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NPY signaling through Y₁ receptors modulates thalamic oscillations

Julia Brill, Gunnar Kwakye, and John R. Huguenard

Department of Neurology and Neurological Sciences, Stanford University School of Medicine, 300 Pasteur Drive, Room M016, Stanford, CA 94305

Abstract

Neuropeptide Y is the ligand of a family of G-protein coupled receptors (Y₁ to Y₆). In the thalamus, exogenous and endogenously released NPY can shorten the duration of thalamic oscillations in brain slices from P13 to P15 rats, an in vitro model of absence seizures. Here, we examine which Y receptors are involved in this modulation. Application of the Y₁ receptor agonist Leu₃₁Pro₃₄NPY caused a reversible reduction in the duration of thalamic oscillations (−26.6 ± 7.8%), while the Y₂ receptor agonist peptide YY_(3–36) and the Y₅ receptor agonist BWX-46 did not exert a significant effect. No Y receptor agonist affected oscillation period. Application of antagonists of Y₁, Y₂ and Y₅ receptors (BIBP3226, BIIE0246 and L152,806, respectively) produced results consistent with those obtained from agonists. BIBP3226 caused a reversible disinhibition, an effect that increases oscillation duration (18.2 ± 9.7%) while BIIE0246 and L152,806 had no significant effect. Expression of NPY is limited to neurons in the reticular thalamic nucleus (nRt), but Y₁ receptors are expressed in both nRt and adjacent thalamic relay nuclei. Thus, intra-nRt or nRt to relay nucleus NPY release could cause Y₁ receptor mediated inhibition of thalamic oscillations.

Keywords

Neuropeptide Y; absence seizures; spike-wave discharges; nRt; thalamus; thalamic oscillations

1. INTRODUCTION

Neuropeptide Y is abundantly expressed throughout the CNS [1] and involved in a multitude of physiological and neuronal processes, such as feeding behavior, angiogenesis, neuronal proliferation [8,9,11] and the modulation of seizure activity. In the hippocampus, increased NPY expression is thought to be a compensatory effect of kindling and epileptogenesis [19, 22,30,31], *in vivo* experiments in GEARS rats demonstrate that NPY suppresses absence seizures [23] and the anticonvulsant valproic acid has been proposed to act at least in part via an upregulation of NPY expression in hippocampus and nRt [2].

NPY signals through a family of six Y receptors (Y₁ – Y₆, reviewed in 7). In thalamic neurons, Y₁ receptor activation leads to opening of postsynaptically located inwardly rectifying K⁺ (GIRK) channels, which results in a long lasting hyperpolarization. Y₂ receptor activation

¹ Corresponding author: John R. Huguenard, 300 Pasteur Drive, Room M016, Stanford, CA 94305, e-mail: john.huguenard@stanford.edu, Phone: 650-723-5522, Fax: 650-723-1080

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causes inhibition of presynaptic calcium channels. Both mechanisms decrease neuronal excitability [24,26].

Thalamic relay neurons and interneurons of the nucleus reticularis thalami (nRt) are reciprocally connected and can generate and sustain network oscillations [Fig. 1A; reviewed in 13,15]. Relay cells, which are glutamatergic, can cause excitation of nRt neurons. nRt neurons in turn send inhibitory synaptic output back to relay cells. The resulting inhibition leads to rebound excitation through deinactivation of T-type calcium channels, which causes a new cycle of nRt-relay-excitation. Thalamic oscillations underlie both sleep spindles and spike wave seizures. Blockade of GABA_A receptors transforms sleep spindle like oscillations into slower, spike-wave like activity. Experimentally, ~3 Hz, spike-wave like oscillations can readily be evoked in thalamic slices from young rats (P13–P15) by delivering single extracellular stimuli to the internal capsule, the main corticothalamic and thalamocortical fiber tract [14]. 3 Hz thalamic oscillations are an *in vitro* model of absence (spike-wave) seizures in rats as well as in primates [28,29]. Indispensable components for generation of these oscillations are GABA_B and AMPA receptors as well as T-type calcium channels [4], however, many other factors modulate oscillatory activity, *e.g.* HCN channels, neuropeptides and electrical synapses [3,16,17,18,27,34]. Neuropeptide Y (NPY) is an inhibitor of absence seizures [23] and thalamic oscillations [25]. Subsets of NPY receptors (Y₁, Y₂ and Y₅ receptors) are expressed in nRt as well as relay nuclei [20,33].

In this report we investigate the involvement of the three Y receptor subtypes expressed in thalamus in the modulation of thalamic oscillations. We have previously reported that Y₁ receptor blockade disinhibits thalamic network oscillations [25]. Little is known about the effectors of Y₅ receptor activation. The fact that they are expressed in thalamic nuclei makes them a potential modulator of thalamic network oscillations.

