Neuropeptide Y$_5$ Receptors Reduce Synaptic Excitation in Proximal Subiculum, But Not Epileptiform Activity in Rat Hippocampal Slices

MELISA W. Y. HO, ANNETTE G. BECK-SICKINGER, and WILLIAM F. COLMERS

INTRODUCTION

Neuropeptide Y (NPY) has profound actions on excitatory synaptic transmission in rat and human hippocampus (Colmers and Bleakman 1994; Colmers et al. 1997; Vezzani et al. 1999). NPY inhibits glutamate release presynaptically (McQuiston and Colmers 1996), mediated by an inhibition of presynaptic Ca$_{2+}$ influx (Qian et al. 1997). In the absence of selective antagonists, experiments with selective agonists strongly suggest that this action of NPY is mediated via Y$_2$ receptors (Klapstein and Colmers 1997; McQuiston and Colmers 1996). The presynaptic NPY receptor appears important in the control of excitability in the hippocampus. NPY knockout mice are more prone to spontaneous seizures, but are profoundly sensitive to kainate-induced status epilepticus, failing to recover and dying from this procedure, whereas their heterozygous littermates recovered completely. NPY knockout animals were rescued from kainate-induced status epilepticus by intracerebroventricular (icv) pretreatment with NPY (Baraban et al. 1997). In vitro, NPY Y$_2$ receptors are capable of interrupting interictal events caused by removal of Mg$_{2+}$ from the extracellular solution or by addition of the GABA$_A$ receptor blocker, picrotoxin. Furthermore, NPY and Y$_2$ receptor–preferring or -selective agonists, but not the Y$_1$ agonist, Leu$_{31}^{31}$ Pro$_{32}^{32}$ NPY were shown to effectively prevent ictal bursts induced in hippocampal slices by a series of stimulus trains (stimulus train–induced bursting (STIB)) (Colmers and Bleakman 1994; Klapstein and Colmers 1997). These and data from other laboratories (Smialowska et al. 1996) suggested that Y$_2$ receptors are important in the control of excitability in the hippocampus.

An in vivo study has suggested that a newly cloned NPY receptor, designated Y$_5$ (Gerald et al. 1996), may mediate the anticonvulsant actions of NPY in adult rats in vivo (Woldbye et al. 1997). Specifically, seizures induced by kainate injection in adult rats were reduced or abolished by NPY or related agonists injected into the lateral cerebral ventricle. Interestingly, in these experiments, the Y$_1$ receptor agonist, NPY$_{13-36}$ had little or no effect on the seizures induced by kainate in these animals, but NPY$_{3-36}$ (which binds Y$_2$ and Y$_5$ receptors), Leu$_{31}^{31}$ Pro$_{32}^{32}$ NPY (which binds Y$_1$, Y$_4$, and Y$_5$ receptors) (Gerald et al. 1996) and human pancreatic polypeptide (hPP, which binds Y$_3$/PP$_1$, and Y$_5$ receptors) were effective (Woldbye et al. 1997), consistent with the agonist binding profile of the Y$_5$ receptor (Gerald et al. 1996; Michel et al. 1998). These results were surprising, given the response to Y$_5$ agonists in the in vitro preparations. However, recent binding studies, using...
Leu$^{31}$, Pro$^{34}$PYY (which binds to Y$_1$, Y$_4$, and Y$_5$ receptors) in the presence of saturating concentrations of the selective Y$_1$ antagonist, BIBP3226 (Doods et al. 1995) suggested the presence of Y$_5$ receptors in the rat hippocampus (Dumont et al. 1998; Widdowson et al. 1997).

Although we earlier failed to observe effects of the Y$_1$/Y$_5$ agonist, Leu$^{1}$Pro$^{4}$NPy on STIB ictiform events (Klapstein and Colmers 1997), it did reduce the frequency of spontaneous bursts (SB) that occurred between stimulus train–induced afterdischarges. This action of Leu$^{31}$Pro$^{34}$NPy was insensitive to the Y$_1$ antagonist 1229U91. We hypothesized that this might represent a Y$_5$ receptor–mediated action of Leu$^{1}$Pro$^{4}$NPy (Klapstein and Colmers 1997).

Here we tested the hypothesis that Y$_5$ receptors could inhibit excitatory synaptic transmission in rat hippocampus. Because we earlier failed to see a significant effect on STIB, which is initiated and sustained in area CA3 (Bindokas et al. 1998; Stasheff et al. 1985), we concentrated our whole cell patch-clamp recordings in areas CA1 and the subiculum. We synthesized a novel, centrally truncated NPY analogue with a preference for Y$_1$ and Y$_5$ receptors and compared its actions with those of other agonists. Our results suggest that Y$_5$ receptors do mediate some of the actions of NPY in the hippocampus of young rats, but their contribution is relatively small compared with those of Y$_2$ receptors, declines with age, and is insufficient to block or significantly attenuate STIB–induced afterdischarges.

