male mice. In cell-attached patch clamp recordings of GFP-expressing neurons in MS and rPOA slices from male GnRH-GFP mice, we found that bath applications of VIP (1nM–1000nM) caused reversible increases in the spontaneous firing of GnRH neurons (Figure 1A). A total of 82 GnRH neurons were tested with VIP and 51% found to be excited in 16 of 20 mice. In those 16 mice, all VIP concentrations tested elicited a statistically significant effect on GnRH neuron firing (Table 1). The proportion of GnRH neurons excited by VIP \( (P > .66, \text{ Fisher's exact test}) \) (Figure 1B) and the increase

Figure 1. VIP increases spontaneous action potential firing in male GnRH neurons. A, Example traces and corresponding rate meters (20-s bins) illustrating the effect of 2-minute bath applications of VIP (1nM–1000nM) in 3 different GnRH neurons. Most GnRH neurons increased their firing in response to VIP, whereas a subset remained unaffected. B, Percentages of GnRH neurons excited by VIP were similar at all concentrations. C, The effect of VIP was similar at all concentrations. Only GnRH neurons showing an increase in firing were included. Numbers of recordings are indicated at the bottom of each bar.
in action potential firing frequency induced by VIP were similar at all VIP concentrations (\(P = .26\), Kruskal-Wallis test) (Figure 1C).

We next monitored the effect of VIP on \([\text{Ca}^{2+}]_{i}\) in GnRH neurons. We generated a mouse line expressing the genetically encoded calcium indicator GCaMP3 (49) in GnRH neurons (GnRH-GCaMP3) by cross-breeding mice expressing Cre recombinase in GnRH neurons (42) with mice expressing Cre-dependent GCaMP3 (43). As expected, GCaMP3 fluorescence could be detected in GnRH neurons in the MS and rPOA (Figure 2A). The presence of GCaMP3-expressing neurons in the lateral septum in these mice is consistent with previously observed Cre-recombinase activity outside of the normal adult GnRH neuron distribution in GnRH-Cre mice (50). These neurons were not recorded from. Bath applications of VIP (1nM–100nM) caused reversible increases in \([\text{Ca}^{2+}]_{i}\) in GnRH neurons (Figure 2B). The proportion of responding GnRH neurons was not affected by the concentration of VIP (1nM, 36% \(n = 22\) from 4 mice; 10nM, 32% \(n = 19\) from 4 mice; 100nM, 46% \(n = 11\) from 4 mice; \(P > .70\), Fisher’s exact test) (Figure 2C). In responding GnRH neurons, VIP-induced changes in \([\text{Ca}^{2+}]_{i}\) were similar at all VIP concentrations (1nM, 0.78 ± 0.38% \(\Delta F/F\) \(n = 8\) from 4 mice; 10nM, 1.31 ± 0.38% \(\Delta F/F\) \(n = 7\) from 3 mice; 100nM, 0.83 ± 0.26% \(\Delta F/F\) \(n = 5\) from 4 mice; \(P = .35\), Kruskal-Wallis test) (Figure 2D).

VIP excites GnRH neurons via a direct mechanism

We investigated the involvement of VIP receptors in mediating the effect of VIP on GnRH neuron firing. We compared the effect of VIP in the presence and in the absence of the VIP receptor antagonist VIP(6–28) (51). VIP (50nM) significantly increased GnRH neuron firing in control conditions (0.13 ± 0.07 to 0.60 ± 0.18 Hz, \(n = 11\) from 5 mice, \(P < .01\)) but not in the presence of VIP(6–28) (100nM; 0.39 ± 0.15 to 0.56 ± 0.18 Hz, \(n = 10\) from 4 mice; \(P > .05\), Bonferroni’s post hoc test two-way ANOVA) (Figure 3). Of note, a nonsignificant trend towards increased firing rates in the presence of VIP(6–28) was seen in these experiments (\(P > .05\), Bonferroni’s post hoc test two-way ANOVA). Nevertheless, the inability of VIP to exert a significant effect on GnRH neuron firing in

Table 1. Summary Statistics of the Effect of VIP on Male GnRH Neuron Firing at Different Concentrations

