EXCITATORY ACTIONS OF VASOACTIVE INTESTINAL PEPTIDE ON MOUSE THALAMOCORTICAL NEURONS ARE MEDIATED BY VPAC$_2$ RECEPTORS

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Running Head: VPAC2 receptor mediated alterations in excitability of thalamic relay neurons

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ABSTRACT

Thalamic nuclei can generate intrathalamic rhythms similar to those observed at various arousal levels and pathophysiological conditions such as absence epilepsy. These rhythmic activities can be altered by a variety of neuromodulators that arise from brainstem regions as well as those that are intrinsic to the thalamic circuitry. Vasoactive intestinal peptide (VIP) is a neuropeptide localized within the thalamus and strongly attenuates intrathalamic rhythms via an unidentified receptor subtype. We have used transgenic mice lacking a specific VIP receptor, VPAC2, to identify its role in VIP-mediated actions in the thalamus. VIP strongly attenuated both the slow, 2-4 Hz and spindle-like 5-8 Hz rhythmic activities in slices from wild-type mice (VPAC2+/+), but not in slices from VPAC2 receptor knock-out mice (VPAC2−/−) suggesting a major role of VPAC2 receptors in the anti-oscillatory actions of VIP.

Intracellular recordings revealed that VIP depolarized all relay neurons tested from VPAC2+/+ mice; however, in VPAC2−/− mice, VIP produced no membrane depolarization in 80% of neurons tested. In relay neurons from VPAC2+/+ mice, VIP enhanced the hyperpolarization activated mixed cation current, Iₜ, via cyclic AMP activity, but VIP did not alter Iₜ in VPAC2−/− mice. In VPAC2−/− mice, pituitary adenylate cyclase activating-polypeptide (PACAP) depolarized the majority of relay neurons via Iₜ enhancement presumably via PAC1 receptor activation. Our findings suggest that VIP-mediated actions are predominantly mediated by VPAC2 receptors but PAC1 receptors may play a minor role. The excitatory actions of VIP and PACAP suggest these peptides may not only regulate intrathalamic rhythmic activities, but may influence information transfer through thalamocortical circuits.
INTRODUCTION

The thalamus serves as the gateway for relaying peripheral sensory-related information to the neocortex. In addition, the thalamus is capable of generating rhythmic activities similar to those observed during various arousal states and in certain pathophysiological conditions such as absence epilepsy (Steriade and Llinás, 1988; von Krosigk et al., 1993; Steriade et al., 1993; Huguenard and Prince, 1994; Warren and Jones, 1994). The rhythmic activities arise from the reciprocal synaptic connectivity between thalamic relay nuclei and the adjacent thalamic reticular nucleus (TRN) in conjunction with the intrinsic properties of thalamic neurons (Deschénes et al., 1982; Jahnsen and Llinás, 1984a; von Krosigk et al., 1993). While the cellular mechanisms required to maintain such rhythmic activities are well understood, those involved in the initiation and termination of these rhythmic activities are unclear. Certain neuromodulators, which arise from brainstem nuclei, such as acetylcholine, norepinephrine, and serotonin, have been found to attenuate the rhythmic activities (Lee and McCormick, 1997; McCormick, 1992; Lee and McCormick, 1996). This termination of the rhythmic activity is closely related to changes in behavioral states.

There is increasing evidence that another group of neuromodulators, namely neuropeptides, can also alter thalamic activity. A variety of neuropeptides (e.g., cholecystokinin, neuropeptide Y, N/OFQ, somatostatin, substance P, and vasoactive intestinal peptide (VIP)) along with their respective receptors have been localized within the thalamus and have been shown to alter the excitability of thalamic neurons by changing intrinsic properties of these cells such as resting membrane potential, input resistance, membrane conductance, and action potential firing mode or affecting synaptic transmission (Cox et al., 1995; Leresche et al., 2000; Sun et al., 2001; Sun et al., 2002; Lee and Cox, 2003; Sun et al., 2003). Considering that neuropeptides have been found to be colocalized with classical neurotransmitters (i.e., γ-aminobutyric acid (GABA) and glutamate) in other brain regions, released in an activity-dependent manner, and produce long-lasting changes in neuronal excitability, they may play an important role in regulating thalamic activity (Lundberg and Hökfelt, 1983).
VIP and pituitary adenylate cyclase activating-polypeptide (PACAP) belong to the glucagons-secretin-VIP family of peptides. VIP was originally isolated from porcine intestine as a 28 amino acid peptide and is involved in many regulatory functions, including vasodilation, gastrointestinal secretion and motility, and glycogenolysis (Gozes and Brenneman, 1989). VIP is also widely distributed in the central and peripheral nervous system where it has been found to produce a variety of actions (Gozes and Brenneman, 1989). PACAP is a 38 amino acid peptide that shares 68% identity with VIP and was originally isolated from ovine hypothalamus and found to increase cyclic AMP accumulation in anterior pituitary cells (Miyata et al., 1989). PACAP is also localized within a variety of brain areas and peripheral organs and exerts a broad range of physiological actions, including the regulation of anterior pituitary cell function, adrenal gland activity, endocrine and exocrine pancreas secretions, and testicular spermatogenesis (Arimura and Shioda, 1995).

VIP and PACAP can activate three different subtypes of receptors in the central nervous system: VPAC₁, VPAC₂, and PAC₁ (Gozes and Brenneman, 1989; Harmar et al., 1998). VIP and PACAP have similar affinities for VPAC₁ and VPAC₂ receptors; however, PACAP has a relatively higher affinity than VIP for PAC₁ receptors. All three receptor subtypes are GTP-dependent protein coupled receptors and are coupled to cyclic AMP and/or inositol phosphate turnover (Ishihara et al., 1992; Deutsch and Sun, 1992; Spengler et al., 1993; Van Rampelbergh et al., 1997; MacKenzie et al., 2001). These receptor subtypes are differentially distributed throughout the brain: VPAC₁ receptors are most abundant in the cerebral cortex and hippocampus (Ishihara et al., 1992), VPAC₂ receptors are primarily found in the thalamus and suprachiasmatic nucleus (Usdin et al., 1994; Sheward et al., 1995), and PAC₁ receptors are more evenly distributed in various brain regions with relatively higher concentrations within olfactory bulb, thalamus, hypothalamus, hippocampus, and cerebellum.

VIP is localized within thalamic reticular nucleus (TRN) neurons, and these neurons in turn innervate almost all thalamic nuclei (Jones, 1985). Both VPAC₂ and PAC₁ receptors are localized within primary sensory thalamic nuclei including the ventrobasal nucleus (VB) and the dorsal lateral geniculate nucleus (Usdin et al., 1994; Sheward et al., 1995; Vertongen et al., 1997; Burgunder et al., 1999); however,
VPAC₁ receptors have not been localized within the thalamus (Usdin et al., 1994). We have previously shown that VIP, via an unidentified receptor subtype, selectively depolarizes VB relay neurons and attenuates slow intrathalamic oscillations (Lee and Cox, 2003; Sun et al., 2003). In a previous study, the VIP and PACAP depolarization of thalamic neurons was claimed to be via activation of PAC₁ receptors based upon agonist concentrations used (Sun et al., 2003). However, these pharmacological agents are not particularly selective. Considering the availability of transgenic mice lacking the VPAC₂ receptor, we can determine the role of VPAC₂ receptor-mediated actions as well as PAC₁ receptor-mediated actions on relay neurons. Our results indicate that VIP depolarizes relay neurons and attenuates intrathalamic oscillation via activation of VPAC₂ receptors in mouse thalamus. PACAP, presumably through the activation of PAC₁ receptors, also depolarizes relay neurons via a common mechanism, the enhancement of the hyperpolarization activated mixed cation current, Iₜ. Finally, the VPAC₂ receptor-mediated actions require the activation of the cyclic AMP (cAMP)-dependent second messenger pathways. Some of these findings have been presented in abstract form (Lee et al., 2003).
MATERIALS AND METHODS

