The anti-epileptic actions of neuropeptide Y in the hippocampus are mediated by \( Y_2 \) and not \( Y_5 \) receptors

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Abstract

Neuropeptide Y (NPY) potently inhibits glutamate release and seizure activity in rodent hippocampus in vitro and in vivo, but the nature of the receptor(s) mediating this action is controversial. In hippocampal slices from rats and several wild-type mice, a \( Y_2 \)-preferring agonist mimicked, and the \( Y_2 \)-specific antagonist BIIE0246 blocked, the NPY-mediated inhibition both of glutamatergic transmission and of epileptiform discharges in two different slice models of temporal lobe epilepsy, stimulus train-induced bursting (STIB) and \( 0\)-Mg\(^{2+}\) bursting. Whereas \( Y_5 \) receptor-preferring agonists had small but significant effects in vitro, they were blocked by BIIE0246, and a \( Y_5 \) receptor-specific antagonist did not affect responses to any agonist tested in any preparation. In slices from \( Y_2 \) mice, NPY was without effect on evoked potentials or in either of the two slice seizure models. In vivo, intrahippocampal injections of \( Y_2 \)- or \( Y_5 \)-preferring agonists inhibited seizures caused by intrahippocampal kainate, but again the \( Y_5 \) agonist effects were insensitive to a \( Y_5 \) antagonist. Neither \( Y_2 \)- nor \( Y_5 \)-preferring agonists affected kainate seizures in \( Y_2 \) mice. A \( Y_5 \)-specific antagonist did not displace the binding of two different NPY ligands in WT or \( Y_1 \) mouse, whereas all NPY binding was eliminated in the \( Y_2 \) mouse. Thus, we show that \( Y_2 \) receptors alone mediate all the anti-excitatory actions of NPY seen in the hippocampus, whereas our findings do not support a role for \( Y_5 \) receptors either in vitro or in vivo. The results suggest that agonists targeting the \( Y_2 \) receptor may be useful anticonvulsants.

Introduction

The neuropeptide Y (NPY) system in the hippocampus and cortex has been widely suggested to be involved in epileptogenesis and epilepsy. Although NPY is distributed broadly throughout the nervous system, seizure-related changes in NPY and NPY-receptor expression are seen mostly in brain areas, such as the hippocampus, involved in the initiation and propagation of epileptic discharges (Sperk et al., 1992; Schwarzer et al., 1995). Furthermore, application of NPY potently and selectively inhibits excitatory synaptic transmission in the hippocampus, making the receptors mediating this of interest as anticonvulsant drug targets.

NPY acts via at least five known receptor subtypes, all of which belong to the \( G \) protein-coupled receptor superfamily (Michel et al., 1998). Extensive studies in the hippocampus in vitro have demonstrated that NPY selectively inhibits glutamatergic synaptic transmission in areas CA1 and CA3 (Colmers et al., 1987, 1991; Klapstein & Colmers, 1992; Greber et al., 1994; McQuiston & Colmers, 1996), and suppresses glutamate release (Greber et al., 1994; Silva et al., 2003). This action results from the suppression of voltage-dependent Ca\(^{2+}\) influx in presynaptic nerve terminals (Toth et al., 1993; Qian et al., 1997).

The NPY system has been proposed to act as an endogenous anticonvulsant (Vezzani & Sperk, 2004; Tu et al., 2005). Thus, in vivo seizures up-regulate NPY and \( Y_2 \) receptor levels (Sperk et al., 1992; Gobbi et al., 1998; Schwarzer et al., 1998; Vezzani et al., 1999) but down-regulate \( Y_1 \) receptors (Kofler et al., 1997), and elevating NPY in the hippocampus elevates seizure thresholds, delays kindling epileptogenesis, and results in fewer and briefer kindled seizures (Vezzani et al., 2002; Reibel et al., 2003; Richichi et al., 2004). NPY-deficient mice occasionally develop mild, spontaneous seizures and exhibit markedly enhanced susceptibility to motor seizures induced by convulsant agents (Erickson et al., 1996; Baraban et al., 1997). Elevations in \( Y_2 \) receptor levels have been observed in patients with temporal lobe epilepsy (Fürtinger et al., 2001), and NPY application suppresses epileptiform activity in human epileptic dentate gyrus (Colmers et al., 1997; Patryo et al., 1999).