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Control (Hz)</th>
<th>VIP (Hz)</th>
<th>(\Delta F/F) (Hz)</th>
<th>Number of Cells/Number of Mice</th>
<th>(P) Value (Wilcoxon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1nM</td>
<td>0.08 ± 0.03</td>
<td>0.90 ± 0.22</td>
<td>0.82 ± 0.21</td>
<td>8/6</td>
<td>.008</td>
</tr>
<tr>
<td>10nM</td>
<td>0.07 ± 0.03</td>
<td>0.60 ± 0.23</td>
<td>0.53 ± 0.21</td>
<td>6/4</td>
<td>.31</td>
</tr>
<tr>
<td>50nM</td>
<td>0.06 ± 0.03</td>
<td>1.00 ± 0.20</td>
<td>0.94 ± 0.20</td>
<td>6/3</td>
<td>.31</td>
</tr>
<tr>
<td>100nM</td>
<td>0.05 ± 0.02</td>
<td>0.74 ± 0.17</td>
<td>0.69 ± 0.16</td>
<td>8/3</td>
<td>.008</td>
</tr>
<tr>
<td>500nM</td>
<td>0.31 ± 0.15</td>
<td>0.71 ± 0.27</td>
<td>0.39 ± 0.13</td>
<td>6/4</td>
<td>.031</td>
</tr>
<tr>
<td>1000nM</td>
<td>0.15 ± 0.05</td>
<td>1.07 ± 0.25</td>
<td>0.92 ± 0.21</td>
<td>8/5</td>
<td>.008</td>
</tr>
</tbody>
</table>

Only responding GnRH neurons are included.

VIP excites GnRH neurons via a direct mechanism

We investigated the involvement of VIP receptors in mediating the effect of VIP on GnRH neuron firing. We compared the effect of VIP in the presence and in the absence of the VIP receptor antagonist VIP(6–28) (51). VIP (50nM) significantly increased GnRH neuron firing in control conditions (0.13 ± 0.07 to 0.60 ± 0.18 Hz, \(n = 11\) from 5 mice, \(P < .01\)) but not in the presence of VIP(6–28) (100nM; 0.39 ± 0.15 to 0.56 ± 0.18 Hz, \(n = 10\) from 4 mice; \(P > .05\), Bonferroni’s post hoc test two-way ANOVA) (Figure 3). Of note, a nonsignificant trend towards increased firing rates in the presence of VIP(6–28) was seen in these experiments (\(P > .05\), Bonferroni’s post hoc test two-way ANOVA). Nevertheless, the inability of VIP to exert a significant effect on GnRH neuron firing in

Figure 2. VIP effect on \([\text{Ca}^{2+}]_{i}\) in male GnRH neurons. A, Average projection image of a slice from a GnRH-GCaMP3 mouse. B, Example traces illustrating the effect of different concentrations of VIP on \([\text{Ca}^{2+}]_{i}\) in GnRH neurons. C, Proportions of GnRH neurons exhibiting elevated \([\text{Ca}^{2+}]_{i}\) in response to 1nM, 10nM, or 100nM VIP. D, The magnitude of elevated \([\text{Ca}^{2+}]_{i}\) was similar at all VIP concentrations. Numbers of recordings are indicated at the bottom of each bar.
the presence of VIP<sub>(6–28)</sub> suggests that the effect of VIP may be mediated by VIP receptors.

We examined whether this occurred at the GnRH neuron or indirectly via activation of other neurons within the slice. To do so, we tested the effect of VIP in the presence of a cocktail of CNQX (10 μM), AP5 (40 μM), and gabazine (5 μM) to inhibit AMPA, NMDA, and GABA<sub>A</sub> receptors, respectively. VIP (100 nM) enhanced GnRH neuron firing both in control conditions (0.06 ± 0.02 to 0.56 ± 0.15 Hz, n = 11 from 4 mice; *P* < .01 and n.s., not significant; two-way ANOVA with Bonferroni’s test).