The general procedures used in these experiments were similar to those previously described (Lee and Cox, 2003). Mice deficient in VPAC2 receptor (VPAC2−/−) were originally generated by Dr. Anthony Harmer (Harmar et al., 2002). The University of Illinois colony of VPAC2−/− and VPAC2+/+ mice was derived from five breeding pairs of heterozygous mice (C57BL/6J) kindly provided by Dr. Harmar. Procedures used for genotyping the mice in these experiments were similar to those previously described (Harmar et al., 2002). Young VPAC2−/− and VPAC2+/+ mice (postnatal age 10-21 days) were deeply anesthetized with sodium pentobarbital (50 mg/kg) and decapitated. The brain was quickly removed and placed into cold, oxygenated slicing medium containing (in mM): 2.5 KCl, 10.0 MgCl₂, 0.5 CaCl₂, 1.25 NaH₂PO₄, 26.0 NaHCO₃, 11.0 glucose, and 234.0 sucrose. Tissue slices (300-400 µm thickness) were cut in the horizontal plane using a vibrating tissue slicer, transferred to a holding chamber, and incubated at least 1 hour prior to recording. Individual slices were then transferred to a recording chamber, and continuously superfused with warm (30°C), oxygenated physiological saline containing (in mM): 126.0 NaCl, 2.5 KCl, 1.25 MgCl₂, 2.0 CaCl₂, 1.25 NaH₂PO₄, 26.0 NaHCO₃, and 10.0 glucose. This solution was gassed with 95% O₂/5% CO₂ to a final pH of 7.4.

Intracellular recording procedures

Intracellular recordings using the whole-cell configuration were obtained with the visual aid of an Axioskop 2FS equipped with differential interference contrast optics (Zeiss Instruments, Thornwood NY). Individual slices were transferred to a submersion-type recording chamber. A low power objective (5×) was used to identify specific thalamic nuclei, and a high-power water immersion objective (63×) was used to visualize individual neurons. Recording pipettes were pulled from 1.5 mm outer diameter capillary tubing and had tip resistances of 3-6 MΩ when filled with the following intracellular solution (in mM): 117 K-glucuronate, 13 KCl, 1.0 MgCl₂, 0.07 CaCl₂, 0.1 EGTA, 10.0 HEPES, 2.0 Na₂-ATP, and 0.4 Na-GTP. The pH was adjusted to 7.3 and osmolarity was adjusted to 290-300 mosm. Initial access resistances following break-in typically ranged
from 10 to 25 MΩ and remained stable during most recordings included for analyses in this study. An Axoclamp2B amplifier (Axon Instrument, Foster City, CA) was used in bridge mode for voltage recordings (current clamp mode) or switching single electrode voltage clamp mode for current recordings. Current and voltage protocols were generated using pClamp software (Axon Instruments, Foster City, CA) and data were stored on computer. For current clamp recordings, an active bridge circuit was continuously adjusted to balance the drop in potential produced by passing current through the recording electrode. The apparent input resistances of individual neurons were calculated from the linear slope of the voltage-current relationship obtained by applying constant current pulses ranging from -100 to +40 pA (800 ms duration). During VIP and PACAP38 application, changes in input resistance were determined by membrane responses to single-intensity constant current hyperpolarizing pulses (5-40 pA, 500 ms, 0.2 Hz). For voltage clamp recordings, the amplifier was used in discontinuous mode. In these recordings, the switching frequency ranged from 2.5 to 3.5 kHz with a gain of 150 to 800 pA/mV, and the headstage was continually monitored to ensure that the current transients had completely decayed before voltage measurements. Voltage-clamp recordings were limited to neurons that had a stable access resistance less than 30 MΩ.

*Extracellular recording procedures*

Extracellular multiple unit recordings were obtained using sharpened tungsten microelectrodes (Frederick Haer, Inc., Bowdoinham, ME). All data were digitized (1-2 kHz) and stored using pClamp software (Axon Instruments, Foster City, CA). Monopolar electrical stimulation was applied to either TRN or internal capsule using sharpened tungsten electrodes (200-600 kΩ). Analyses of intrathalamic rhythmic activities were similar to those described previously (Lee and Cox, 2003). Briefly, autocorrelograms were constructed from the extracellular multiple-unit data over a period of 3-9 seconds with a bin size of 30 ms to quantify degree of synchrony and duration of intrathalamic oscillations (Minianalysis, Synaptosoft Inc., Fort Lee, NJ). Three measures were used to quantify oscillatory activity in autocorrelograms: number of peaks, oscillation amplitude (Amp_{osc}), and frequency of oscillation. Alterations in Amp_{osc} indicate a change in the number of unit discharges in the rhythmic activity. The number
of peaks indicates the number of cycles in the rhythm and the oscillation frequency reflects the principle frequency of the rhythmic activity.

**Pharmacological agents**

Concentrated stock solutions of VIP and PACAP<sub>38</sub> were prepared in distilled water and diluted in physiological saline to a concentration of 0.4-4.0 µM. VIP and PACAP<sub>38</sub> were applied by injecting a bolus into the input line of the chamber using a motorized syringe pump. Based on the rate of agonist injection and the rate of chamber perfusion, the final bath concentration of drugs was estimated to be one-eighth of the concentration introduced in the flow line and this dilution is reflected within the results (Cox et al., 1995; Lee and Cox, 2003). Control injections of physiological saline did not alter the intrathalamic activity during extracellular recording or intrinsic properties (e.g., membrane potential, input resistance) of neurons during current-clamp recordings, suggesting that the temporary increase in flow rate during the bolus injections had no effect on the recordings. VIP and PACAP<sub>38</sub> were purchased from Calbiochem (San Diego, CA) and ZD7288 from Tocris (Ellisville, MO). All remaining compounds were purchased from Sigma (St. Louis, MO).

**Statistics**

Data are presented as mean ± standard deviation. Most statistical analyses consist of Mann-Whitney U test, and when appropriate, the Wilcoxon test for paired samples. In some noted instances, a students’ t-test or paired t-test were used for testing statistical significance. Results were considered statistically significant when p<0.05.
RESULTS

VIP attenuates intrathalamic rhythms via activation of VPAC2 receptors

Extracellular recording electrodes were placed in VB to monitor intrathalamic rhythmic activity that arises from the reciprocal synaptic connectivity between VB and the adjacent TRN. Single electrical stimuli in or near TRN typically evoked a spindle-like rhythmic activity (5-8 Hz) in slices from VPAC2⁺/+ and VPAC2⁻/- mice that could last for many seconds (Figure 1Ai). These spindle-like rhythms were produced in 76% of slices from VPAC2⁺/+ mice (13/17 slices) and 78% of slices from VPAC2⁻/- mice (14/18 slices). This incidence rate of obtaining the rhythmic activity was not significantly different between the two groups (p>0.5, Fisher’s Exact test). The autocorrelogram clearly indicates a highly synchronized response with duration of approximately 2 seconds (~10 cycles) in a slice from a VPAC2⁺/+ mouse (Figure 1B, left panel, black line) and a similar duration (2 s, ~12 cycles) in a slice from a VPAC2⁻/- mouse (Figure 1B, right panel, black line). Under these conditions, the frequency and duration of the rhythmic activity was stable from trial to trial. From the autocorrelograms, we quantified the number of peaks and interburst frequency. The interburst periods of the spindle-like activity produced in slices from VPAC2⁻/- mice (170 ± 35 ms, n=12) were significantly shorter than those evoked in slices from VPAC2⁺/+ mice (213 ± 45 ms, n=10; p<0.05, Mann-Whitney U test), but there was no significant difference in the number of peaks (Figure 1C, D).