The nature of the NPY receptor mediating its inhibitory and anticonvulsant actions has been controversial. In vitro studies in rodents and humans indicate a major role for the \( Y_2 \) receptor (Colmers et al., 1991, 1997; El Bahh et al., 2002; Vezzani & Sperk, 2004).
2004), but some *in vivo* experiments, using agonist pharmacology (Woldbye *et al.*, 1997) and knockout strategies (Marsh *et al.*, 1999; Barabani, 2002), have implicated the Y₂ receptor. Here, we combine *in vitro* and *in vivo* approaches in rats, and a spectrum of wild-type (WT) and Y-receptor knockout mice to study the site(s) and nature(s) of Y receptors that regulate glutamate release and, by extension, limbic seizures. Evidence here from all approaches taken, including pharmacological agonists and antagonists, *in vitro* and *in vivo*, in rats, WT and receptor knockout mice, consistently supports a key role for the Y₂ receptor, but provides no evidence for any hippocampal NPY responses that were verifiably mediated by the Y₃ receptor.

**Methods**

**Experimental procedures**

All animal procedures were in accordance with Canadian Council of Animal Care guidelines (protocol approved by the University of Alberta Health Sciences Laboratory Animal Committee) and relevant international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, 12 December 1987; Guide for the Care & Use of Laboratory Animals, US National Research Council, 1996).

**Animals**

The generation of Y₂⁻⁻ knockout mice on a mixed C57BL/6 × 129/Sv background was described previously (Sainsbury *et al.*, 2002). Y₂⁻⁻ mice on an inbred 129/Sv background were generated using homologous recombination techniques as described by Marsh *et al.* (1999) and maintained in a breeding colony at UCSF, and were a generous gift of Dr R. Palmier. WT animals used in the *in vitro* studies were male Sprague-Dawley rats and C57BL/6 (both from the University of Alberta colonies). WT 129/SvJ mice, of the same genetic background as the Y₂⁻⁻ animals, were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and C57BL/6 × 129/SvJ came from the Garvan Institute (Sydney, Australia) and were identical to WT animals described previously (Sainsbury *et al.*, 2002). The C57BL/6 mice used in the *in vivo* experiments were from Charles River (Calco, Italy).

**In vitro experiments**

**Slice preparation**

Transverse hippocampal slices (400 μm) were prepared from young (3–5 weeks) animals, after decapitation, as described previously (Klapstein & Colmers, 1997; Ho *et al.*, 2000; El Bahh *et al.*, 2002) using a vibriscriner HR-2 (Sigmann-Elektronik, Hüffenhardt, Germany), and submerged in a continuous flow (2–3 mL/min) of artificial cerebrospinal fluid (ACSF), composed of (mm): 124 NaCl, 3 KCl, 1.4 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 1.5 CaCl₂, 10 glucose, saturated with 5% CO₂, 95%O₂ (carbogen) at 32–34 °C. For stimulus-train-induced bursting (STIB) experiments, slicing procedures were identical, except that slices were cut at 600 μm thickness (Klapstein & Colmers, 1997; Ho *et al.*, 2000; El Bahh *et al.*, 2002).

**Electrophysiology**

Extracellular recordings of hippocampal areas CA1 and CA3 were performed as previously detailed (Klapstein & Colmers, 1992, 1997; Ho *et al.*, 2000; El Bahh *et al.*, 2002). Orthodromic population excitatory postsynaptic potentials (pEPSPs) were evoked via electrodes placed in stratum radiatum of CA2 or the mossy fiber pathway. Field potentials were digitally averaged (n = 3 for each data point) and analysed with pClamp 9 software (Axon Instruments, Union City, CA, USA). The initial linear slope of the pEPSP (50–80% of the maximum response in a slice) was measured (Klapstein & Colmers, 1992).

The procedures both for 0-Mg²⁺ bursting and for STIB experiments have been detailed previously (Stasheff *et al.*, 1985; Klapstein & Colmers, 1997; Ho *et al.*, 2000; El Bahh *et al.*, 2002).