METHODS

Synthesis and preparation of a centrally truncated peptide analogue

The peptide was synthesized by automated multiple solid phase peptide synthesis using Fmoc/tert. butyl strategy as described previously (Rist et al. 1995, 1998). The peptides were analyzed and purified to homogeneity >94% by reversed-phase HPLC. Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto).

Membrane preparation

SMS-KAN cells (Y$_5$ receptor-expressing) were grown in 50% nutrient mixture Ham’s F12/50% Dulbecco’s modified Eagle medium with 15% fetal calf serum, 2 mM glutamine, and nonessential amino acids at 37°C and 5% CO$_2$ until they were confluent. SK-N-MC cells (Y$_1$ receptor-expressing) were grown under the same conditions using MEM Earle’s salts medium with 10% fetal calf serum, while BHK/Y5 cells (Y$_5$ receptor-expressing) were raised in DMEM (4.5 g/l glucose) with 10% fetal calf serum, 1% PENStrep, and 1 mg/ml Geneticin. Membrane preparation was performed as described before (Beck-Sickinger et al. 1994; Ingenhoven and Beck-Sickinger 1997). After determination of the protein concentration, inhibitors were added accordingly. Aliquots of the membrane suspension of 1 ml were stored at −80°C.

All cell culture media and supplements were purchased from Gibco, Pefabloc SC from Serva, and Bacitracin from Sigma.

Receptor binding

Membrane preparations of SK-N-MC, SMS-KAN, or BHK/Y5 cells were diluted in incubation buffer (MEM/25 mM HEPES, 1% bovine serum albumin, 50 µM Pefabloc SC, 0.1% bacitracin, 3.75 mM CaCl$_2$). Two hundred microliters of the suspension containing 20 µg protein were incubated with 25 µl 1.2 nM $^3$H-propionyl-NPY (3.18 TBq/mmol; Amersham) and 25 µl of solutions of the analogues in increasing concentrations to give a total volume of 250 µl. After 1.5 h at room temperature the incubation was terminated by centrifugation of the samples for 10 min at 3,000 $\times$ g and 4°C. The pellets were washed with PBS, resuspended in PBS, and mixed with scintillation cocktail, and radioactivity was determined (Ingenhoven and Beck-Sickinger 1997). Nonspecific binding was defined in the presence of 1 µM NPY. All experiments were performed in triplicate, and data are provided as means ± SE.

Brain slice preparation

Male Sprague-Dawley rats were used for both whole cell intracellular recording (ages 7–35 days old) and extracellular recording experiments (ages 15–22 days) in conjunction with stimulus train–induced bursting (STIB). Animals were decapitated without prior anesthesia according to Canadian Council on Animal Care guidelines in a protocol approved by the University of Alberta Health Sciences Laboratory Animal Care Committee. Brains were rapidly removed and placed in cold (2–4°C) artificial cerebrospinal fluid (ACSF) bubbled continuously with 95% O$_2$–5% CO$_2$ (carbogen). A block of tissue containing a hippocampus and overlying cortex, including the entorhinal cortex, was mounted in a chamber and sliced transversely with a Vibratome (TPI, St. Louis, MO) to a thickness of 350 and 600 µm for whole cell and STIB experiments, respectively. Slices containing both hippocampus and entorhinal cortex were equilibrated at 32°C or room temperature for 30–60 min for whole cell and STIB experiments, respectively. Slices were then transferred to a perfusion chamber on the stage of an upright microscope (Zeiss Axioskop FS) and perfused, submerged, with warm (34 ± 0.5°C), carbogenated ACSF (flow rate ~ 2–2.5 ml/min) and equilibrated for a minimum of 10 min before the beginning of experiments.

Whole cell recordings

Composition of the ACSF used for dissection and storage of hippocampal slices was (in mM) 124 NaCl, 3 KCl, 1.3 MgSO$_4$, 4 MgCl$_2$, 2 CaCl$_2$, 1.4 NaH$_2$PO$_4$, 26 NaHCO$_3$, and 10 glucose. The ACSF used for recording was identical to the one used for dissection with the exception that MgCl$_2$ was reduced to 2 mM and 0.5 mM CaCl$_2$ was added. Whole cell recordings were performed with fiber-fill borosilicate patch pipettes (4–6 MΩ) filled with saline containing (in mM) 130 potassium gluconate, 2 KCl, 5 HEPES, 5 MgATP, 1 NaGTP, and 1.1 BAPTA tetrapotassium salt. Potassium hydroxide was used to adjust the pH of the pipette saline to 7.22–7.25, and the final osmolarity for the pipette saline was adjusted to 270–285 mOsm.