As with other species tested (Huguenard and Prince, 1994;Bal et al., 1995;McCormick et al., 1995), addition of the GABA_A receptor antagonist, bicuculline methiodide, (BMI, 10 µM), slowed the 5-8 Hz activity to a 2-4 Hz rhythmic activity in all slices tested (Figure 1A, 2A, n=20). Under these conditions, the frequency and duration of the rhythmic activity was very stable from trial to trial (Figure 1Aii, 2Aii). The autocorrelograms clearly indicate a highly synchronized response that lasts 4 cycles (~1.5 s) in a slice from a VPAC2⁺/+ mouse (Figure 1B, left panel, gray line) and 3 cycles (~1.5 s) in a slice from a VPAC2⁻/- mouse (Figure 1B, right panel, gray line). The interburst periods and the number of peaks of the rhythmic activity did not significantly differ in slices from VPAC2⁺/+ and VPAC2⁻/- mice (Figure 1C, D; p>0.05; Mann-Whitney U test).

We next tested the effects of VIP on intrathalamic rhythmic activity in slices from VPAC2⁺/+ mice (n=6) and from VPAC2⁻/- mice (n=5). VIP (0.5 µM, 60s duration)
dramatically suppressed the spindle-like rhythmic activity in all slices from VPAC$_2^{+/+}$ mice (Figure 2Ai, left column). This VIP concentration was used because it produced a maximal depolarization in rat thalamic relay neurons (Lee and Cox, 2003). The contour plot illustrates that the maximum effect occurred 120 s after VIP treatment, and recovered near pre-drug levels within 5 minutes following VIP application (Figure 2Aii, left column). VIP significantly reduced the number of peaks and Amp$_{osc}$ in all VPAC$_2^{+/+}$ slices (Figure 2B, p<0.05; Mann-Whitney U test). In addition to the suppression of the rhythmic activity, the interburst period was shortened from 220 ms to 150 ms after VIP application in two slices in which VIP severely attenuated but did not eliminate the rhythmic activity. Following the addition of BMI (10 µM) to transform the spindle-like rhythm to a slow wave rhythm, VIP (0.5 µM, 60 s) also dramatically suppressed the slow rhythmic activity in a reversible manner in all slices tested from VPAC$_2^{+/+}$ mice (Figure 2Ai, ii, left panel, n=7). In slices from VPAC$_2^{-/-}$ mice, this relatively high concentration of VIP (0.5 µM) did not alter the spindle-like or slow wave rhythmic activity (Figure 2Ai, ii, iii). Our population data indicate that VIP did not significantly alter the number of peaks, Amp$_{osc}$, or interburst period in slices from VPAC$_2^{-/-}$ mice (Figure 2B, p's>0.1, Mann-Whitney U test). Our data indicate that the anti-oscillatory action of VIP is mediated through the activation of VPAC$_2$ receptors.

**VIP depolarizes relay neurons in VPAC$_2^{+/+}$ slices, not in VPAC$_2^{-/-}$ slices**

We next examined the effects of VIP on thalamic relay neurons in slices from VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ mice. Intracellular recordings were obtained from 68 relay neurons from VPAC$_2^{+/+}$ mice and 61 relay neurons from VPAC$_2^{-/-}$ mice. The average resting membrane potential (VPAC$_2^{+/+}$: -67.7 ± 3.1 mV; VPAC$_2^{-/-}$: -68.7 ± 2.5 mV) and apparent input resistance (VPAC$_2^{+/+}$: 210.0 ± 82.5 MΩ; VPAC$_2^{-/-}$: 218.2 ± 87.0 MΩ) of relay neurons from VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ mice did not differ significantly (p>0.1, Mann-Whitney U test).

Similar to what we had previously observed in rat thalamic relay neurons, VIP (0.5 µM) consistently depolarizes thalamic relay neurons from wild type mice (Figure 3A). This VIP concentration (0.5 µM) depolarized all relay neurons tested from VPAC$_2^{+/+}$ mice with an average amplitude of 3.9 ± 1.5 mV (n=23). The latency to peak
of the VIP-mediated depolarization occurred 115 ± 26 s (n=23) after VIP application, and the depolarization lasted an average of 333 ± 71 seconds in 12 of 17 neurons that completely recovered to the pre-VIP membrane potential. A smaller depolarization was produced by lower VIP concentration (0.1 μM, 3.4 ± 0.8 mV, n=3, data not shown). A decrease in the apparent input resistance was commonly associated with the VIP-mediated membrane depolarization (Figure 3A). In a subset of neurons in which the membrane potential was manually repolarized to pre-drug levels during the peak of the VIP-mediated depolarization, the input resistance was decreased by 36 ± 4 % (n=4).

In slices from knock out (VPAC2−/−) animals, VIP (0.5 μM) did not alter the membrane potential in the majority of neurons (8/10 cells, Figure 3Bi); however, a much smaller depolarization was observed in 2 neurons (Figure 3Bii, 1.7 ± 0.8). The scatter plot in Figure 3C illustrates the range of responses to VIP (0.5 μM) in neurons from VPAC2+/+ (●) and VPAC2−/− (▲) animals. Clearly, the excitatory action of VIP on thalamic relay neurons is predominantly mediated via activation of VPAC2 receptors.

In our previous study in rat thalamus, we found that VIP enhances Ih in relay neurons (Lee and Cox, 2003). We wanted to extend this finding to the mouse thalamus, but more importantly, determine if the alteration of Ih is abolished in the VPAC2−/− animals. Voltage-clamp recordings were used to characterize the VIP-mediated conductance changes in relay neurons from VPAC2+/+ and VPAC2−/− animals. Long duration (2.4 s) voltage step commands (-50 to -120 mV; 10 mV increments; 0.125 Hz) were used to activate Ih in relay neurons. The current responses to the step commands consisted of an initial instantaneous response followed by a slow inward current that typically reached a steady state after a couple of seconds (Figure 4A). We calculated Ih as the difference between the initial instantaneous response (I_{ins}) and the steady state level (I_{ss}). Little Ih was evoked in response to the initial small voltage commands, but with stronger hyperpolarizing voltage commands, there was an increase in the slow inward current (Figure 4A, black line). Following VIP application (0.5 μM; 60 s duration), Ih was enhanced similar to that observed in rat thalamic relay neurons (Figure 4A, VPAC2+/+, gray lines). VIP produced a statistically significant shift in the depolarizing direction of the membrane potential at half-maximal activation in a reversible manner (6.6 ± 3.5 mV, n=5, p<0.02; paired t-test, Figure 4B VPAC2+/+). In contrast, VIP did not
alter I_h in relay neurons from VPAC2−/− mice (Figure 4A,B VPAC2−/−, n=6). It is important to note that despite our negative findings with VIP, I_h was present in the VPAC2−/− animals and was actually slightly greater in amplitude than that recorded from neurons of VPAC2+/+ animals (Figure 4C). Furthermore, the voltage dependence of I_h did not differ in the two different types of animals (Figure 4D). The membrane potential at half-maximal activation was -96.4 ± 2.0 mV (n=10) and -97.8 ± 3.0 mV (n=13) in relay neurons from VPAC2+/+ and VPAC2−/− mice, respectively (p>0.2, Mann-Whitney U test; Figure 4D). These data support the role of VPAC2 receptors underlying the VIP-mediated enhancement of I_h and membrane depolarization in thalamic relay neurons.