**0-Mg²⁺-bursting**

In brief, 400-μm-thick transverse hippocampal slices were prepared as above, transferred to the perfusion chamber and an extracellular recording electrode placed in stratum pyramidale of area CA3. In some experiments, a bipolar stimulating electrode was placed on the mossy fiber tract in the dentate hilus to deliver single stimuli (0.05 Hz) to optimize the placement of the recording electrode; stimuli were not applied via this electrode during the remainder of the experiment. The perfusion medium was then changed to one in which the MgSO₄ was omitted (0-Mg²⁺ ACSF), and the slice monitored for the occurrence of spontaneous bursts (SBs). Experiments were initiated once the SB frequency was stable (Klapstein & Colmers, 1997; El Bahh *et al.*, 2002).

**Stimulus train-induced bursting**

Briefly, stratum radiatum of CA2 was stimulated and recordings made in area CA3 in 600-μm-thick transverse hippocampal slices. Slices were perfused with ACSF composed of (mm): 120 NaCl, 3.3 KCl, 0.9 MgSO₄, 1.6 CaCl₂, 1.23 NaH₂PO₄, 25 NaHCO₃, 10 glucose. After 30 min perfusion, afterdischarges were elicited every 5 min by brief stimulus trains (four stimuli, 30 V, 0.1 ms, 100 Hz repeated at 5 Hz). The number of stimulus trains was set just above that needed to elicit an afterdischarge reliably (Klapstein & Colmers, 1997; Ho *et al.*, 2000; El Bahh *et al.*, 2002).

**Drugs**

Human NPY (hNPY) was purchased from Peptidex Technologies (Pierrefonds, Quebec, Canada). The receptor-preferring agonists: [ahx⁵⁻²⁴]hNPY, F8⁵ʰ⁻hNPY and [hPP₁⁷⁻Ala¹³, Aib²¹]hNPY (Ala-AibhNPY), were made by solid-state synthesis, as described previously (Rist *et al.*, 1995; Cabrele *et al.*, 2000; Soll *et al.*, 2001). The ‘scrambled’ NPY used as a control was similarly synthesized and contained the same amino acids as NPY but in random sequence: SKPQORDANEPR-TRYAIDYSNPDIEHLYRPAAYLNGH₂. BIIE0246 ((S)-N²-[1-[2-[4-[[R(S),S]-5,11-dihydro-6(6H)-oxoobenz[b,e]azepin-11-yl]-1-piperazine-2-oxoethyl]cyclopentyl]acyl]N-2-[1,2-dihydro-3,5(4H)-dioxo-1,2-diphenyl-3H-I,2,4-triazol-4-yl]ethyl]arginimid] (Doods *et al.*, 1999) was a generous gift of Dr Henri Doods (Boehringer-Ingehelm). Novartis 1 ((trans-2-nitrobenzene-2-sulfonic acid (4-(2-naphthylmethyl) amino) methyl) cylohexyl methyl) amide) (Pronchuk *et al.*, 2002) was a generous gift of Dr P. Hippskind (Lilly Research Laboratories), and GW438014A ([N-1-(2-phenylethyl)-1H-benzimidazol-2-yl]benzamide methanesulfonate) (Daniels *et al.*, 2002) was a generous gift of Dr Alex Daniels (GlaxoSmithKline). All other chemicals were obtained from BDH Inc. (Toronto, Canada).

All pharmacological agents were applied via bath perfusion. All peptides and most drugs were stored as concentrated stock solutions and diluted in ACSF prior to application, with the exception of BIIE0246, which was dissolved in ethanol as a 1 mM stock solution (El Bahh *et al.*, 2002) and diluted at least 10 000-fold in ACSF before application.
The agonist and antagonist concentrations used throughout these experiments were chosen to maximize our opportunity to observe specifically responses that were not mediated by Y$_2$ receptors. In general, peptide agonists were tested at a concentration of 1 μM, which, although relatively high in comparison with binding affinities, was never above the maximal effective concentration demonstrated for an NPY-related agonist in the hippocampus in vitro (Colmers et al., 1991; El Bahh et al., 2002). With agonists we had not tested in the hippocampus before (specifically AlaAibNPY and F$^{5,34}$NPY), we maintained a similar relationship between agonist concentration (1 μM) and the relative affinities of these agonists for their intended receptors (1 nM at Y$_2$ and >1 nM at Y$_1$, respectively, Cabrele et al., 2000; Soll et al., 2001). Agonists were applied either alone or in the presence of antagonists in 10 mL of saline. At the flow rates used, agonist application lasted between 3 and 5 min, while washout was continued until the reversal of agonist effects or a minimum of 20 min.