All whole cell experiments were performed on neurons in either CA1 or the subicular regions of the hippocampal slice. Neurons in the cell body layer of area CA1 or the subiculum having typical pyramidal cell morphology were identified using a water immersion objective (Zeiss ×40) with an infrared filter and differential interference contrast (DIC) optics. The patch pipette was guided to the identified cell under visual control using infrared illumination and a television monitor (DAGE-MTI, Michigan City, IN), a gigohm seal (>2 GΩ) was established and the patch ruptured to gain access to the cell. Membrane potential and action potential waveform were initially monitored to confirm pyramidal cell properties, then the cell in voltage clamp was held near their resting potentials (~58 to ~70 mV). Synaptic currents were evoked via a sharpened tungsten, monopolar stimulating electrode placed in stratum radiatum of area CA1 or the subiculum, using a paired pulse protocol (5–40 V, 100–200 µs, 40-ms interstimulus interval), delivered from a stimulus isolation unit (Isoflex, AMPI, Jerusalem). The intensity of the stimuli was adjusted until a submaximal and stable synaptic current was induced. Such paired stimuli were used to demonstrate that stimulus amplitudes were not close to saturating the synaptic responses and to emphasize the presynaptic nature of the receptor actions. All whole cell currents were...
recorded using a single-electrode voltage-clamp amplifier (NPI SEC 05 1, NPI, Tamm, Germany) with a switching frequency of 35–38 kHz. Care was taken with clamp gain and capacity compensation settings to ensure proper square-wave headstage voltage throughout the duration of a voltage-clamp experiment. Whole cell synaptic currents were filtered at 0.7–1.3 kHz, and each current trace was the digital average of three successive responses, evoked at 0.1 Hz. In most cases, a voltage step (50 ms, 10–20 mV negative to rest) was performed during the protocol, after the synaptic stimulus, to monitor for changes in access resistance. Passive postsynaptic membrane properties near rest were also tested with a slow (2 s), 35 mV, positive-going voltage ramp starting 20 mV negative to rest. Data were acquired and membrane potential controlled using pClamp (Axon Instruments, Foster City, CA). Results from experiments in which whole cell access resistance had changed significantly (>10%) were discarded.

All NPY agonists were stored as aqueous solutions of 100 μM to 1 mM kept frozen (−20°C) in small aliquots until immediately before use, then diluted to their final concentration with 10 ml ACSF and perfused through the recording chamber in 4–5 min. In antagonist experiments, slices were first perfused with ACSF containing a blocking concentration of the antagonist for 4–5 min before either [ahx₈₋₂₀]Pro³⁴NPY or n-Trp³¹NPY was added to the antagonist-containing ACSF. All reported effects of different NPY agonists were calculated as the percent inhibition of the peak EPSC amplitude of the first of the paired synaptic responses. Statistical comparisons were made using paired t-tests in most cases, with neurons serving as their own controls.

**STIB recordings**

Composition of the ACSF used for dissection, storage of slices, and testing for field potential was (in mM) 120 NaCl, 3.3 KCl, 1.2 MgSO₄, 1.8 CaCl₂, 1.23 NaH₂PO₄, 25 NaHCO₃, and 10 glucose. All experiments were recorded with glass pipette (5–10 MΩ) filled with ACSF, connected to the headstage of the voltage-clamp amplifier used in the bridge current-clamp mode. Field potentials were evoked from a monopolar stimulating electrode placed in stratum radiatum of CA2/CA3a. A 30-V, 100-μs pulse was used to optimize placement of recording and stimulating electrodes. Field potentials were recorded from stratum pyramidale of CA3a, CA3b, or CA3c, or the subiculum. Recordings in area CA3 were made for comparison with previous results (Klapstein and Colmers 1997), and electrophysiological (Bagdon et al. 1992) and imaging experiments (Bindokas et al. 1998) suggested that in STIB, activity in area CA1 follows that in area CA3. Once the evoked field potential response was stable, slices were incubated without synaptic stimulation in the modified ACSF (identical with the above with the exception of MgCl₂ being reduced to 0.9 mM and CaCl₂ being reduced to 1.6 mM) for 20–40 min before the beginning of the STIB experiments.

Stimulus trains were delivered every 8 min and 5–6 min to CA3 and the subiculum, respectively. Each stimulus train contained four stimuli (30 V, 100–200 μs) at 100 Hz, repeated 20–25 times at 5 Hz. Once a stable primary ictal afterdischarge (1°AD) was recorded, the duration remained constant within ~10% (Klapstein and Colmers 1997). The number of stimulus trains were reduced until the 1°AD failed to occur, and was then raised gradually until at least three consecutive, stable 1°ADs were elicited. The average number of stimulus trains at threshold needed to elicit stable 1°AD was 13 ± 2.5 (mean ± SE, n = 14). The field afterdischarge potential was routinely amplified with an AC-coupled amplifier (×10 to ×100), filtered at 1–3 kHz, and recorded with a rectilinear chart recorder (Gould RS3200). Different NPY agonists were applied to slices via the perfusate during the last 4 min of a stimulus train cycle. A positive effect of an agonist on AD was determined as at least 50% reduction in 1°AD duration. All data were taken from slices in which the effect of an agonist reversed completely, i.e., the 1°AD duration remained stable for three successive stimulus train cycles on wash out. All STIB experiments were performed at 32 ± 0.5°C.

**Materials**

[ahx₈₋₂₀]NPY, [ahx₅₋₂₄]NPY (Rist et al. 1995) and [ahx₈₋₂₀]Pro³⁴NPY were all synthesized in Zürich as detailed above. n-Trp³¹NPY was purchased from Bachem California (Torrance, CA). BIBP3226 was purchased from Peninsulal Laboratories (Belmont, CA). Porcine sequence NPY was purchased from Dr. S. St-Pierre, Peptide Technologies, St-Laurent, Quebec.