**PACAP38 depolarizes relay neurons in VPAC2−/− and VPAC2+/+ slices**

The fact that VIP produced a small depolarization in 2 of 10 neurons in slices from VPAC2−/− mice suggests VIP may also act on other receptor subtypes (i.e., VPAC1, PAC1). Both VPAC2 and PAC1 receptors are localized in the thalamus (Masuo et al., 1992; Hashimoto et al., 1996; Shioda et al., 1997). Considering VIP has high affinity for both VPAC1 and VPAC2 receptors, but a much lower affinity (~1000-fold less) for the PAC1 receptor, it is possible that the VIP concentrations used in our current study (0.5 µM) could also activate PAC1 receptors. But it is important to note that this VIP mediated action was only in a small minority of neurons in the VPAC2−/− slices. To probe for PAC1 receptor mediated actions, we next used the agonist, PACAP38, which has a relatively higher affinity for PAC1 receptors.

In neurons from VPAC2−/− mice, PACAP38 (0.5 µM) depolarized 10 of 11 neurons tested (Figure 5A,C). The average response to PACAP38 (0.5 µM) was 2.6 ± 0.9 mV (n = 10). Of the 10 cells tested, 5 of them were in control physiological solution, and the remaining 5 in TTX (1.0 µM). The amplitudes of the PACAP38-mediated responses did not significantly differ between these two conditions (p>0.1; Mann-Whitney U test), and thus these data have been combined. The latency to peak of the PACAP38-mediated depolarization occurred 176 ± 48 s (n = 10) after application, and the membrane potential partially recovered to pre-drug levels. Similar to VIP, the PACAP38-mediated membrane depolarization was associated with a decrease in apparent input resistance (17 ± 16%, n = 5).
Because PACAP38 has a similar affinity for VPAC2 and PAC1 receptors, PACAP38 application may equally activate both of these two receptor subtypes (Gottschall et al., 1990; Lam and Srivastava, 1990). In slices from VPAC2+/+ mice, PACAP38 (0.5 μM) produced a depolarization in 9 of 11 neurons that averaged 3.4 ± 0.9 mV (n=9; Figure 5B,C). Of the 9 cells tested, 4 of them were in control ACSF, and the remaining 5 in 1.0 μM TTX. The amplitude of the PACAP38-mediated depolarization did not differ between these two conditions, and thus these data have been combined (p>0.1, t-test). The latency to peak of the PACAP38-mediated depolarization occurred 169 ± 42 s (n = 9) after application and the membrane potential partially recovered to pre-drug levels. The PACAP38-mediated membrane depolarization was associated with a decrease in apparent input resistance (32 ± 18 %, n=5; p<0.05, paired t-test).

Considering that relay neurons from VPAC2+/+ mice may have more receptors (i.e., VPAC2, PAC1), it is interesting that the PACAP38-mediated depolarization in relay neurons from VPAC2+/+ mice did not significantly differ from that in relay neurons from VPAC2−/− mice (p>0.1; Mann-Whitney U test). Thus, we next tested whether VIP and PACAP38 share identical pathways involved in membrane depolarization. We attempted whether VIP could occlude the membrane depolarization produced by PACAP38. As illustrated in Figure 5D, VIP (0.5 μM) depolarized all relay neurons tested from VPAC2+/+ slices (n=6, 4.2 ± 1.1 mV). At the peak of VIP effect, the membrane potential was manually adjusted to resting levels and PACAP38 (0.5 μM) was applied. Under these conditions, PACAP38 produced no obvious depolarization in all cells tested (n=6; Figure 5D). Following washout of PACAP38 and VIP, a subsequent PACAP38 application (16 minute interval) produced membrane depolarizations in 4 of 6 neurons tested (n = 4, 1.9 ± 0.1 mV).

**PACAP38 enhances Ih in relay neurons from VPAC2−/− slices**

Our initial focus in this study was to identify the specific receptor subtype underlying the VIP-mediated depolarization in thalamic relay cells; however, given the small population of neurons in which VIP still depolarizes neurons in the VPAC2−/− animal, this animal model also provides a unique tool to study the role of PAC1 receptors in the thalamus. Voltage-clamp recordings were used to characterize the conductance
changes produced by PACAP$_{38}$ in relay neurons from VPAC$_2^{-/-}$ mice. Analogous to our current clamp recordings above, PACAP$_{38}$ (0.05-0.5 µM) produced an inward current that partially recovered to pre-drug holding current levels (Figure 6A). In TTX (1.0 µM), PACAP$_{38}$ (0.05-0.5 µM) produced an inward current that averaged $18 \pm 15$ pA ($n = 8$), and the time to peak of the inward current averaged $198 \pm 56$ seconds, similar to our current clamp recordings. Slow voltage command ramps (-60 mV to -110 mV, 2 s duration, 0.1 Hz) were used to determine the voltage dependence of the PACAP$_{38}$-mediated alteration in conductance (Figure 6A, B). In the pre-drug condition, the current response to the ramped voltage command is non-linear; during the ramped voltage command there is an increase in conductance at more hyperpolarized potentials, which is likely a result of $I_h$ activation. In order to quantify changes in the “resting” conductance of the neuron, we analyzed the initial portion of the current response near resting membrane potential (-60 to -80 mV). The resting conductance of the neurons prior to PACAP$_{38}$ averaged $4.7 \pm 2.1$ nS. Following PACAP$_{38}$ application, the conductance was significantly increased to $5.6 \pm 2.6$ nS ($n = 9$; p<0.02, Wilcoxon test). Of the 9 cells tested, 0.1 µM PACAP$_{38}$ was applied to 4 cells, 0.5 µM to 4 cells, and 0.05 µM to the remaining cell, and these data have been combined for this analysis.

In order to determine the voltage dependence of the conductance altered by PACAP$_{38}$, we calculated the difference in current responses before and after PACAP$_{38}$ application (Figure 6C). The PACAP$_{38}$-mediated conductance ($I_{\text{diff}}$) was usually linear over the voltage range of -60 mV to -90 mV. Extrapolating the linear fit of $I_{\text{diff}}$ indicated that the conductance sensitive to PACAP$_{38}$ had a reversal potential of $-47 \pm 4$ mV ($n = 6$, Figure 6C). These results are consistent with our previous study testing VIP on rat thalamic relay neurons (Lee and Cox, 2003). Considering our previous work demonstrating that the VIP-mediated response was completely attenuated by the $I_h$ blocker, ZD7288, we next tested whether the PACAP$_{38}$-mediated response was also sensitive to this agent. In ZD7288 (100 µM), PACAP$_{38}$ (0.5 µM) did not produce inward current, nor was there any change in the current response to the ramped voltage commands (Figure 6D,E, n=5). Our data suggest that PACAP$_{38}$ enhances $I_h$ in relay neurons from VPAC$_2^{-/-}$ animals.
Role of cAMP in VIP-mediated actions