Concentrations of antagonists used were determined from previous experiments (BIIE0246 – El Bahh et al., 2002; Novartis 1 – Pronchuk et al., 2002), and were initially based on the known affinities of the antagonist for the target receptor. In the present experiments, we used a comparatively high (1 μM) concentration of the relatively potent Novartis 1 (2 nM affinity for Y$_5$ receptors, see below) to ensure blockade of Y$_5$ receptors, whereas we used substantially lower concentrations (30–100 nM) of BIIE0246, which has a similar (3 nM) affinity for the Y$_2$ receptor. To assess antagonist effects, we first established the effect of the agonist on a given preparation, then an antagonist was pre-applied for 15 min (in pEPSP experiments) or for at least 30 min (for STIB experiments), before agonists were again tested in the presence of the antagonist (El Bahh et al., 2002).

**Statistical analyses**

Each preparation served as its own control for comparison of agonist actions. Statistical comparisons were performed where appropriate using a paired t-test (Graphpad Prism 4.0). For antagonist experiments, comparisons were made between responses taken after the equilibration period of the antagonist, immediately before application of the agonist. We never observed significant changes in synaptic responses with antagonist applications alone. Because of the very long duration of the in vitro experiments, usually only one slice per animal could be analysed.

**In vivo experiments**

**Animals**

Male C57BL/6, C57x129 and Y$_2$$^{−/−}$ mice (~60 days old) were used for assessing seizure susceptibility to kainic acid (KA) and in experiments involving intracerebral application of Y$_2$ or Y$_5$ receptor ligands.

**Intrahippocampal injections and EEG recordings**

For intrahippocampal injections, mice were anesthetized with Equithesin (3.5 mL/kg: 1% phenobarbital/4% chloral hydrate, Sigma, St Louis, MO, USA), and an injection guide cannula was secured to the skull with acrylic dental cement. Mice were allowed 3–5 days to recover from the surgical procedure before the start of the study (Vezzani et al., 2000).

**Treatments**

Drugs were dissolved in their respective vehicles and injected in a volume of 0.5 μL. The time course of drug injection was 1 min, after which the needle was left in place for a further 1 min before removal (to avoid backflux). NPY agonists were infused intrahippocampally, 5 min before a local injection of 33 pmol/0.5 μL KA (Sigma) in freely moving mice, a dose which induces EEG seizures but not damage (Vezzani et al., 2000). GW438014A (Daniels et al., 2002) was injected at 10 mg/kg intraperitoneally in a fine suspension of 0.5% methylcellulose containing 0.1% Tween 80, 30 min before administration of the Y$_5$ receptor agonist and/or KA. Although considerably less potent than Novartis 1, GW438014A has a high selectivity and moderate potency for NPY-Y$_5$ receptors (211 nM for Y$_5$ vs. >10 000 nM for Y$_1$, Y$_2$ and Y$_4$), and has demonstrated bioavailability when administered intraperitoneally (Daniels et al., 2002), which Novartis 1 does not. The dose and timing of the administration of this antagonist has previously been shown to inhibit feeding mediated by centrally injected NPY (Daniels et al., 2002). Control mice were injected intrahippocampally and/or systemically with the corresponding volumes of vehicle or scrambled NPY.

**Seizure assessment**

EEG recordings were carried out in the hippocampus of freely moving mice, as previously described (Vezzani et al., 2000). After baseline recording of spontaneous EEG, drugs were injected through a needle extending 1.5 mm below the guide cannula into dorsal hippocampus. Continuous EEG recordings were made for at least 90 min after drug injection; EEG recordings were ended only if no seizures were observed for at least 30 min. Ictal episodes were characterized by high-frequency and/or multispike complexes and/or high-voltage synchronized spikes simultaneously occurring in both hippocampi. Seizures were quantitated by determining the latency to the first seizure (onset), the number of seizures, and the time spent in ictal activity and spiking (interictal activity) (Vezzani et al., 2000).