2. MATERIALS AND METHODS

2.1. Slice preparation

Experiments were conducted according to protocols approved by the Stanford Institutional Animal Care and Use Committee. Rats (postnatal day 12–15) were deeply anesthetized with 50 mg/kg sodium pentobarbital and decapitated. Brains were transferred into ice cold sucrose solution containing, in mM: 234 sucrose, 11 glucose, 24 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, and 0.5 CaCl₂, equilibrated with 95 O₂ / 5% CO₂. 400 μm horizontal slices were cut on a VT 1000S vibratome (Leica) at 4°C in sucrose solution and trimmed to exclude hippocampus and cortex. Slices were incubated in a holding chamber filled with artificial cerebrospinal fluid (ACSF) containing, in mM: 126 NaCl, 26 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 2 MgCl₂, and 10 glucose, equilibrated with 95% O₂ / 5% CO₂, pH 7.4 at 32°C for 1 h and subsequently in the same solution at room temperature.

2.2. Extracellular recordings

Extracellular recordings were performed as described previously [14,25]. Briefly, thalamic slices were transferred to an interface recording chamber and superfused with modified ACSF containing 1 mM MgCl₂, 0.02 mM bicuculline methiodide and 0.5 mM L-glutamine (both Sigma). After waiting at least 5 minutes to allow slices to equilibrate, extracellular multiple-unit activities in nRt were recorded using a monopolar tungsten electrode (0.2–2 MΩ; Frederick Haer) and a Grass amplifier (bandwidth 0.03–3 kHz). Data were digitized at 10 kHz and stored using pClamp software (Axon Instruments). Single extracellular 40 s stimuli were delivered to the internal capsule through a bipolar sharpened tungsten electrode at intervals between 30 and 60 seconds. Y receptor agonists and antagonists were bath applied at 600 nM (agonists) or 1 μM (antagonists) for 5 to 10 minutes followed by drug washout for at least the same amount

of time. Agonists used were: Leu₃₁Pro₃₄NPY (Y₁ receptor agonist, Tocris), Peptide YY_{3–36} (PYY_{3–36}, Y₂ receptor agonist, Tocris) and BWX-46 (Y₅ receptor agonist, Tocris). Antagonists used were: BIBP3226 (Y₁ receptor, Bachem), BIIE0246 (Y₂ receptor, Tocris), L152,806 (Y₅ receptor, Tocris).

2.3. Data analysis

The degree of synchrony and the duration of thalamic oscillations were calculated from autocorrelograms constructed from the spikes count and fitted using a modified Gabor function:

$$y = A_G (e^{|x|/\tau_G})^{1.5} (\cos(x\pi/p))^6 + A_R e^{|x|/\tau_R}$$

A_G, A_R: amplitudes of oscillatory and non-oscillatory components; τ_G, τ_R: decay constants of oscillatory and non-oscillatory components; p: period of the oscillatory component, x: time. Recordings in which τ_G during washout was not within 66 to 140 % of baseline (ACSF) conditions were rejected.

2.4. Immunohistochemistry

Rats were deeply anesthetized with 50 mg/kg sodium pentobarbital and transcardially perfused with 100 ml saline, followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. After postfixing overnight at 4°C in paraformaldehyde and cryoprotection in 30% sucrose for up to 3 days at 4°C, brains were sectioned at 40 μm on a cryotome (HM 400, Microm). Sections were processed as described previously [25]. Briefly, free floating sections were rinsed in 75% ethanol and PBS prior to blocking in 10% normal goat serum/PBS for 2 hours. Next, sections were incubated on a rotary shaker for 16 hr at 4°C in rabbit polyclonal NPY or Y₁ receptor antiserum (1:1000, Sigma) and monoclonal mouse NeuN antibodies (1:1000, Chemicon) in PBS/0.2% Triton X-100. Finally, sections were incubated at room temperature with fluorescence labeled goat anti rabbit (Alexa Fluor 488, 2 g/ml, Invitrogen) and goat anti mouse IgG antibodies (Alexa Fluor 568, 2 g/ml, Invitrogen) for 1 hour. Sections were mounted and coverslipped and images were acquired using a laser confocal microscope (LSM 510 Confocal Laser Scanning Microscope, Zeiss).

3. RESULTS

We assessed the effects of Y receptor agonists and antagonists on thalamic oscillations in horizontal slices from P13–P15 rats. An extracellular recording electrode was placed in nRt and oscillations evoked by single 40 s pulses to the internal capsule.