Previous studies have demonstrated that increases in cAMP activity can enhance Ih in thalamic relay neurons (Budde et al., 1997; Lüthi and McCormick, 1999; Sun et al., 2003). Given the absence of the VIP-mediated enhancement of Ih in neurons from VPAC2<sup>+/−</sup> animals, we tested whether this pathway may have been disrupted by the genetic manipulation. Voltage steps commands were used to activate Ih in neurons of slices from VPAC2<sup>+/+</sup> and VPAC2<sup>−/−</sup> animals (Figure 7). Similar to VIP, the membrane permeable, cAMP analogue, 8-cpt-cAMP (1 mM; 60 s application) produced a statistically significant depolarizing shift in the Ih activation curve (4.8 ± 0.4 mV, n = 5) in neurons from VPAC2<sup>+/+</sup> mice (Figure 7B, p<0.01; paired t-test). In relay neurons from VPAC2<sup>−/−</sup> mice, 8-cpt-cAMP (1 mM) also produced a significant depolarizing shift in the Ih activation function (4.2 ± 1.1 mV, n = 5; p<0.02; paired t-test, Figure 7B). These results indicate that the deletion of VPAC2 receptor gene did not disrupt the modulation of Ih by cAMP, thereby suggesting that the lack of VIP mediated responses in the VPAC2<sup>−/−</sup> mice is not due to a disruption of the second messenger pathway.

Because VIP receptors are linked to G-proteins that activate adenylate cyclase, we next tested if exogenous application of membrane-permeable cAMP analogs would reproduce and/or occlude VIP-mediated membrane depolarizations. In TTX (1 μM), 8-cpt-cAMP (10-1000 μM) produced a membrane depolarization in 20 of 25 relay neurons from cells tested from VPAC2<sup>+/+</sup> mice (Figure 8A,B). 8-cpt-cAMP (100 μM) produced a reversible depolarization that averaged 1.1 ± 0.6 mV (n=9). The latency to peak of the 8-cpt-cAMP-mediated depolarization occurred 124 ± 28 s after application with an average duration of 324 ± 79 s for 6 neurons that completely recovered to pre-drug levels. The 8-cpt-cAMP depolarization was associated with a decrease in apparent input resistance (Figure 8A,B). We next determined whether the 8-cpt-cAMP-mediated depolarization was concentration dependent. Low 8-cpt-cAMP concentration (10 μM) depolarized one of four relay neurons. Increasing the 8-cpt-cAMP concentration (500 and 1000 μM) produced larger membrane depolarizations (2.2 ± 1.4 mV; n=12; Figure 8B,C). The average duration of the depolarization was 527 ± 203 s for 6 neurons that completely recovered to the pre8-cpt-cAMP membrane potential. At these higher concentrations, 8-
cpt-cAMP reduced the input resistance by 18 ± 12 % (n = 5; p<0.03). Our population data indicate a dose dependent increase in membrane depolarization by 8-cpt-cAMP (Figure 8C). In eight neurons, repeated applications of 8-cpt-cAMP, low-concentration (10 or 100 µM) followed 12 minutes later by a higher 8-cpt-cAMP concentration (100 or 1000 µM) resulted in a larger depolarization by the higher concentration in all cells (Figure 8D).

We next attempted to test whether activation of the cAMP pathway by 8-cpt-cAMP could occlude the membrane depolarization produced by VIP. As illustrated in Figure 8E, VIP (0.1 µM) depolarized a relay neuron from a VPAC₂⁺/+ slice. Following VIP washout, 8-cpt-cAMP (500 µM) was bath-applied and subsequently depolarized the neuron. The membrane potential was then manually adjusted to resting levels and VIP was reapplied in the presence of 8-cpt-cAMP. In this case, VIP produced no obvious depolarization (Figure 8E). Following washout of 8-cpt-cAMP (20 minutes), VIP once again depolarized the neuron. For this experiment, higher concentration of 8-cpt-cAMP (1000 µM) was used in four neurons in which 0.5 µM VIP was applied, and a lower concentration of 8-cpt-cAMP (500 µM) was used in the remaining four cells. In all cells tested, the response to VIP-mediated depolarization was significantly attenuated in the presence of 8-cpt-cAMP (Figure 8F, n=8, p<0.01, Wilcoxon test).
DISCUSSION

Our study provides several novel findings regarding peptide actions on thalamic neurons and intrathalamic circuit activity. First, we provide evidence that both spindle-like (5-8 Hz) and slow wave (2-4 Hz) rhythmic activity can be produced in the mouse in vitro slice preparation. VIP attenuates both types of intrathalamic rhythmic activities, and this action is mediated through the activation of VPAC₂ receptors. The VIP-mediated depolarization of relay neurons requires the activation of VPAC₂ receptors and engages cAMP-dependent mechanisms leading to the enhancement of Iₜ. Finally, we provide evidence that activation of PAC₁ receptors produces excitatory responses in thalamic relay neurons via a similar mechanism as that with VPAC₂ receptor activation. Key aspects of our findings are illustrated in Figure 9. VIP primarily binds to VPAC₂ receptors, which in turn activates adenylate cyclase and increases cytosolic cAMP concentrations leading to an enhancement of Iₜ and ultimately depolarization of the relay neurons. In addition, PACAP₃₈ depolarizes thalamic relay neurons in mice lacking the VPAC₂ receptor, presumably via activation of PAC₁ receptors. Activation of PAC₁ receptors also enhances Iₜ in a similar manner as for VPAC₂ receptors. Our current working hypothesis regarding VIP-mediated actions in the thalamus is that burst activity of VIP-containing TRN neurons, which is common during rhythmic activity, causes synaptic release of VIP. The VIP in turn activates VPAC₂ receptors on relay neurons and enhances Iₜ via a cAMP-dependent process thereby producing a membrane depolarization in relay neurons. This depolarization biases the thalamic relay neurons towards tonic firing mode thereby attenuating the rhythmic activity. Thus, VIP may act as an endogenous modulator of intrathalamic circuit activity.

Antioscillatory actions of VIP

Intrathalamic rhythmic activities result from the intrinsic properties of thalamic neurons and the reciprocal synaptic connections between thalamic relay nuclei and the TRN (Steriade and Llinás, 1988;von Krosigk et al., 1993;Steriade et al., 1993;Huguenard and Prince, 1994;Warren and Jones, 1994). Spindle-like and slow wave rhythms have been observed in vivo in many animal species, but in vitro such rhythms have clearly
been observed in ferrets, guinea pigs, and to a lesser extent in rats. Despite the presence of slow wave rhythms in mice, spindle like rhythms have not been obvious (Warren et al., 1994; Huntsman et al., 1999). Our study provides clear evidence that the mouse thalamus is clearly capable of both spindle-like rhythmic activities, and attenuating GABA_A receptor-mediated activity transformed the spindle-like rhythmic activity to a slow wave rhythmic activity similar to that observe in other animal models (Huguenard and Prince, 1994; Bal et al., 1995; McCormick et al., 1995). The presence of these intrathalamic rhythms in wild type mice allows the power of transgenic approaches to be utilized for the studies of these activities, in a manner similar to our use with the VPAC_2^-/- mouse.

In our previous study, we found that VIP attenuates slow wave rhythmic activity via an unidentified receptor subtype in rat thalamic slices (Lee and Cox, 2003). In the present study, we found that VIP attenuates both spindle-like and slow 2-4 Hz intrathalamic rhythms in slices from wild type (VPAC_2^{+/+}) mice. In slices from VPAC_2 receptor knock out animals (VPAC_2^{-/-}), VIP did not alter the rhythmic activity. These data clearly indicate that the anti-oscillatory actions of VIP are mediated via activation of VPAC_2 receptors.