**RESULTS**

**Binding of [ahx₈₋₂₀]Pro³⁴ NPY to Y₁, Y₂, and Y₅ receptors**

Binding curves were constructed for NPY and [ahx₈₋₂₀]Pro³⁴NPY in membrane preparations containing and Y₁, Y₂, and Y₅ receptors. Figure 1 illustrates competitive binding profiles for Y₁ and Y₅ receptors for NPY and [ahx₈₋₂₀]Pro³⁴NPY. Although NPY has approximately the same affinity for both receptors in this assay, [ahx₈₋₂₀]Pro³⁴NPY had about 10-fold less affinity for Y₁ receptors, and about 200-fold less affinity for Y₅ receptors than the native peptide. Although previous experiments show NPY binding to the Y₅ receptor with an affinity of 0.04 nM (Rist et al. 1996), [ahx₈₋₂₀]Pro³⁴NPY had essentially no affinity for Y₂ receptors (IC₅₀ > 10,000 nM, not illustrated).

**Whole cell recordings**

To investigate the possible involvement of Y₅ receptors in the regulation of synaptic transmission, we first tested the action of the Y₅-preferring agonists, n-Trp³¹NPY, or [ahx₈₋₂₀]Pro³⁴ NPY on excitatory synaptic responses evoked in pyramidal neurons of area CA1. In whole cell patch-clamp recordings from 33 neurons in area CA1, only 7 cells exhibited significant responses to applications of either Y₅-preferring agonist (not illustrated). We noticed that the responsive cells of area CA1 lay distal, close to the subiculum. We next hypothesized that synaptic inputs to neurons of the subiculum might respond to Y₅-preferring agonists, and we made whole cell recordings of neurons within the pyramidal cell layer of the subiculum. For these experiments, to best make comparisons with pyramidal cells in area CA1, we chose neurons (mostly in the proximal subiculum) that did not respond to stimulation with bursting (Greene and Totterdell 1997). Figure 2 illustrates typical excitatory postsynaptic currents (EPSCs) recorded under control conditions in such a subicular neuron; paired-pulse stimulation demonstrated facilitation of the second response. Bath application of the Y₅ receptor–preferring agonist, [ahx₈₋₂₀]Pro³⁴ NPY (1 μM) caused a 67.4% reduction in the amplitude of the first EPSC response, with an accompanying increase in the facilitation ratio of the first to the second EPSC response (1.6 vs. 2.4). At the same time, the response to a −20-mV voltage step (Fig. 2B) or a 35-mV voltage ramp (Fig. 2C) was not significantly affected by the application of the peptide agonist. The response reversed on agonist wash out (Fig. 2A).

Synaptic responses of subicular neurons were sensitive to both Y₅-preferring agonists tested, in addition to less selective agonists. Figure 3, A and B, illustrates the response of one subicular pyramidal cell to both [ahx₈₋₂₀]Pro³⁴NPY (3 μM)
and d-Trp^{32}NPY (1 μM). Both agonists were effective in reducing EPSC in this neuron. [ahl^{8–20}]Pro^{34}NPY inhibited the EPSC by 41.8%, and d-Trp^{32}NPY inhibited it by 23.0%. The synaptic response reversed almost completely on wash out of the agonists. In this cell, the response recovered stably to 80% of control values within 5 min wash out of [ahl^{8–20}]Pro^{34}NPY, and to 100% of control within 25 min wash out of d-Trp^{32}NPY (Figs. 3A and 2B). Effects of the centrally truncated agonist, [ahl^{8–20}]Pro^{34}NPY usually recovered within 5–10 min wash, whereas recovery from the effects of the full-length agonist, l-Trp^{32}NPY required between 20 and 40 min. The difference in wash out rates between centrally truncated and full-length NPY agonists is similar to results reported earlier in the hippocampal slice (Klapstein and Colmers 1997; McQuiston and Colmers 1996).

To compare the relative potency of these Y₅-preferring agonists with that of a full agonist at this synapse, we tested the effect of [ahl^{8–20}]NPY, a relatively nonselective, centrally truncated agonist on which the more selective agonist examined here was based (Rist et al. 1995), on subicular neurons. Figure 3C shows a typical response of the synaptic input to a subicular neuron to 1 μM [ahl^{8–20}]NPY. In this cell, [ahl^{8–20}]NPY caused an 88.5% reduction of the EPSC amplitude, revealing an inhibitory postsynaptic current (IPSC) underlying the first synaptic response. The inhibition of the EPSC recovered within 12 min of wash. Recovery from the effects of this concentration of [ahl^{8–20}]NPY generally occurred within 10–20 min.