Figure 3. The excitatory effect of VIP is prevented by a VIP receptor antagonist. A, Example traces and corresponding rate meters (20-s bins) of the effect of 50 nM VIP on GnRH neuron spontaneous firing in the absence (top) and in the presence (bottom) of the VIP receptor antagonist VIP<sub>(6–28)</sub> (100 nM). B, Bar graph summarizing the effect of VIP in the absence and in the presence of VIP<sub>(6–28)</sub>; **, *P* < .01 and n.s., not significant; two-way ANOVA with Bonferroni’s test.

![Figure 3](https://example.com/figure3.png)

Figure 4. The excitatory effect of VIP is independent on fast synaptic transmission in the slice. A, Example trace and corresponding rate meter (20-s bins) illustrating the effect of VIP (100 nM) in the presence of a cocktail of ionotropic glutamate and GABA receptor antagonists including CNQX (10 μM), AP5 (40 μM), and gabazine (5 μM). B, Bar graph summarizing the effect of VIP in the absence and the presence (n = 11 each) of the cocktail; **, *P* < .01, two-way ANOVA with Bonferroni’s test.

![Figure 4](https://example.com/figure4.png)

Figure 4 was similar in this experiment to that obtained in separate control experiments where slices were challenged with 2 consecutive VIP applications (0.77 ± 0.09, n = 12 from 5 mice; *P* = .57, Mann-Whitney test) (Figure 5B).

Taken together, these results show that VIP increases GnRH neuron activity by directly activating VIP receptors expressed by GnRH neurons.

VIP excites GnRH neurons in ovary-intact female mice independent of estrous cycle stage

Previous work in OVX GnRH-GFP mice has revealed that VIP can excite GnRH neurons in an estradiol- and time of day-dependent manner (39). This suggests a cellular mechanism through which SCN neurons may control the activity of GnRH neurons to trigger the preovulatory surge in GnRH release. However, the fluctuations in circulating estrogen that drive the generation of the GnRH/LH surge are abolished in estradiol-treated OVX mice. Whether VIP receptor function in GnRH neurons will be dependent on the variations in circulating ovarian steroid hormones occurring during the estrous cycle remains unknown. To address this issue, we compared the effect of VIP (10 nM–100 nM) in diestrous and proestrous female GnRH-GFP mice.

As in males, bath applications of VIP increased action potential firing in 42 of 78 female GnRH neurons (54%) (Figure 6A). When examined at the level of the animal,
excitations were seen in 6 out of 7 and in 7 out of 10 diestrous and proestrous mice, respectively (P/H11005/.60, Fisher’s exact test). In those animals, VIP caused significant enhancements in GnRH neuron firing in both diestrus and proestrus (Table 2). The proportion of GnRH neurons excited by VIP was similar in diestrous (72%, n = 18 out of 25 from 6 mice) and in proestrous mice (69%, n = 24 out of 35 from 7 mice; P = 1.00, Fisher’s exact test) (Figure 6B). In addition, the magnitude of the VIP effect on GnRH neuron firing was similar in diestrous (1.00 ± 0.25 Hz) and in proestrous mice (1.16 ± 0.19 Hz; P = .27, Mann-Whitney test) (Figure 6C). When considering the time of day slices were prepared, VIP caused significant increases in GnRH neuron firing at ZT5–ZT6 and at ZT10 in both diestrous and proestrous mice (Figure 6, D and E, and Table 2). The proportions of GnRH neurons excited by VIP were comparable in all groups (60%–80%, P > .28, Fisher’s exact test) (Figure 6D). Similarly, there were no significant differences in the effect of VIP across all groups (dimestru, ZT5–ZT6: 0.78 ± 0.39 Hz and ZT10: 1.21 ± 0.31 Hz n = 9 from 3 mice each; proestru, ZT5–ZT6: 1.07 ± 0.26 Hz and ZT10: 1.25 ± 0.28 Hz n = 12 each from 4 and 3 mice, respectively; P = .33 time of day and P = .59 cycle stage two-way ANOVA) (Figure 6E).

Discussion

Using electrophysiology and GCaMP3 calcium imaging we report here that VIP increases the activity of GnRH neurons in acute brain slices prepared from adult male and female mice. The effect of VIP was found to be direct at the GnRH neuron and likely mediated by high-affinity VPAC receptors. Unexpectedly, we found that the percentage of GnRH neurons responding to VIP, and the magnitude of their response, was the same at ZT5–ZT6 and ZT10 in both diestrous and proestrous mice. These results indicate that, in ovary-intact females, VIP receptor function in GnRH neurons is relatively constant through the days of diestrus and proestrus leading up to the preovulatory LH surge.