The anti-oscillatory actions of VIP are likely a consequence of the depolarizing actions of VIP on thalamic relay neurons. Within mouse thalamic relay neurons, we found that VIP produced a long-lasting robust membrane depolarization. Despite numerous reports of VIP-mediated membrane depolarizations in different regions of the central nervous system, the identification of the specific receptors underlying these actions has remained elusive due to poorly selective pharmacological agents for VIP/PACAP receptors (Phillis et al., 1978; Jef tinija et al., 1982; Kohlmeier and Reiner, 1999; Liu and Morris, 1999; Lee and Cox, 2003; Sun et al., 2003). The development of this VPAC_2^{-/-} mouse may serve as a valuable tool to investigate the functional role of VPAC_2 receptors in various brain regions (Harmar et al., 2002; Aton et al., 2005). Immunocytochemical studies indicate that VPAC_2 and PAC_1 are present within thalamic nuclei, however distinguishing the functional consequences of these specific receptor subtypes has been difficult with current pharmacological agents (Usdin et al., 1994; Sheward et al., 1995; Vertongen et al., 1997; Burgunder et al., 1999).
The integral role of VPAC\textsubscript{2} receptors

The VPAC\textsubscript{2} \textsuperscript{−/−} mice have served as a very important tool in further understanding of VIP mediated actions in the thalamus. In slices from VPAC\textsubscript{2} \textsuperscript{+/+} mice, we have found that in relay neurons, VIP produces a membrane depolarization that is associated with a decreased input resistance. Voltage clamp recordings clearly indicate that VIP enhances \(I_h\), similar to what has been observed in rat thalamic neurons (Lee and Cox, 2003; Sun et al., 2003). However, in slices from VPAC\textsubscript{2} \textsuperscript{−/−} mice, the VIP-mediated depolarization in response to a relatively high VIP concentration (0.5 \(\mu\text{M}\)) is absent in 80% of the neurons, and is severely reduced in amplitude in the remaining cells. In addition, VIP does not alter \(I_h\) in relay neurons in slices from VPAC\textsubscript{2} \textsuperscript{−/−} mice. Thus, our data strongly support the notion that the excitatory actions of VIP are predominantly via VPAC\textsubscript{2} receptors. Our results differ from the conclusions of Sun et. al. (2003) in which they concluded that the predominant action of VIP/PACAP was due to PAC\textsubscript{1} receptors based on 1) the concentration dependence of their agonists responses (PACAP more potent than VIP), and 2) the VPAC\textsubscript{1} receptor agonist, [Ala\textsuperscript{11,22,28}] VIP, did not depolarize thalamic relay neurons suggesting the lack of VPAC\textsubscript{1} receptor mediated actions on thalamic relay neurons (Nicole et al., 2000; Sun et al., 2003). Our results strongly indicate the role of the VPAC\textsubscript{2} receptor unless there is a complete remodeling of the VIP/PACAP receptor distribution as a result of the VPAC\textsubscript{2} knockout, but such a remodeling has not been observed in other brain regions of these VPAC\textsubscript{2} \textsuperscript{−/−} mice and thus would be specific only to the thalamic nuclei (Aton et al., 2005).

As mentioned above, despite the majority of relay neurons from VPAC\textsubscript{2} \textsuperscript{−/−} mice did not respond to VIP, in the remaining 20% of neurons, VIP produced a smaller depolarization. Considering VIP has a lower affinity for PAC\textsubscript{1} receptors relative to VPAC\textsubscript{2} receptors, the VIP concentration (0.5 \(\mu\text{M}\)) used in this study could possibly activate PAC\textsubscript{1} receptors. Our finding supports such a conclusion because the agonist, PACAP\textsubscript{38}, which has a higher affinity for PAC\textsubscript{1} receptors than VIP, depolarized the majority of neurons from VPAC\textsubscript{2} \textsuperscript{−/−} mice. In light of the anatomical data indicating the presence of VPAC\textsubscript{2} and PAC\textsubscript{1} receptors in thalamic relay nuclei, the VPAC\textsubscript{2} \textsuperscript{−/−} mice serve as a potentially useful model to understand PAC\textsubscript{1}-mediated actions within thalamic neurons. We found that PACAP\textsubscript{38} not only depolarized thalamic neurons from VPAC\textsubscript{2} \textsuperscript{−/−}
mice, but our voltage clamp recordings also reveal that PACAP$_{38}$, also increases the conductance of relay neurons and appears to also enhance I$_h$, similar to that observed by VIP via VPAC$_2$ receptors. Thus, our data clearly indicate that activation of PAC$_1$ receptors can enhance I$_h$, but perhaps more importantly suggest that these two different receptors may converge onto a common effector mechanism to alter the excitability of thalamic neurons. Consistent with this idea, our data strongly suggest that VIP occludes PACAP38-mediated membrane depolarization in thalamic neurons from VPAC$_2^{+/+}$ mice.

**The dependence of VIP-mediated response on cAMP**

VIP-sensitive receptors are G-protein dependent receptors that have been found to engage cAMP synthesis as well as inositol phosphate turnover pathways (Ishihara et al., 1992; Deutsch and Sun, 1992; Spengler et al., 1993; Van Rampelbergh et al., 1997; MacKenzie et al., 2001). In addition, hyperpolarization-activated cyclic nucleotide-gated (HCN) channels that underlie I$_h$, are modulated by alteration in cAMP concentrations (Beaumont and Zucker, 2000; Wainger et al., 2001). Several neuromodulators within the thalamus (e.g., norepinephrine, serotonin, VIP, PACAP), which activate receptors coupled to cAMP dependent pathways, enhance I$_h$ and thereby depolarize thalamic neurons (present study, (McCormick and Pape, 1990; Pape, 1996; Sun et al., 2003; Frere and Luthi, 2004). Our data demonstrate that cAMP analogues enhance I$_h$, and produce membrane depolarizations similar to VIP. Furthermore, we have found that activation of cAMP can occlude the VIP-mediated membrane depolarization indicating the dependence of the depolarizing action on cAMP dependent processes. Our findings are consistent with previous reports that VPAC$_2$ receptors are primarily coupled to cAMP dependent pathways (Lutz et al., 1993; Lutz et al., 1999; Itri and Colwell, 2003).

**Functional role of VIP/PACAP in the thalamus**

Thalamic neurons discharge action potentials in two basic firing modes: tonic and burst. The firing mode of thalamic neurons is dependent on the low threshold, transient calcium current, I$_T$ (Jahnsen and Llinás, 1984a; Steriade and Llinás, 1988; Crunelli et al., 1989). At relatively hyperpolarized membrane potentials, channels underlying I$_T$ are deinactivated, and with membrane depolarization, I$_T$ can be activated giving rise to a
transient Ca\(^{2+}\)-dependent depolarization, a low threshold calcium spike (LTS), upon which a short-duration (100-200 ms), high frequency burst (>200 Hz) of Na\(^+\)-dependent action potentials can occur (Jahnsen and Llinás, 1984a; Steriade and Llinás, 1988; Crunelli et al., 1989). At relatively depolarized membrane potentials, \(I_T\) is inactivated, and subsequent depolarization of the membrane potential will result in tonic action potential discharge at a frequency that is relatively linear with the degree of membrane depolarization (Jahnsen and Llinás, 1984a; Jahnsen and Llinás, 1984b; Huguenard and McCormick, 1992; Zhan et al., 1999).