Here, we refer to the repetitive alternating firing of thalamocortical relay and nRt neurons as “thalamic oscillations”. These oscillations are made up of bursts of spikes that correspond to the near synchronous firing of populations of neurons. Oscillations can be described on the basis of their amplitude, duration and period. Autocorrelograms were calculated from extracellular spike peristimulus time histograms and fitted to a modified Gabor equation (see Materials and Methods) to quantify these parameters. The amplitude of an oscillation is the initial fitted value of the oscillatory component and its values correlates with the numbers of spikes within bursts. Oscillations grow weaker over time and eventually stop (see traces in Fig 2 and 3 A₂, B₂ and C₂). In the autocorrelogram, this is reflected in decrease in the amplitude of subsequent peaks and is fitted to a single exponential decay. We used the decay constant, τ, as a measure of oscillation duration. The oscillation period is the time between bursts; i.e. the inverse of frequency. A shortened period corresponds to shorter intervals between bursts and thus a faster oscillation.

3.1. Y receptor agonists

We used specific peptide agonists of Y_1 receptors (Leu₃₁Pro₃₄NPY), Y_2 receptors (PYY₃₋₃₆) and Y_5 receptors (BW_X-46). After recording 10 to 20 evoked oscillations under baseline conditions, we superfused slices with ACSF containing specific Y receptor agonists and recorded at least 10 evoked oscillations under these conditions. Finally, we recorded a minimum of 20 evoked oscillations while superfusing control ACSF to ensure that changes in oscillation duration were reversible, i.e. not due to rundown or other changes within the slice. Fig. 2A1, B1 and C1 show contour plots that depict all oscillations in a representative recording for each of the three Y receptor agonists. Fig. 2A2, B2 and C2 shows representative sweeps within these recordings for each of the three conditions, i.e. a baseline oscillation (bottom), an oscillation in the presence of respective agonists (middle; Y_1 in Fig. 2A2, Y_2 in Fig. 2B2, Y_5 in Fig. 2C2), and during agonist washout (top). Application of the Y_1 receptor agonist Leu₃₁Pro₃₄NPY resulted in shortening of the oscillation, while application of the Y_2 receptor agonist PYY₃₋₃₆ and the Y_5 receptor agonist BW_X-46 had no obvious effect. We used agonist concentrations of 0.6 M in the experiments shown here. Leu₃₁Pro₃₄NPY had similar effects on thalamic oscillations at concentrations between 0.2 and 1.0 M (data not shown). Fig. 2D shows oscillation decay constants of all relevant recordings, normalized to baseline. Leu₃₁Pro₃₄NPY caused a reversible reduction in the oscillation decay in all cases, while no significant trend could be discerned for PYY₃₋₃₆ and BW_X-46. Fig 2E1 depicts a summary of the results, and shows a significant reduction in oscillation decay time constant in the presence of Leu₃₁Pro₃₄NPY ($-26.6 \pm 7.8\%$, $p < 0.01$, $n = 6$), but no significant changes in the presence of PYY₃₋₃₆ and BW_X-46. Oscillation amplitude, another measure of oscillatory strength, was not significantly altered in the presence of Y_1 , Y_2 or Y_5 receptor agonists and showed a much higher degree of apparently random variation during recordings. Leu₃₁Pro₃₄NPY application decreased oscillatory amplitude ($-18.0 \pm 18.8\%$), unlike under any other condition, however, this trend was not significant (Fig. 2E2). Oscillation period, a third major indicator of oscillatory behavior, remained nearly constant under all experimental conditions (Fig. 2E3), including during the significantly shortened oscillations in the presence of Leu₃₁Pro₃₄NPY. Thus, only Y_1 receptor activation had a significant inhibitory effect on the duration of evoked thalamic oscillations under the conditions assayed.

3.2. Y receptor antagonists

Exogenously applied receptor agonists are present at potentially non-physiological concentrations uniformly throughout the slice and thus are not necessarily representative of the degree and location of endogenous receptor activation. We have previously shown that NPY is released endogenously during thalamic oscillations and that Y_1 receptor blockade results in lengthening of evoked oscillations, i.e. thalamic disinhibition [25]. These data are consistent with the shortening of evoked oscillations upon Y_1 receptor activation reported here. Here, we expand on these results by also including Y_2 and Y_5 receptor antagonists. Experiments were carried out under identical conditions as those outlined above, using the following antagonists at concentrations of 1 M: BIBP3226 (Y_1 receptor antagonist), BIIE0246 (Y_2 receptor antagonist) and L152,806 (Y_5 receptor antagonist). Antagonist concentrations between 0.5 and 2 M did not result in a qualitative alteration of their effects on thalamic oscillations (data not shown). Fig. 3A1, B1 and C1 show contour plots of representative recordings for the three antagonists; Fig. 3A2, B2 and C2 show single oscillations under all experimental conditions. Results were consistent with our previously reported data and with the agonist data reported above. Y_1 receptor blockade resulted in significant increase of the oscillation decay constant ($+18.2 \pm 9.7\%$, $p < 0.05$, $n = 5$) while Y_2 and Y_5 receptor blockade had no significant effects (Fig. 3D and 3E1). Oscillation amplitude was again highly variable and showed a trend towards increase upon BIBP3226 application ($+32.8 \pm 14.7\%$) which was not statistically significant (Fig. 3E2). Oscillation period remained constant under all