Three days after seizure assessments, mice were decapitated and their brains postfixed in 4% buffered paraformaldehyde, cryoprotected by immersion in 30% sucrose in phosphate-buffered saline, and Nissl stained to assess the correct positioning of the injection cannula and electrodes. We observed nonspecific damage due to mechanical insertion of the electrodes and cannula only in KA-injected mice. This pattern was similar to that observed in mice receiving NPY agonists or antagonists.

**Statistical analysis**

Data from experiments examining the actions of the Y$_2$-selective agonist [Ahx$^{5,24}$]NPY on kainate-induced seizures were compared using Student’s t-test. Data from experiments examining the actions of the Y$_5$ agonist and antagonist were compared using a one-way ANOVA followed by Tukey’s test. Data examined the effects of Y$_2$ and Y$_5$ agonists in the Y$_2$ knockout mouse were compared using a two-way ANOVA followed by Tukey’s test.

**Binding studies**

Novartis 1 (Y$_5$ antagonist) characterization

Binding assays were performed with crude membranes isolated from cells transfected with cDNA constructs for the human Y$_1$, Y$_2$, Y$_4$ or...
TABLE 1. Characterization of Novartis 1 affinity at human NPY/PP receptors

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_i$ (± SEM) (nM)</th>
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<tbody>
<tr>
<td>hY$_1$</td>
<td>&gt; 1000</td>
<td>2</td>
</tr>
<tr>
<td>hY$_2$</td>
<td>&gt; 1000</td>
<td>2</td>
</tr>
<tr>
<td>hY$_4$</td>
<td>&gt; 1000</td>
<td>2</td>
</tr>
<tr>
<td>hY$_5$</td>
<td>1.72 ± 0.73</td>
<td>4</td>
</tr>
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Y$_5$ receptors. Cells were scraped from the culture plates into PBS and pelleted in tubes. The homogenate binding studies were conducted as previously described (Gehrert et al., 1996, 2001). The cell pellets were resuspended using a glass homogenizer in a 25 μm HEPES (pH 7.4) buffer containing 2.5 mM CaCl$_2$, 1 mM MgCl$_2$ and 2 g/L bacitracin. Cell membranes were incubated in a final volume of 200 μL containing 0.1 nM $^{[125]}$IhPYY for Y$_1$, Y$_2$ and Y$_5$ (SA 2200 Ci/mmol, DuPont-NEN, Boston, MA, USA) or 0.1 nM $^{[125]}$IPPY (SA 2200 Ci/mmol, DuPont-NEN) for 2 h at room temperature. Nonspecific binding was defined as the amount of radioactivity remaining bound after incubating in the presence of 1 μM human NPY (Y$_1$, Y$_2$ and Y$_5$ – Gehrert et al., 2001) or 1 μM bovine PP (Y4 – Gehrert et al., 1996). Incubations were terminated by rapid filtration through GF/C filters (Wallac, Gaithersburg, MD, USA), which had been presoaked in 0.3% polyethyleneimine (Sigma), using a TOMTEC (Orange, CT, USA) cell harvester. The filters were washed with 5 mL of 50 mM Tris (pH 7.4) at 4 °C and rapidly dried at 60 °C. The dried filters were treated with MeltiLex A (Wallac), melt-on scintillator sheets, and the radioactivity retained on the filters counted using the Wallac 1205 Betaplate counter. The results were analysed using the Excel (Microsoft Corp., Redmond, WA, USA) or Prism software packages (Graphpad, San Diego, CA, USA). Protein concentrations were measured with Comassie Protein Assay Reagent (Pierce, Rockford, IL, USA) using BSA for standards. This antagonist had no significant affinity for any other Y receptor except Y$_5$, where the $K_i$ was about 2 nM (Table 1).