Several neuromodulators depolarize thalamic neurons, predisposing the neurons to a tonic mode and thereby terminating the intrathalamic rhythmic activity. Interestingly, many of these modulators alter the excitability of thalamic neurons via common effector mechanisms. Two obvious candidates are the resting K current, \(K_{\text{leak}}\), and \(I_h\). Within thalamic neurons, acetylcholine, glutamate (via metabotropic receptors), norepinephrine, serotonin, cholecystokinin, as well as histamine depolarize thalamic neurons by decreasing a resting leak potassium current, and thereby alter the action potential discharge mode of these neurons and ultimately terminating the rhythmic activity (McCormick and Prince, 1986; McCormick and Prince, 1987; McCormick and Prince, 1988; McCormick, 1989; McCormick and Williamson, 1991; McCormick and von Krosigk, 1992; McCormick, 1992; Cox et al., 1995; Cox et al., 1997; Cox and Sherman, 1999). Furthermore, norepinephrine, serotonin, and histamine have been found to enhance \(I_h\), leading to membrane depolarization and ultimately terminating the intrathalamic oscillation (Pape and McCormick, 1989; McCormick and Pape, 1990; McCormick and Williamson, 1991). Our data strongly indicate VIP and PACAP\(_{38}\) depolarize relay neurons via an enhancement of \(I_h\) (Lee and Cox, 2003; Sun et al., 2003). A common aspect of these modulators is their ability to engage particular intracellular second-messenger pathways, namely cAMP, which ultimately alters \(I_h\).

Despite sharing a common effector mechanism, a key to understanding the functional aspects of VIP and PACAP likely depends on the source of these peptides. VIP-containing neurons are in the thalamic reticular nucleus and therefore VIP is endogenous to the intrathalamic circuit, and its release is likely correlated to intrathalamic circuit activity (Burgunder et al., 1999). Other neuropeptides (e.g.,
cholecystokinin, somatostatin, neuropeptide Y, and VIP) can also alter the firing mode of thalamic neurons and attenuate intrathalamic rhythmic activity (Cox et al., 1997; Leresche et al., 2000; Sun et al., 2002; Lee and Cox, 2003; Sun et al., 2003). Similar to VIP, these other neuropeptides are localized within the thalamic neurons and may play a potentially important role in regulating thalamic neuron excitability, but further studies are needed. In contrast to the above-mentioned neuropeptides, PACAP-containing neurons that innervate the thalamus originate from brain stem regions in a somewhat similar manner as other known modulators such as acetylcholine, norepinephrine, serotonin, and histamine (Hashimoto et al., 1996; Shioda et al., 1997; Hannibal, 2002). The activity of neurons in these areas is closely associated with levels of arousal and sleep-wake states (Steriade et al., 1993; McCormick and Bal, 1997). Thus, the functional distinction between peptides such as VIP and PACAP, which appear to share common cellular mechanisms, likely involves their sites of origin. In the case of these two peptides, VIP appears to be endogenous to the thalamic circuitry whereas PACAP would presumably be released by increase activity of brainstem neurons.

Our working hypothesis is that high frequency burst activity of TRN required for intrathalamic rhythmic activity may be optimal for peptide release and therefore produce the release of VIP. This synaptically released VIP activates VPAC$_2$ receptors on relay neurons, increases cAMP activity, enhancing $I_h$, and thereby depolarizes the relay neurons. This depolarization and decrease of membrane resistance biases the cells to tonic-discharge mode and may contribute to the termination of rhythmic activity. We speculate that VIP acts as an intrinsic modulator of intrathalamic oscillation that could regulate the duration of rhythmic activity. We predict that overall decreases in VIP release could lead to prolonged durations of intrathalamic rhythmic activities, similar to that during absence seizure activity. The role of neuropeptides in the thalamus may not only influence rhythmic activities, but considering their long-lasting actions, these compounds may also play an important role in regulating the overall information transfer through thalamocortical circuits.
FIGURE LEGENDS

**Figure 1:** Intrathalamic rhythmic activity recorded from *in vitro* thalamic slices from VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ mice. **A.** Multiple-unit extracellular recordings in VB from slices of VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ animals. **Ai.** In normal ACSF, single stimulus (•) in internal capsule evokes a 5–8 Hz rhythmic activity. Three consecutive sweeps are presented in each condition. **Aii.** The addition of BMI (10 µM) transforms the spindle-like rhythm into a slower 2–4 Hz rhythm. **B.** Autocorrelogram of experiment in A illustrating the highly synchronized rhythmic responses in normal ACSF (black trace) and BMI (gray trace). **C, D.** Summary of the characteristics of spindle-like and slow intrathalamic rhythmic activity recorded from *in vitro* thalamic slice from VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ mice. The number in each bar indicates the number of slices tested. Error bars represent standard deviation. *, p<0.05.

**Figure 2:** VIP strongly attenuates intrathalamic oscillations in VPAC$_2^{+/+}$ slices, but not in VPAC$_2^{-/-}$ slices. **Ai.** In control conditions, single stimulus (•) in internal capsule evokes 5–8 Hz rhythmic activities in slices from VPAC$_2^{+/+}$ and VPAC$_2^{-/-}$ mice. VIP (0.5 µM) strongly attenuates this oscillation in VPAC$_2^{+/+}$ slices; but does not alter activity in VPAC$_2^{-/-}$ slices. The addition of BMI (10 µM) transforms the fast rhythmic activity to a slower 2–4 Hz rhythmic activity in slices from both animal types. VIP strongly attenuates this slow oscillation in VPAC$_2^{+/+}$ slices, but not in VPAC$_2^{-/-}$ slices. **Aii.** Contour plots of experiments in Ai illustrating the time course of VIP effect on intrathalamic rhythmic activity. **Aiii.** The degree of synchrony and duration of the rhythmic activity were quantified from autocorrelograms constructed from the raw data. The autocorrelograms illustrate the synchronized rhythmic activity in prior to (black trace) and following (gray trace) VIP application. **B.** Summary of VIP effects on number of peaks, oscillation amplitude (Amposc), and oscillation period as calculated from the autocorrelograms. The number in each bar indicates the number of slices tested. *, p<0.05.

**Figure 3:** VIP-mediated depolarization dependent on VPAC$_2$ receptor. **A.** In a VB neuron from a VPAC$_2^{+/+}$ animal, intracellular recording reveals that VIP (0.5 µM) evokes a long lasting, membrane depolarization associated with a decrease in input resistance.
The downward deflections are membrane responses to hyperpolarizing current steps (500 ms duration) and are used to quantify changes in apparent input resistance of the neuron.

**Bi.** In a VB neuron from VPAC$_2^{+/−}$ animal, VIP (0.5 μM) does not alter the membrane potential or input resistance. **Bii.** In a different VB neuron, VIP (0.5 μM) produces a small depolarization. **C.** Scatter plot illustrating the VIP-mediated change in membrane potential in VB neurons from VPAC$_2^{+/+}$ (n = 23) and VPAC$_2^{−/−}$ (n = 10) animals. The open symbols denote specific cells illustrated in A and Bii.