In all three experiments in this figure, voltage ramps were applied to the cell in the absence of synaptic stimulation in the

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**FIG. 1.** Binding of neuropeptide Y (NPY) and [ahl^{8–20}]Pro^{34}NPY to membranes containing Y1 (A) and Y5 (B) receptors. IC₅₀ values are given in the bottom left of each panel. The binding affinities for these and the Y₂ receptor are given in the text.
absence or presence of the agonist, after peak effects on the EPSC were observed (insets in Fig. 3, A–C). As can be seen, there were no significant changes in somatic conductance that accompanied the significant changes in synaptic response amplitudes, consistent with previous observations of NPY action in hippocampal pyramidal cells (McQuiston and Colmers 1992, 1996). These results are consistent with an entirely presynaptic action of the NPY agonists in the subiculum.

At the respective concentrations tested, [ahx\(^{8-20}\)]Pro\(^{34}\)NPY and d-Trp\(^{32}\)NPY were roughly equipotent at inhibiting the EPSC in pyramidal cells of the subiculum. The average effect of 1 \(\mu\)M d-Trp\(^{32}\)NPY was an inhibition of 44 ± 5.7%, whereas 3 \(\mu\)M [ahx\(^{8-20}\)]Pro\(^{34}\)NPY inhibited the EPSC on average by 51.3 ± 3.5%. However, [ahx\(^{8-20}\)]Pro\(^{34}\)NPY was more frequently effective at inhibiting the EPSC in subicular pyramidal cells (26/45 cells tested) than was d-Trp\(^{32}\)NPY (17/46 cells tested). By contrast, the nonselective NPY agonist, [ahx\(^{8-20}\)]NPY (1 \(\mu\)M), induced an even greater inhibition in the subiculum (66.4 ± 4%), in a far greater proportion of neurons (18/21 cells tested). The average ages for the animals from which neurons showing responses to [ahx\(^{8-20}\)]Pro\(^{34}\)NPY were observed were 19.1 ± 0.79 days (n = 21) and was significantly greater than either [ahx\(^{8-20}\)]Pro\(^{34}\)NPY– (P = 0.001) or d-Trp\(^{32}\)NPY–responsive cells (P < 0.001).

We tested the hypothesis that the expression of presynaptic responses to Y\(_5\) agonists was a function of the age of the animal (Fig. 4). In general, we more commonly observed a Y\(_5\) agonist–induced inhibition of the subicular EPSC in slices prepared from younger animals, whereas the effect of the nonselective agonist, [ahx\(^{8-20}\)]NPY, was equally potent among each age group (Fig. 4). Although the degree of inhibition by either [ahx\(^{8-20}\)]Pro\(^{34}\)NPY or d-Trp\(^{32}\)NPY within each age group was highly variable (Fig. 4), regression analysis showed that the reductions with age in Y\(_5\)–agonist responses were statistically significant (\(R^2 = 0.277\), P < 0.0005 for d-Trp\(^{32}\) and \(R^2 = 0.271\), P < 0.0003 for [ahx\(^{8-20}\)]Pro\(^{34}\)NPY), but there was no significant relationship with age for nonselective agonist, [ahx\(^{8-20}\)]NPY.

Because the centrally truncated Y\(_5\) agonist we tested also has a high affinity for Y\(_1\) receptors, we next tested the hypothesis that the response in the subiculum was mediated by a Y\(_1\) receptor. We thus compared the action of [ahx\(^{8-20}\)]Pro\(^{34}\)NPY
alone and in the presence of a blocking concentration of the Y₁-specific antagonist BIBP3226 (Doods et al. 1995) on the EPSC recorded in subicular neurons. In Fig. 5A, BIBP3226 alone had no effect on the EPSC in this neuron, whereas co-application of [ahx⁸–²⁰]Pro³⁴NPY with BIBP3226 caused a reduction in EPSC as observed with [ahx⁸–²⁰]Pro³⁴NPY alone. Neither BIBP3226 alone nor [ahx⁸–²⁰]Pro³⁴NPY in the presence of BIBP3226 affected the membrane response to a voltage ramp (Fig. 5B). Results of these experiments are summarized in Fig. 5C. Similar results were also observed with D-Trp³²NPY and BIBP3226 (not illustrated).

**STIB recordings**

To assess the anticonvulsant effects of the Y₅ agonists [ahx⁸–²⁰]Pro³⁴NPY and D-Trp³²NPY in the in vitro slice preparation, we performed extracellular field potential recording in either area CA3 or the subiculum, using the STIB model for epilepsy (Klapstein and Colmers 1997; Stasheff et al. 1985).

Figure 6A shows a typical field potential recording from stratum pyramidale of CA3 after stable 1°AD was obtained. Once a 1°AD developed in response to stimulation, it remained stable for as long as 5 h under our experimental conditions. Afterdischarges generally occurred after a short delay from the end of a stimulus train and were generally clonic or tonic-clonic in nature (Klapstein and Colmers 1997).

