Neuropeptide Y5 Receptors Reduce Synaptic Excitation in Proximal Subiculum, But Not Epileptiform Activity in Rat Hippocampal Slices

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INTRODUCTION

Neuropeptide Y (NPY) has profound actions on excitatory synaptic transmission in rat and human hippocampus (Colmers and Bleakman 1994; Colmers et al. 1987; Vezzani et al. 1999). NPY inhibits glutamate release presynaptically (McQuiston and Colmers 1996), mediated by an inhibition of presynaptic Ca2+ 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 Y2 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 Y2 receptors are capable of interrupting interictal events caused by removal of Mg2+ from the extracellular solution or by addition of the GABA_A receptor blocker, picrotoxin. Furthermore, NPY and Y2 receptor–preferring or –selective agonists, but not the Y1 agonist, Leu31,Pro34NPY were shown to effectively prevent ictiform 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 Y2 receptors are important in the control of excitability in the hippocampus.

An in vivo study has suggested that a newly cloned NPY receptor, designated Y5 (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 Y2 receptor agonist, NPY13–36 had little or no effect on the seizures induced by kainate in these animals, but NPY3–36 (which binds Y2 and Y5 receptors), Leu31,Pro34NPY (which binds Y1, Y4, and Y5 receptors) (Gerald et al. 1996) and human pancreatic polypeptide (hPP, which binds Y4/PP, and Y5 receptors) were effective (Woldbye et al. 1997), consistent with the agonist binding profile of the Y5 receptor (Gerald et al. 1996; Michel et al. 1998). These results were surprising, given the response to Y2 agonists in the in vitro preparations. However, recent binding studies, using...
Leu\textsuperscript{31}, Pro\textsuperscript{34}PYY (which binds to Y\textsubscript{1}, Y\textsubscript{4}, and Y\textsubscript{5} receptors) in the presence of saturating concentrations of the selective Y\textsubscript{1} antagonist, BIBP3226 (Doods et al. 1995) suggested the presence of Y\textsubscript{5} receptors in the rat hippocampus (Dumont et al. 1998; Widdowson et al. 1997).

Although we earlier failed to observe effects of the Y\textsubscript{1}/Y\textsubscript{5} agonist, Leu\textsuperscript{31}Pro\textsuperscript{34}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\textsuperscript{31}Pro\textsuperscript{34}NPY was insensitive to the Y\textsubscript{1} antagonist 1229U91. We hypothesized that this might represent a Y\textsubscript{5} receptor–mediated action of Leu\textsuperscript{31}Pro\textsuperscript{34}NPY (Klapstein and Colmers 1997).

Here we tested the hypothesis that Y\textsubscript{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\textsubscript{1} and Y\textsubscript{5} receptors and compared its actions with those of other agonists. Our results suggest that Y\textsubscript{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\textsubscript{2} receptors, declines with age, and is insufficient to block or significantly attenuate STIB–induced afterdischarges.

\section*{METHODS}

\subsection*{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 \textgreater94\% by reversed-phase HPLC. Correct mass was identified by ion-spray mass spectrometry (API III, Sciex, Toronto).

\subsection*{Membrane preparation}

SMS-KAN cells (Y\textsubscript{2} 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\textsubscript{2} until they were confluent. SK-N-MC cells (Y\textsubscript{1} receptor-expressing) were grown under the same conditions using MEM Earle’s salts medium with 10% fetal calf serum, while BHKY5 cells (Y\textsubscript{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 determined for the protein concentration, inhibitors were added accordingly. Aliquots of the membrane suspension of 1 ml were used for recording 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.

\subsection*{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\textsubscript{4}, 4 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 1.4 NaH\textsubscript{2}PO\textsubscript{4}, 26 NaHCO\textsubscript{3}, and 10 glucose. The ACSF used for recording was identical to the one used for dissection with the exception that MgCl\textsubscript{2} was reduced to 2 mM and 0.5 mM CaCl\textsubscript{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 osmolality 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 (\textgreater2 MΩ) 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 its resting potentials (−58 to −70 mV). Somatic evoked 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 inter-stimulus 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