The proportion of GnRH neurons increasing their [Ca2+]i in response to VIP (~40%) was to some extent lower than that of GnRH neurons increasing their firing (50%–80%). This likely reflects differences in the sensitivity of cell-attached electrophysiology compared with that of [Ca2+]i imaging. In the latter, we suspect that subtle or microdomain-restricted changes in fluorescence might have been missed by imaging over entire GnRH neuron somata, which may explain the slightly lower proportions of GnRH neurons showing increases in [Ca2+]i in response to VIP. Immunohistochemical studies have reported that approximately 30% of female mouse GnRH neurons show VIP-ir appositions (52). That figure is less than the percentage of GnRH neurons responding to VIP we report here, suggesting a mismatch between the functional expression of VIP receptors in GnRH neurons and immunohistochemical detection of VIP innervation. This may, in part, result from anatomical investigations of VIP appositions being restricted to only the GnRH cell soma and very proximal dendrite. Studies have now shown that action potentials are initiated in regions of the GnRH neuron dendrite that lie some distance (100–150 μm) from the cell body (53, 54).

VIP can bind VPAC1 and VPAC2 receptors with high affinity and pituitary adenylate cyclase-activating polypeptide receptor 1 with low affinity (55). In our experiments, VIP increased GnRH neuron action potential firing and [Ca2+]i, at low nanomolar concentrations, and this was inhibited by VIP(6–28), a VPAC receptor antagonist.
This suggested that the effects of VIP might primarily be mediated via activation of high affinity VPAC receptors. This result is in agreement with an earlier report that GnRH neurons in female rats express the VPAC2 receptor (27). The lack of dose response relationship in the present experiments using a range of 1nM–1000nM suggests that even low nanomolar concentrations of VIP may saturate the receptor(s) mediating the effects of VIP. Moreover, because higher VIP concentrations did not produce additional effects, it is likely that the effect of VIP we observed is mediated solely via activation of VPAC and not pituitary adenylyl cyclase-activating polypeptide receptors.

The physiological role of VIP inputs to GnRH neurons in males is unknown. A previous study reported that VIP-ir fibers were apposed to GnRH neurons in male rats (40), and we now show here that over half of all GnRH neurons are activated in a direct manner by VIP. To our knowledge, there is no evidence for the circadian regulation of GnRH or LH secretion in male rodents. The impact of clock genes on male fertility is not well documented (56). The Bmal1-null male has reproductive deficits but this has been attributed to testicular defects (57), whereas Clock mutant and VPAC2 receptor knockout mice only exhibit very mild reductions in fertility (58). Thus, any role of a direct SCN VIP input to male GnRH neurons may be subtle. An alternative explanation for the presence of VIP receptors in GnRH neurons is that they are innervated by VIP neurons located outside the SCN.

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An important role for VIP in regulating GnRH neuron activity at the time of the preovulatory LH surge in female rodents has been indicated by several studies. Anatomical tracing revealed that VIP-producing neurons in the SCN project monosynaptically to GnRH neurons (18, 24). Importantly, VIP-ir fibers are found preferentially apposed to GnRH neuron somata expressing c-Fos at the time of the

### Table 2. Summary Statistics of the Effect of VIP (10nM–100nM) on Female GnRH Neuron Firing in Diestrus and Proestrus at Different Times of Day