**AREA CA3.** Neither [ahx⁸–²⁰]Pro³⁴NPY (3 μM) nor D-Trp³²NPY (1 μM) were significantly effective in suppressing or shortening the 1°AD in area CA3 (Fig. 6, B and C). Of 10 slices tested, 1°ADs recorded in area CA3 were totally suppressed by D-Trp³²NPY in only two, whereas only one slice responded strongly to [ahx⁸–²⁰]Pro³⁴NPY (1 μM) totally abolished the evoked EPSC, also without affecting the neuron’s postsynaptic properties. Note the underlying outward (upward) synaptic current, presumably representing a GABAₐ-mediated inhibitory postsynaptic current (IPSC), which is revealed in the presence of the agonist. The EPSC recovered to >90% of control amplitude within 12 min wash.
Pro 34 NPY effect on the EPSC also declined with age (52). It was more difficult to obtain spike waveforms (Fig. 7). Unlike the Y5 agonists, [ahx 5–24]NPY was effective in most slices (3/4 slices; Fig. 8). The response of the one slice to [ahx 5–24]NPY recovered within one cycle of washing (Fig. 7E). [ahx 8–20]NPY (1 μM) effect on 1°AD was more frequently observed in the subiculum (4/4; Fig. 8), and its effect also recovered within one cycle of washing (Fig. 7D). Finally, NPY was a potent inhibitor, and its effect on 1°AD was consistently observed in the subiculum (3/4; Fig. 8). However, NPY’s effect in the subiculum was not as readily reversed as in CA3 (Fig. 7F). Differences in response to various NPY agonists in these two areas could not be simply explained by the ages of animals used, because the mean ages were 19.08 ± 0.69 days (area CA3) and 17.0 ± 0.41 days (subiculum; P = 0.14).

**DISCUSSION**

We have shown here that Y5 receptors can mediate presynaptic inhibition of stratum radiatum–evoked glutamatergic responses in some pyramidal neurons of area CA1 and the proximal subiculum, in vitro, although this response declines rapidly with age. On the basis of these results, we then tested the hypothesis that Y5 receptors can control hyperexcitability in the hippocampal STIB model of epileptiform activity, using slices from animals in the age range where Y5 inhibitory responses were significant. Although earlier results demonstrated that NPY receptors, predominantly of the Y5 subtype, can inhibit epileptiform activity in this model (Klapstein and Colmers 1997), we were unable to demonstrate a significant action of Y5 receptors in this model, compared with the potent and prolonged effects of NPY itself. We conclude that, although Y5 receptors can inhibit the synaptic excitation of some neurons in area CA1 and the proximal subiculum in young rats, they do not appear to contribute significantly to the overall action of NPY in regulating hyperexcitability in the hippocampal formation, at any age tested.

**Whole cell recordings**

We first attempted to demonstrate that Y5 receptor agonists affected hippocampal synaptic responses already known to be sensitive to NPY and Y2 agonists. In the absence of consistent, Y5-mediated responses in area CA1, we examined the sensitivity of excitatory synaptic responses in neurons of the proximal subiculum to Y5 agonists. These cells exhibited typical pyramidal morphology and did not fire bursts on stimulation, in agreement with observations of Greene and Totterdell (1997) that nonbursting neurons were most often found in this area. However, such typical morphology was not often observed in rats younger than 12 days of age. In these young rats, the subicular neurons usually appeared round using infrared DIC videomicroscopy, with a less prominent or invisible apical dendrite. Nonetheless, we could stably evoke EPSCs in these neurons on stimulation of stratum radiatum, and their action potentials appeared like those of pyramidal cells from older animals.

In many of these experiments, we utilized a centrally truncated analogue of NPY, [ahx 8–20]Pro 34 NPY. We had originally...
designed this molecule to be a Y₁-selective agonist, but as we demonstrate here, it also shows a reasonably high-affinity binding for Y₅ (but not Y₂) receptors. The central truncation conferred the advantage of rapid wash out for this peptide in the hippocampal slice (Klapstein and Colmers 1997; McQuiston and Colmers 1996). The specificity of [ahx₈₋₂₀]Pro³⁴ NPY for Y₁ and Y₅ receptors allowed us to attribute the contribution of these two receptors to the effects of this agonist. Both the centrally truncated peptide [ahx₈₋₂₀]Pro³⁴ NPY (3 μM), and d-Trp³² NPY (1 μM), a full-length, specific, but weak Y₅ agonist (Gerald et al. 1996) were effective in inhibiting to a greater or lesser degree the evoked EPSCs in most subicular neurons. However, we did not observe any synaptic inhibition by these Y₅ agonists at concentrations below those used throughout our experiments. As expected, the effect of [ahx₈₋₂₀]Pro³⁴ NPY was short lived in comparison with d-Trp³² NPY.

Before attributing the actions of [ahx₈₋₂₀]Pro³⁴ NPY to the activation of Y₅ receptors, we had to ensure that the effects we observed were not mediated by Y₁ receptors, by comparing its action in the absence and presence of the specific Y₁ receptor antagonist, BIBP3226 (Rudolf et al. 1994). BIBP3226 has no significant affinity for Y₂ (Jacques et al. 1995; Rudolf et al. 1994), Y₄ (Bard et al. 1995), or Y₅ (Gerald et al. 1996) receptor subtypes. A blocking concentration of the antagonist (1 μM) had no effect on the action of [ahx₈₋₂₀]Pro³⁴ NPY, ruling out a Y₁ receptor as mediating the effect we observed. Although the expression of moderate levels of Y₁ receptor mRNA has been reported in the pyramidal layer of area CA1 and the subiculum (Larsen et al. 1993), based on the present results there is no evidence for a Y₁ response in this region. We conclude that this centrally truncated agonist acts predominantly at Y₅ receptors in this preparation.