\subsection*{Receptor binding}

Membrane preparations of SK-N-MC, SMS-KAN, or BHKY5 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\textsubscript{2}). Two hundred microliters of the suspension containing 20 μg protein were incubated with 25 μl 1.2 nM \textsuperscript{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.
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 α-Trp32NPY 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 Na2HPO4, 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 (Bradon et al. 1992) and imaging experiments (Bindokas et al. 1998) suggested that in STIB, activity in area CA1 follows that in area CA3 consistently. 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, meaning 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]-Pro34 NPY were all synthesized in Zürich as detailed above, α-Trp32 NPY was purchased from Bachem California (Torrance, CA). BIBP3226 was purchased from Peninsula 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 Yp, 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 Y2 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, α-Trp32NPY, or [ahx8–20] Pro34NPY 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] Pro34NPY (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. [ahx^{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 [ahx^{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, [ahx^{8–20}]Pro^{34}NPY usually recovered within 5–10 min wash, whereas recovery from the effects of the full-length agonist, d-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 Y_{5}-preferring agonists with that of a full agonist at this synapse, we tested the effect of [ahx^{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 [ahx^{8–20}] NPY. In this cell, [ahx^{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 [ahx^{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
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, [ahx8–20]Pro34NPY and D-Trp32NPY were roughly equipotent at inhibiting the EPSC in pyramidal cells of the subiculum. The average effect of 1 μM D-Trp32NPY was an inhibition of 44.6 ± 5.7%, whereas 3 μM [ahx8–20]Pro34NPY inhibited the EPSC on average by 51.3 ± 3.5%. However, [ahx8–20]Pro34NPY was more frequently effective at inhibiting the EPSC in subicular pyramidal cells (26/45 cells tested) than was D-Trp32NPY (17/46 cells tested). By contrast, the nonselective NPY agonist, [ahx8–20]NPY (1 μM), 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 [ahx8–20]NPY were observed was 19.1 ± 0.79 days (n = 21) and was significantly greater than either [ahx8–20]Pro34NPY– (P = 0.001) or D-Trp32NPY–responsive cells (P < 0.001).

We tested the hypothesis that the expression of presynaptic responses to Y5 agonists was a function of the age of the animal (Fig. 4). In general, we more commonly observed a Y5 agonist–induced inhibition of the subicular EPSC in slices prepared from younger animals, whereas the effect of the nonselective agonist, [ahx8–20]NPY, was equally potent among each age group (Fig. 4). Although the degree of inhibition by either [ahx8–20]Pro34NPY or D-Trp32NPY within each age group was highly variable (Fig. 4), regression analysis showed that the reductions with age in Y5-agonist responses were statistically significant (R² = 0.277, P < 0.0005 for D-Trp32 and R² = 0.271, P < 0.0003 for [ahx8–20]Pro34NPY), but there was no significant relationship with age for nonselective agonist, [ahx8–20]NPY.

Because the centrally truncated Y5 agonist we tested also has a high affinity for Y1 receptors, we next tested the hypothesis that the response in the subiculum was mediated by a Y1 receptor. We thus compared the action of [ahx8–20]Pro34NPY
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 (Fig. 8). By contrast, the Y₂-selective agonist, [ahx₅₋₂₄]NPY (1 μM), totally abolished 1°AD in most slices tested, but in some cases did not abolish the spontaneous bursts that followed after the stimulus train (Fig. 6D). The effect of [ahx₅₋₂₄]NPY on 1°AD usually reversed within one stimulus cycle after washing (Fig. 6E).
Pro 34: NPY effect on the EPSC also declined with age (significant affected by the animal’s age (5, P<0.0002). It was also more difficult to obtain A spike waveforms (Fig. 7briefer and smaller in amplitude and rarely exhibited multiple stimulation of stratum radiatum in area CA2/3 was generally inhibitory agonist, [ahx 8–20]NPY (1 M), but not all slices (Fig. 8). Similarly, the nonselective analogue of NPY, [ahx 8–20]Pro 34 NPY (1 M), was also effective in inhibiting 1°AD duration (not illustrated), and its effect was recovered within one cycle of washing (Fig. 7D). NPY was a potent inhibitor, and its effect on 1°AD was consistently observed in the subiculum (3/3; 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 19.0 ± 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 Y3 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 $\mathrm{Y}_1$-selective agonist, but as we demonstrate here, it also shows a reasonably high-affinity binding for $\mathrm{Y}_5$ (but not $\mathrm{Y}_2$) 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 [ahx$^{8-20}$]Pro$^{34}$NPY for $\mathrm{Y}_1$ and $\mathrm{Y}_5$ receptors allowed us to attribute the contribution of these two receptors to the effects of this agonist. Both the centrally truncated peptide [ahx$^{8-20}$]Pro$^{34}$NPY (3 $\mu$M), and d-Trp$^{32}$NPY (1 $\mu$M), a full-length, specific, but weak $\mathrm{Y}_5$ 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 $\mathrm{Y}_5$ agonists at concentrations below those used throughout our experiments. As expected, the effect of [ahx$^{8-20}$]Pro$^{34}$NPY was short lived in comparison with d-Trp$^{32}$NPY.