<table>
<thead>
<tr>
<th>Control (Hz)</th>
<th>VIP (Hz)</th>
<th>Number of Cells/Number of Mice</th>
<th>P Value (Wilcoxon)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diestrus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All ZTs</td>
<td>All ZTs</td>
<td>All ZTs</td>
<td>All ZTs</td>
</tr>
<tr>
<td>0.20 ± 0.08</td>
<td>1.19 ± 0.27</td>
<td>18/6</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>ZT5–ZT6</td>
<td>ZT10</td>
<td>ZT5–ZT6</td>
<td>ZT10</td>
</tr>
<tr>
<td>0.08 ± 0.05</td>
<td>0.31 ± 0.14</td>
<td>0.86 ± 0.42</td>
<td>1.52 ± 0.31</td>
</tr>
<tr>
<td>Proestrus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All ZTs</td>
<td>All ZTs</td>
<td>All ZTs</td>
<td>All ZTs</td>
</tr>
<tr>
<td>0.18 ± 0.06</td>
<td>1.34 ± 0.22</td>
<td>24/7</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>ZT5–ZT6</td>
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<td>ZT5–ZT6</td>
<td>ZT10</td>
</tr>
<tr>
<td>0.17 ± 0.09</td>
<td>0.19 ± 0.09</td>
<td>1.24 ± 0.28</td>
<td>1.44 ± 0.34</td>
</tr>
</tbody>
</table>

Only responding GnRH neurons are included.
incidence of circadian VIP and estradiol signals essential to the preovulatory GnRH-LH surge. Although this does not discount a role for VIP inputs to GnRH neurons in the LH surge. In agreement with these observations, VPAC2 receptor knockout and VIP knockout mice exhibit disrupted estrous cycles (58, 60), whereas VIP knockout mice show deficits in ovulation (60), suggesting a role for endogenous VIP in regulating the GnRH neuronal network.

In contrast, in vivo studies investigating the effects of centrally administered VIP on LH release have been inconsistent in their findings. Although an early report showed that VIP increased LH release (28), subsequent studies revealed that VIP may decrease the amplitude of the LH surge (31, 61, 62) and the activation of GnRH neurons at the time the of the surge (61, 63). It is worth noting, however, that centrally administered VIP may act at sites other than the GnRH neurons to indirectly alter LH release (59, 64), and may not necessarily reflect the direct actions of VIP on GnRH neurons. Nevertheless, the observation that VIP directly excites GnRH neurons, along with previous in vitro findings (37–39), supports the idea that VIP release from SCN projections participates in the activation of GnRH neurons leading to the generation of the preovulatory surge.

The electrophysiological observations of Christian and Moenter (39) provided important support for a role for VIP in activating GnRH neurons at the time of the surge. In those studies, VIP was only found to activate GnRH neurons at the time of surge onset (~ZT10) in slices prepared from OVX, estradiol-treated mice. Recordings from GnRH neurons at other times, and in OVX mice, demonstrated substantially decreased responsiveness to VIP. Those results are in stark contrast to the present observations that 60%–80% of GnRH neurons responded to VIP at ZT5–ZT6 and ZT10 in brain slices prepared from both diestrous and proestrous mice. We consider that the most likely reason for this discrepancy is the use of an OVX, estradiol-treated mouse model by Christian and Moenter, (33) in which mice exhibit abnormal daily surges of LH secretion (65). Our data now demonstrate that, under normal circumstances in intact mice, VIP receptor signaling in GnRH neurons remains unchanged in the days leading up to the preovulatory GnRH-LH surge. Although this does not discount a role for VIP inputs to GnRH neurons in surge generation, these observations indicate that the coincidence of circadian VIP and estradiol signals essential for the surge does not occur through modulation of VIP receptor function in the GnRH neuron.

To conclude, we provide here evidence that VIP, a neuropeptide central to the circadian clock, directly stimulates mouse GnRH neurons. Somewhat unexpectedly, GnRH neurons respond to VIP in both male and female mice. Our experiments in ovari-intact females reveal that the expression of functional VIP receptors by GnRH neurons is not modulated during the estrous cycle and furthermore is not dependent on time of day. These observations are reminiscent of our previous findings regarding the modulation of RP3V kisspeptin neuron activity by vasopressin (44). Taken together, this suggests a model in which circadian and humoral cues do not converge at the level of VIP and vasopressin receptors in GnRH neurons and RP3V kisspeptin neurons, respectively, to evoke the preovulatory LH surge.

Acknowledgments

We thank Dr Karl Iremonger for commenting on an earlier draft of this manuscript, Rob Porteous for technical assistance, and Antoine Fraissenon for his valuable help with R scripts.

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This work was supported by a grant from the Health Research Council of New Zealand.

Disclosure Summary: The authors have nothing to disclose.

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