Receptor autoradiography

This performed as described previously (Fürtinger et al., 2001). Animals used in these studies (both Sprague–Dawley rats and mouse strains as indicated) were between 2 and 3 months of age. $^{[125]}$IhPYY$_{3-36}$ (Y$_2$ + Y$_5$) and $^{[125]}$IPhPYY (Y$_1$ + Y$_5$) (Neosystems, Strasbourg, France) were freshly iodinated and purified by HPLC as described previously (Fürtinger et al., 2001). Frozen sections (20 μm) of the dorsal (coronal sections) and ventral (horizontal sections) hippocampus from WT, Y$_5^-$/Y$_5^-$ (Howell et al., 2003), Y$_2^+$/~ (Sainsbury et al., 2002) and Y$_5^+$/~ (Marsh et al., 1999) mice were preincubated for 30 min at room temperature in 200 mM K$_2$HPO$_4$–H$_2$PO$_4$–Tris buffer (118 mM NaCl, 4.8 mM KCl, 1.3 mM MgSO$_4$, 1.2 mM CaCl$_2$, 50 mM glucose, 15 mM NaHCO$_3$, 1.2 mM KH$_2$PO$_4$, 10 mM Tris, pH 7.3), then were incubated for 2 h in 20 mL buffer supplemented with 0.1% bovine serum albumin, 0.05% bacitracin and 50 μM of the respective radioligand. Displacement studies were performed with BIBO3304 (100 nM), Novartis 1 (30 and 100 nM), [δ-Trp$^{22}$]NPY (100 nM) and AlaAibNPY (30 and 100 nM). Nonspecific binding was determined in the presence of 1 μM NPY. After incubation, sections were dipped twice and then washed for 30 s in ice-cold buffer, dipped in deionized water and rapidly dried under a stream of cold air. They were exposed to BioMax MR films (Amersham Pharmacia Biotech, Bucks, UK) for 2–3 days together with $^{[125]}$I-microscales.

The autoradiograms were developed, digitized and analysed using a computer-assisted image analysis system (Metamorph 3, VisiTron System, Puchheim, Germany) equipped with a Zeiss CCD video camera. Absorbance was measured in the molecular layer and the hilus of the dentate gyrus and in the stratum radiatum of area CA1. Gray values were converted to fmoL/mg wet weight using $^{[125]}$I-microscales as standards. Specific binding was calculated by subtracting the nonspecific from total binding. Binding was performed on three sections from 4–6 animals for each animal and experimental condition studied. Comparisons were made using Student’s t-test.

Results

Evoked synaptic responses

NPY has potent inhibitory effects on evoked, excitatory synaptic transmission in both area CA1 and area CA3 of hippocampal slices prepared from rats and a series of WT mice. Thus, the pan-agonist NPY (1 μM) suppressed pEPSPs in hippocampal area CA1 of rat, and WT mice having either a 129/Sv, C57BL/6 or mixed C57BL/6 × 129/Sv background (C57 × 129) (Sainsbury et al., 2002) by 77–86%, with EC$_{50}$ values ranging between 118 and 162 nM (Fig. 1a–c). In hippocampal area CA3, 1 μM NPY inhibited the pEPSPs by a smaller, but significant amount, 47 ± 4.7% (P < 0.02, n = 7) in rat or WT mice, 32.7 ± 4.1% in 129/SvJ WT (P < 0.01, n = 4) and 31.5 ± 4.4% in C57 × 129 WT (P < 0.02, n = 4).

We also tested the actions of Y$_2$ or Y$_5$ receptor-agonist preferring antagonists in both areas of the hippocampus in rats and three WT strains. In area CA1 of rat and WT (129/SvJ and C57 × 129) mice, the Y$_2$-selective agonist [ahx$_5$–$^{24}$]pNPY (1 μM – Rist et al., 1995; El Bahh et al., 2002) inhibited the pEPSPs by 51–67% (Fig. 1b), whereas the same concentration of the potent, Y$_5$-preferring agonist [hPP$_{1-17}$, Ala$_{31}$Aib$_{32}$]NPY (AlaAibNPY – Cabrele et al., 2000) inhibited the pEPSPs only by 11–14% in rat and all three WT mouse strains (Fig. 1c).