Regardless of their relative potency, the effects of both Y₅ agonists were more often seen in younger animals, suggesting a waning role for the receptor in this brain region with maturity. NPY itself remains effective regardless of age, as does the nonselective agonist, [ahx₈₋₂₀]NPY. We must, however, caution that the present results are based on animals between 1 to 4 wk of age. Immunocytochemical studies of Y₅ receptor distribution in the hippocampus with age would be of significant interest and complement existing pharmacological studies.

We did not investigate the exact mechanism(s) by which NPY receptors reduced synaptic excitation in subicular neurons. However, our voltage-ramp and -step data indicated that there was no change in postsynaptic membrane properties with
application of any agonist tested. This is consistent with previous findings of NPY actions in the hippocampus that demonstrated that they resulted entirely from a presynaptic mechanism (Colmers et al. 1987; McQuiston and Colmers 1996; Qian et al. 1997). Although it is possible that Y5 receptors may also suppress Ca2+ currents in subicular neurons, as has been observed in dentate granule cells (McQuiston et al. 1996), such a mechanism is unlikely to contribute to the suppression of synaptic input observed here, because it did not alter synaptic excitation of rat granule cells (Klapstein and Colmers 1993).

**STIB recordings**

The STIB model has been frequently used to study epileptogenesis in vitro (Klapstein and Colmers 1997; Stasheff et al. 1985). The 1°AD that follows the stimulus trains resembles the electrographic activities that occur during seizures both in human epilepsy patients and in animal models (McNamara 1994) and has been validated to be sensitive to several anticonvulsants when applied at clinically relevant concentrations (Clark and Wilson 1992). Our previous STIB experiments suggested that, although Y2 receptors played a significant role in suppressing 1°AD in CA3, spontaneous bursting was also reduced by the Y1 agonist, Leu31-Pro34NPY, even in the presence of a Y1 antagonist (Klapstein and Colmers 1997). This agonist also has a very high affinity for Y5 receptors (Gerald et al. 1996). Given the recent in vivo studies reporting that Y5 receptors are anticonvulsant in the kainate model (Woldbye et al. 1997) and the present observations in subicular neurons, we hypothesized that Y5 receptors might also play a role in controlling neuronal excitability in the STIB model. Surprisingly, our data do not support this hypothesis.

The present results from area CA3 were consistent with our previous observations that Y2 receptors were mostly responsible for the suppression of the 1°AD in area CA3 (Klapstein and Colmers 1997). The nonselective agonist, [ahx8–20]NPY (1 μM) was as effective as Y2 agonist, [ahx5–24]NPY (1 μM), in suppressing 1°AD. However, neither of the two Y5 agonists, [ahx8–20]Pro34NPY (3 μM) nor D-Trp32NPY (1 μM) was clearly effective in preventing 1°AD expression. Of the 10 slices tested, only two had a measurable response to D-Trp32NPY, whereas only one responded to [ahx8–20]Pro34NPY.

In the subiculum, the Y5-selective agonists, D-Trp32NPY and [ahx8–20]Pro34NPY were not significantly effective in reducing 1°AD duration in the four slices tested. In the same slices, the Y2-selective agonist, [ahx5–24]NPY (1 μM), was unexpectedly not very effective in suppressing 1°AD, whereas the nonselective agonist of any agonist tested. This is consistent with previous findings of NPY actions in the hippocampus that demonstrated that they resulted entirely from a presynaptic mechanism (Colmers et al. 1987; McQuiston and Colmers 1996; Qian et al. 1997). Although it is possible that Y5 receptors may also suppress Ca2+ currents in subicular neurons, as has been observed in dentate granule cells (McQuiston et al. 1996), such a mechanism is unlikely to contribute to the suppression of synaptic input observed here, because it did not alter synaptic excitation of rat granule cells (Klapstein and Colmers 1993).

**FIG. 6.** Effects of different NPY agonists on stimulus train-induced bursting (STIB) induced afterdischarge recorded in area CA3. Stable 1°AD was established in this slice after 25 min incubation in modified artificial cerebrospinal fluid (ACSF). Stimulus trains (13 × 4 stimuli) were delivered every 8 min (see METHODS for details). A: typical 1°AD recorded in CA3, with single (tonic) spikes developing shortly after the end of stimulus train, then gradually developed into multiple (clonic) discharges. B: D-Trp32NPY (1 μM) had no significant effect on the duration of 1°AD. C: [ahx8–20]Pro34NPY (3 μM) also had no effect on 1°AD. D: the Y2 selective agonist, [ahx5–24]NPY (1 μM), suppressed 1°AD but had no effect on the interictal discharge. E: the effect of [ahx8–20]NPY reversed within one cycle (i.e., 8 min) of wash. F: NPY itself (1 μM) strongly suppressed the 1°AD. Some recovery was observed after 70 min wash (not illustrated). All traces were recorded from a single slice from a 15-day-old animal.
Subiculum