experimental conditions (Fig. 3E3). We conclude that endogenously released NPY modulates thalamic oscillations chiefly via Y_1 receptors.

3.3. Y_1 receptor expression in nRt and relay nuclei

We examined NPY and Y_1 receptor expression in nRt and thalamic relay nuclei. NPY was found to be expressed in virtually all neurons within the nRt (Fig. 4A), but no significant expression was found in thalamic relay nuclei [2], which is consistent with the notion that NPY is generally expressed by GABAergic interneurons. Y_1 receptors were found to be expressed at intermediate levels in nRt neurons and at higher levels in neurons of relay nuclei (Fig. 4B). Thus, either intra-nRt or nRt to relay neuron connections could be responsible for Y_1 receptor mediated inhibition of thalamic oscillations.

4. DISCUSSION

We show that modulation of 3 Hz thalamic oscillations by NPY occurs mainly via Y_1 receptor activation, which causes a shortening of evoked oscillations, but no significant changes in oscillation amplitude or period. Previous results by our group have shown that in thalamic nuclei Y_2 receptor activation inhibits presynaptic calcium channels. As a consequence, reduced neurotransmitter release depresses neuronal excitability [24,26]. Here, we found that neither Y_2 receptor agonists nor antagonists modulated the duration of thalamic oscillations. We cannot however rule out more subtle effects on oscillation parameters exerted by activation of Y_2 (or Y_5) receptors, or effects on parameters we did not assay for. Among those might be the initiation or spread of oscillations [6]. Furthermore, these receptors might play different roles *in vivo*, as circuitry relevant for seizure modulation may not be preserved in the thalamic slice preparation used here.

NPY was shown to be expressed exclusively by nRt neurons, while Y_1 receptors were expressed by both nRt and relay neurons. There are thus two main mechanisms by which Y_1 receptor activation might influence the duration of thalamic oscillations. Firstly, NPY released from nRt onto nRt neurons might activate GIRK channels [24,26]. The resulting hyperpolarization would decrease the likelihood that AMPA receptor mediated EPSPs will depolarize nRt neurons above threshold for action potential generation (Fig. 1B). Consistent with this hypothesis, GluR2 knockout mice have a decreased susceptibility to pharmacologically evoked absence seizures [12] and AMPA receptor antagonism reduces the incidence of spike wave discharges in rats [21].

The second potential mechanism involves NPY released by nRt neurons onto relay cells. Again, Y_1 receptor activation would result in hyperpolarization; similar to the concurrent $GABA_B$ receptor activation, but potentially with a slower and longer time course [25]. Thus, after the $GABA_B$ receptor mediated IPSP has decayed, Y_1 receptor activation might keep the membrane potential below the threshold for T-type calcium channel activation. This effect would prevent rebound excitation (Fig. 1C) or might cause the rate of relay cell depolarization to be too low for T-type calcium channel activation [10]. Low threshold calcium channels are concentrated on relay cell dendrites [5], and nRt axons synapse onto relay cell dendrites [32], which could allow for effective T-type calcium channel modulation.

Our results underscore that NPY can function as an endogenous anticonvulsant in spike-wave seizures of absence epilepsy [2,23,25]. Understanding the modulatory action of NPY and specifying which Y receptors mediate a given effect contributes towards a better understanding of thalamic oscillations and may possibly highlight novel routes for treatment of absence seizures.

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FIGURE 1. Basic thalamic circuitry and electrophysiology

A. Relay neurons (black) send excitatory glutamatergic collaterals (Glu) from thalamocortical projections onto nRt neurons (grey). nRt neurons send inhibitory GABAergic projections back to relay neurons as well as onto other nRt neurons (GABA). nRt neurons can also release NPY through these projections (NPY). In the presence of GABA_A receptor blockers, GABAergic transmission between nRt neurons is essentially abolished; while transmission at nRt-relay neuron synapses persists and is mediated by GABA_B receptors. Activation of T-type calcium channels on relay neurons causes rebound spiking after GABAergic inhibition.