**Figure 4:** VIP enhances $I_h$ in relay neurons from VPAC$_2^{+/+}$, not VPAC$_2^{−/−}$ mice. **A.** To test the VIP actions on $I_h$ activation, membrane currents are evoked by voltage commands (-50 mV to -120 mV, 10 mV increments) from an initial $V_{hold}$ of -50 mV. In control conditions (VPAC$_2^{+/+}$ and VPAC$_2^{−/−}$; black traces), increasing amplitude of hyperpolarizing voltage commands evokes a larger long latency inward current. VIP (0.5 μM, gray traces) increases the current response to mid-ranged voltage commands (-80 to -110 mV) in VPAC$_2^{+/+}$ slices. VIP produced no detectable changes in $I_h$ of VPAC$_2^{−/−}$ slices. **B.** The data were quantified by calculating the difference in steady state current near the end of the voltage command ($I_{ss}$ in A) and instantaneous current ($I_{ins}$ in A). There is a clear increase in putative $I_h$ in the range of -80 mV to -100 mV (VPAC$_2^{+/+}$; n = 5). However, there is no increase in VPAC$_2^{−/−}$ (n = 6). **C.** Summary of the amplitude of $I_h$ in relay neurons from VPAC$_2^{+/+}$ (n = 10) and VPAC$_2^{−/−}$ slices (n = 13). **D.** Summary of the voltage dependence of $I_h$ in relay neurons from VPAC$_2^{+/+}$ (n = 10) and VPAC$_2^{−/−}$ slices (n = 13).

**Figure 5:** PACAP$_{38}$ depolarizes relay neurons in VPAC$_2^{−/−}$ and VPAC$_2^{+/+}$ slices. **A.** Intracellular recording from a neuron in VPAC$_2^{−/−}$ slice reveals that PACAP$_{38}$ (0.5 μM) evokes a long lasting, membrane depolarization associated with a decrease in input resistance. **B.** In a different relay neuron from VPAC$_2^{+/+}$ mice, PACAP$_{38}$ (0.5 μM) evokes a similar depolarization. **C.** Scattered plot of PACAP$_{38}$-mediated membrane depolarizations produced in VPAC$_2^{−/−}$ and VPAC$_2^{+/+}$ slices. The responses of cells in A and B are indicated by the open symbols. **D.** VIP occludes PACAP$_{38}$-mediated membrane depolarization. VIP (0.5 μM) produces a depolarization in a relay neuron from VPAC$_2^{+/+}$
slice. At the peak of VIP-mediated depolarization, the membrane potential is manually adjusted to resting levels. PACAP38 (0.5 µM) produces no changes in membrane potential 3 minutes after washout of VIP. PACAP38 is re-applied 16 minutes following the first PACAP38 and produces a depolarization in this neuron.

**Figure 6:** PACAP38 enhances I_h in relay neurons from VPAC₂⁻/⁻ mice. A. PACAP38 produces an inward current associated with increases in membrane conductance in a relay neuron from a VPAC₂⁻/⁻ slice. In voltage clamp recordings from a VB neuron, slow ramped voltage commands (-60 to -110 mV, 4 s duration) are used to measure conductance before and after agonist application. PACAP38 (0.5 µM) produces a small inward current in a partially reversible manner. B. Expanded traces of the membrane response to the ramped voltage command reveal not only the inward current, but also the conductance increase by PACAP38 (gray trace). Each trace consists of an average of 3 subsequent responses prior to and at the peak of the PACAP38-mediated inward current. C. The difference between the PACAP38 (B, gray trace) and Pre-drug (B, black trace) is indicative of the PACAP38-sensitive current (I_diff). Extrapolation of the linear portion of this current indicates that the PACAP38-mediated current has a reversal potential (E_rev) of -44 mV. The dotted line represents the slope calculated from the linear fit of current response. It is important note that the linear portion of I_diff in the PACAP38-sensitive current ranged from -60 to -90 mV, but this could vary across neurons. D. ZD7288 attenuates the PACAP38 receptor-mediated inward current in VPAC₂⁻/⁻ slices. In TTX (1.0 µM) and ZD7288 (100 µM), PACAP38 produces neither an inward current nor change in membrane conductance. E. The difference between the PACAP38 and pre-drug clearly indicates the lack of PACAP38 effect in ZD7288.

**Figure 7:** Cyclic AMP analogues enhance I_h in VPAC₂⁻/⁻ neurons and VPAC₂⁺/+ neurons. A. To test cAMP analogues actions on I_h, membrane currents are evoked by voltage commands (-50 mV to -120 mV, 10 mV increments) from V_hold of -50 mV. In control conditions (VPAC₂⁺/+ and VPAC₂⁻/⁻; black traces), increasing amplitude of hyperpolarizing voltage commands evokes a larger long latency inward current. The membrane permeable cAMP analogue, 8-cpt-cAMP (1 mM) enhances I_h in VPAC₂⁺/+
neurons as well as VPAC₂⁺/⁻ neurons. B. Data were quantified by calculating the difference in steady state current near the end of the voltage command (Iₘₜ in A) and instantaneous current (Iₘₜ in A). There is a clear increase in putative Iₜ in the range of -80 mV to -100 mV in VPAC₂⁺/+ neurons (n = 5) and VPAC₂⁻/⁻ (n = 5).

**Figure 8:** cAMP analogue depolarizes relay neurons in VPAC₂⁺/+ slices. A. In TTX, 8-cpt-cAMP (0.01, 0.1 mM) produces small reversible membrane depolarizations in relay neurons from VPAC₂⁺/+ slices. B. In a different neuron, 8-cpt-cAMP (0.1, 1 mM) produces small depolarizations. C. Summary of 8-cpt-cAMP-mediated membrane depolarizations in VB neurons. Cell counts for each concentration are listed in parentheses. D. Scatter plot indicating the effect of multiple 8-cpt-cAMP doses to individual neurons. In eight neurons, low 8-cpt-cAMP concentration (0.01, 0.1 mM) application was followed 10 minutes later by a higher 8-cpt-cAMP concentration (0.1, 1 mM) application. Note the dose-dependent increases in depolarizations produced by 8-cpt-cAMP. E. cAMP analogue occludes VIP-mediated membrane depolarization. VIP produces a reversible depolarization in a relay neuron from VPAC₂⁺/+ slice. After washout of VIP, 8-cpt-cAMP (0.5 mM) is applied and depolarizes the neuron. The membrane potential is manually adjusted to resting levels and VIP is applied again. This time VIP produces no obvious depolarization. Following washout of 8-cpt-cAMP, VIP once again depolarizes the neuron. F. Summary of VIP-mediated membrane depolarization in thalamic neurons. Cell counts for each condition are listed in parenthesis.

**Figure 9:** Simplified schematic illustrating VIP/PACAP receptors in relay neurons from VPAC₂⁻/⁻ and VPAC₂⁺/+ mice and their common actions on Iₜ. In relay neurons from VPAC₂⁺/+ mice, VIP predominantly binds to VPAC₂ receptors that activate adenylate cyclase, leading to an increase of cyclic AMP concentration in cytosol and ultimately enhancing Iₜ. However, PACAP₃₈ activates PAC₁ and VPAC₂ receptors with the similar affinity and enhances Iₜ via cyclic AMP-dependent second messenger systems. In relay neurons from VPAC₂⁻/⁻ mice, VIP binds PAC₁ receptors with a low affinity and do not enhances Iₜ. However, PACAP₃₈ exerts excitatory actions on relay neurons from VPAC₂⁻
mice presumably via PAC1 receptors. Note thick arrows indicate high affinity or strong activation and thin arrows indicate low affinity or weak activation. Abbreviations. AC, adenylate cyclase; cAMP, cyclic AMP; HCN, hyperpolarization activated cyclic nucleotide-gate channels.
Reference List


Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9