Before attributing the actions of [ahx$^{8-20}$]Pro$^{34}$NPY to the activation of $\mathrm{Y}_5$ receptors, we had to ensure that the effects we observed were not mediated by $\mathrm{Y}_1$ receptors, by comparing its action in the absence and presence of the specific $\mathrm{Y}_1$ receptor antagonist, BIBP3226 (Rudolf et al. 1994). BIBP3226 has no significant affinity for $\mathrm{Y}_2$ (Jacques et al. 1995; Rudolf et al. 1994), $\mathrm{Y}_4$ (Bard et al. 1995), or $\mathrm{Y}_5$ (Gerald et al. 1996) receptor subtypes. A blocking concentration of the antagonist (1 $\mu$M) had no effect on the action of [ahx$^{8-20}$]Pro$^{34}$NPY, ruling out a $\mathrm{Y}_1$ receptor as mediating the effect we observed. Although the expression of moderate levels of $\mathrm{Y}_1$ 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 $\mathrm{Y}_1$ response in this region. We conclude that this centrally truncated agonist acts predominantly at $\mathrm{Y}_5$ receptors in this preparation.

Regardless of their relative potency, the effects of both $\mathrm{Y}_5$ 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, [ahx$^{8-20}$]NPY. We must, however, caution that the present results are based on animals between 1 to 4 wk of age. Immunocytochemical studies of $\mathrm{Y}_5$ 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 Ca\(^{2+}\) 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; Stasseff et al. 1985). The 1\(^{st}\)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\(^{st}\)AD in CA3, spontaneous bursting was also reduced by the Y1 agonist, Leu\(^{31-}\)Pro\(^{34}\)NPY, 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\(^{st}\)AD in area CA3 (Klapstein and Colmers 1997). The nonselective agonist, [ahx\(^{8-20}\)NPY (1 \(\mu\)M), was as effective as Y2 agonist, [ahx\(^{5-24}\)NPY (1 \(\mu\)M), in suppressing 1\(^{st}\)AD. However, neither of the two Y5 agonists, [ahx\(^{8-20}\)Pro\(^{34}\)NPY (3 \(\mu\)M) nor d-Trp\(^{32}\)NPY (1 \(\mu\)M) was clearly effective in preventing 1\(^{st}\)AD expression. Of the 10 slices tested, only two had a measurable response to d-Trp\(^{32}\)NPY, whereas only one responded to [ahx\(^{8-20}\)Pro\(^{34}\)NPY.

In the subiculum, the Y5-selective agonists, d-Trp\(^{32}\)NPY and [ahx\(^{8-20}\)Pro\(^{34}\)NPY were not significantly effective in reducing 1\(^{st}\)AD duration in the four slices tested. In the same slices, the Y5-selective agonist, [ahx\(^{5-24}\)NPY (1 \(\mu\)M), was unexpectedly not very effective in suppressing 1\(^{st}\)AD, whereas the nonselect-
tive agonist \([\text{ahx}^{8-20}]\text{Pro}^{34}\text{NPY}\) (1 \(\mu\text{M}\)) was as potent as NPY (1 \(\mu\text{M}\)) in inhibiting 1°AD expression in this region. Such differences in distribution of \(Y_2\) 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 \(Y_2\) receptors in this region. Gustafson et al. (1997) reported a concentration of \(Y_2\) receptor mRNA in area CA3, presumably reflecting the high concentration of \(Y_2\) receptors we have observed functionally in the Schaffer collaterals of area CA1 (Klapstein and Colmers 1993; McQuiston and Colmers 1996). However, lower levels of \(Y_2\) 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 \(Y_3\) 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 \(Y_3\) 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 \(Y_2\)-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 \(Y_3\) agonists encountered here reflects the actual receptor distribution, then the poor response of the STIB preparation to \(Y_3\) agonists may result from an insufficient density of \(Y_3\) receptors to suppress this regenerative network event. Alternatively, or in addition, the concentration of \(Y_3\) 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 \(Y_3\) 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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