B. Hypothetical mechanism of NPY/Y₁ receptor (Y₁R) mediated network inhibition and schematic traces. Y₁R-GIRK channel mediated hyperpolarization in nRt neurons will cause a larger proportion of fast EPSPs to become sub-threshold, and reduce action potential firing.

C. In relay neurons, long lasting hyperpolarization and slower repolarization may reduce activation of T-type Ca²⁺ channels, which will limit rebound excitation.

FIGURE 2. Effects of Y receptor agonists on evoked thalamic oscillation: Leu₃₁Pro₃₄NPY inhibits thalamic bursting responses, while PYY₃₋₃₆ and BWX-46 have no significant effect

Contour plots (A₁, B₁, C₁) and representative multiunit recordings of thalamic oscillations in nRt evoked by single extracellular stimuli in the internal capsule (A₂, B₂, C₂). Effects of the Y₁ receptor agonist Leu₃₁Pro₃₄NPY (A₁, A₂), the Y₂ receptor agonist PYY₃₋₃₆ (B₁, B₂) and the Y₅ receptor agonist BWX-46 (C₁, C₂).

A₁, B₁, C₁: Contour plots representing ratemeter of spikes. The x-axis represents time within each evoked oscillation, the y-axis represents the time course throughout the experiment (i.e. number of sweeps, recorded every 30 seconds). Darker grey scale levels (z-axis) correspond

to greater numbers of spikes within 50 ms bins. Dotted lines indicate transition between control, agonist superfusion and washout. Black triangles indicate time of stimulus.

A2, B2, C2: Oscillation under control conditions (ACSF, bottom trace), during agonist superfusion (middle trace in grey) and during washout (wash, upper trace). Black triangles indicate time of stimulus. Scale bar = 1 sec, 20 V.

D: Oscillation decay constants normalized to control for each recording (a), effect of agonist application (b) and return to 66–140% of baseline during washout (c). Leu₃₁Pro₃₄NPY: 6 slices, PYY_{3–36}: 4 slices, BWX-46: 5 slices.

E1, E2, E3: Averaged normalized decay constants (E1), oscillation amplitudes (E2) and period (E3) during baseline measurement (a), Y receptor agonist superfusion (b) and washout (c). Light grey bars: Leu₃₁Pro₃₄NPY; medium grey bars: PYY_{3–36}; dark grey bars: BWX-46. Asterisk indicates value that differs significantly from control.

FIGURE 3. Effects of Y receptor antagonists on evoked thalamic oscillations: BIBP3226 application disinhibits thalamic bursting responses, while BIIE0246 and L152,806 have no obvious effect
 Contour plots (A₁, B₁, C₁) and representative multiunit recordings of thalamic oscillations in nRt evoked by single extracellular stimuli in the internal capsule (A₂, B₂, C₂). Effects of the Y₁ receptor blocker BIBP3226 (A₁, A₂) the Y₂ receptor blocker BIIE0246 (B₁, B₂) and the Y₅ receptor Y₅ receptor blocker L152,806 (C₁, C₂).

A₁, B₁, C₁: Contour plots representing ratemeter of spikes; see legend to Fig. 2.

A₂, B₂, C₂: Oscillations under control conditions (ACSF, bottom trace), during antagonist superfusion (middle trace in grey) and during washout (wash, upper trace). Black triangles indicate time of stimulus. Scale bar = 1 sec, 20 V.

D: Oscillation decay constants normalized to control for each recording (a), effect of antagonist application (b) and return to 66–140% of baseline during washout (c). BIBP3226: 5 slices, BIEE0246 and L152,806: 4 slices.

E1, E2, E3: Averaged normalized decay constants (E1), oscillation amplitudes (E2) and period (E3) during baseline measurement (a), Y receptor antagonist superfusion (b) and washout (c). Light grey bars: BIBP3226; medium grey bars: BIEE0246; dark grey bars: L152,806. Asterisk indicates value that differs significantly from control (average of baseline and washout).

FIGURE 4. NPY and Y₁ receptor (Y₁R) expression in thalamic nuclei

Immunohistochemical co-staining of coronal slices from P24 animals with antibodies against the neuronal marker protein NeuN (Left panels in A and B; red immunofluorescence) and NPY (A, middle panel) or Y₁ receptor (B, middle panel); green immunofluorescence. The right panels in A and B shows merged images of the red and green immunofluorescence. NPY expressing neurons are restricted to the nRt, while Y₁ receptor is expressed in nRt as well as VB. Scale bar: 50 m.