![Graph of different NPY agonists on STIB-induced afterdischarges recorded in the subiculum.](Image)

**FIG. 7.** Effects of different NPY agonists on STIB-induced afterdischarges recorded in the subiculum. Stable 1°AD was established in this slice after 40 min incubation in modified ACSF. Stimulus trains (10 × 4) were delivered every 5 min. **A:** typical 1°AD recorded in the subiculum. Unlike in CA3, the 1°AD in the subiculum usually consisted of single spikes throughout its duration. B and C: neither [ahx<sup>8–20</sup>Pro<sup>34</sup>NPY (3 μM; B) nor D-Trp<sup>32</sup>NPY (1 μM; C) had any significant effect on the duration of the subicular 1°AD. D: as in area CA3, the Y<sub>2</sub>-selective agonist, [ahx<sup>5–24</sup>]NPY (1 μM) was also effective in inhibiting the development of the 1°AD recorded in the subiculum, and its effects also reversed rapidly (~5 min) on wash out. E: [ahx<sup>8–20</sup>]NPY (1 μM) was effective in suppressing the 1°AD, which usually returned within a few minutes of wash out. F: NPY (1 μM) completely suppressed the 1°AD, but did not wash out even after 30 min. The response began to recover after 55 min wash (not illustrated). All traces were recorded from a single slice from an 18-day-old animal.

An agonist [ahx<sup>8–20</sup>]NPY (1 μM) was as potent as NPY (1 μM) in inhibiting 1°AD expression in this region. Such differences in distribution of Y<sub>2</sub> receptors in these two areas were not a result of any developmental factors because there was no significant difference (P = 0.14) in the age of animals used for either set of experiments. This may instead reflect the relative density of Y<sub>2</sub> receptors in this region. Gustafson et al. (1997) reported a concentration of Y<sub>2</sub> receptor mRNA in area CA3, presumably reflecting the high concentration of Y<sub>2</sub> receptors we have observed functionally in the Schaffer collaterals of area CA1 (Klapstein and Colmers 1993; McQuiston and Colmers 1996). However, lower levels of Y<sub>2</sub> receptor mRNA expression in CA1 pyramidal cells might be reflected in lower concentrations of functional receptors in their projections to the subiculum.

The experiments thus suggest that there are Y<sub>5</sub> receptors that can inhibit synaptic excitation, particularly in distal area CA1 and the proximal subiculum of young rats. This action is not universal, because the inputs to a majority of neurons tested failed to express a response to the Y<sub>5</sub> agonists. Furthermore, although these agonists share a site (and presumably a mechanism) of action with the nonspecific NPY agonists, they were essentially incapable of mimicking the anticonvulsant actions observed with the Y<sub>2</sub>-selective or nonselective agonists in the STIB model.

Experiments using rhodamine 123 imaging to study patterns of activity and the actions of NPY in the STIB model suggest that subthreshold activity persists even with concentrations of NPY sufficient to prevent 1°AD expression (Bindokas et al. 1998). It is thus tempting to speculate that, if the scarcity of whole cell responses to Y<sub>5</sub> agonists encountered here reflects the actual receptor distribution, then the poor response of the STIB preparation to Y<sub>5</sub> agonists may result from an insufficient density of Y<sub>5</sub> receptors to suppress this regenerative network event. Alternatively, or in addition, the concentration of Y<sub>5</sub> receptors in the subiculum may not be adequate to prevent the regenerative activity of STIB, which relies heavily on reciprocal excitatory connections within area CA3 for its expression (Wong and Traub 1983).

This might explain why our current results contradict an earlier in vivo study of the anticonvulsant action of NPY (Woldbye et al. 1997). These authors used intracerebroventricular preinjections of NPY and related agonists to suppress seizures induced by acute peripheral kainate injection, and concluded that NPY acted via a Y<sub>5</sub> receptor to suppress these seizures. It is possible that, in the in vivo kainate model as used
by these investigators, sufficient Y₅ receptors are located somewhere in the pathways mediating the seizures, but are located outside the hippocampal circuitry included in our in vitro preparation. Alternatively, the mechanism by which putative Y₅ receptors suppress picrotoxin-induced interictiform bursts (Klapstein and Colmers 1997) may have greater significance in vivo. Nonetheless, based on the present data, the Y₅ receptors in young rats appear incapable of significantly contributing to the actions of NPY receptors in suppressing epileptiform activity in hippocampal circuitry even when they are present.

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This work was supported by the Medical Research Council of Canada. Present addresses: M.W.Y. Ho, Inositol Signaling Section, Laboratory of Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27713; A. G. Beck-Sickinger, Institut of Biochemistry, University of Leipzig, Talstr. 33, D 04103 Leipzig, Germany. Address for reprint requests: W. F. Colmers, Dept. of Pharmacology, University of Alberta, 9-36 MSB, Edmonton, Alberta T6G 2H7, Canada.

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