We determined the receptor selectivity of NPY and of the agonists we used by comparing their effects in the absence and presence of antagonists specific to the Y$_2$ or Y$_5$ receptors in both hippocampal areas. The effect of NPY in area CA1 of rat and WT (129/SvJ and C57 × 129) mice was reduced by 60–80% by 30 nM of the Y$_2$-specific antagonist, Y$_5$ receptors.
antagonist BIIE0246 (Doods et al., 1999; El Bahh et al., 2002) \((P < 0.01, n = 4–8)\); the same concentration of BIIE0246 reduced the effect of the Y\(_2\) preferring agonist [ahx\(^{5-24}\)]NPY in rat and these two WT mice by 70–86\% (\(P < 0.01, n = 4–11\); Fig. 1d). By contrast, the Y\(_5\)-specific antagonist Novartis 1 (1 \(\mu\)M–Pronchuk et al., 2002) had no significant influence on the actions of either NPY or AlaAibNPY in rat or in any of the WT mice tested (Fig. 1d and e). Interestingly, the small but significant inhibitory actions of the Y\(_5\) agonist observed in area CA1 of rat and all three WT mice tested were not altered by the

Y\(_5\) antagonist, but were significantly reduced by 30 nM BIIE 0246 (Fig. 1e, \(P < 0.05, n = 4\)).

Similar responses to agonists and antagonists were observed in area CA3 of rat. Thus, whereas there were little or no significant changes caused by Novartis 1 in the responses to 1 \(\mu\)M applications of NPY, [ahx\(^{5-24}\)]NPY, BIIE 0246 (30 nM) significantly reduced responses to both agonists; the responses to 1 \(\mu\)M AlaAib NPY in rat area CA3 were not significant (pEPSP inhibition of 6.7 \(\pm\) 2.9\%, \(n = 6, P > 0.05\)) (Fig. 1f).
We also compared the actions in WT mice of the putatively mixed Y2/Y5-preferring agonist NPY13–36 (Woldbye et al., 1997; Baraban, 2002). NPY13–36 (1 μM) inhibited evoked synaptic responses in area CA1 of 129/SvJ mice and in area CA3 of both 129/SvJ and C57 × 129 mice. However, as observed with the other agonists, the effect of NPY13–36 was not significantly inhibited by the Y2-specific antagonist Novartis 1 (1 μM), but was inhibited strongly by 30 nM BIIE2046 (Fig. 1g). Thus, this mixed agonist also appears to act only through a Y2 receptor.

We next tested NPY and related agonists in areas CA1 and CA3 of the hippocampus from Y2 receptor-deficient mice. In contrast to all WT mice studied, 1 μM concentrations of NPY or agonists selective for the Y2 or Y5 receptor had no significant effect on pEPSPs evoked in both hippocampal areas of Y2–/– mice (Fig. 1h), nor did a Y1 receptor-selective agonist (F13NPY – Soll et al., 2001). However, the GABAβ agonist baclofen (10 μM; Klapstein & Colmers, 1992; Qian et al., 1997) inhibited the pEPSPs in areas CA1 and CA3 of Y2–/– mice by 85% (n = 10) and 50% (n = 5), respectively (Fig. 1h), indicating that presynaptic neuromodulatory mechanisms at these synapses are not altered by the genetic inactivation of the Y2 receptor.

These results indicate that NPY inhibits excitatory synaptic transmission at Schaffer collateral–CA1 and mossy fiber–CA3 synapses of all species tested by activation of Y2 receptors alone.

**In vitro epileptiform discharges**

To assess the contribution of specific receptor subtypes to the anticonvulsant actions of NPY we used two different models of epileptiform activity in vitro, the 0-Mg2+ bursting model (Mody et al., 1987; Klapstein & Colmers, 1997; Marsh et al., 1999) and STIB, an in vitro model of limbic seizures (Stasheff et al., 1985; Klapstein & Colmers, 1997; Ho et al., 2000; El Bahh et al., 2002), which is sensitive to therapeutic concentrations of anticonvulsant drugs effective against partial complex seizures (Clark & Wilson, 1992). In the 0-Mg2+ bursting model, which is insensitive to therapeutic levels of anticonvulsants (Zhang et al., 1995), but which others have used previously to test Y5 agonists (Marsh et al., 1999), we measured the actions of NPY and the Y5 agonist in slices from WT (C57 × 129) mice, in saline or in the presence of either Novartis 1 (1 μM) or BIIE2046 (100 nM). At 1 μM, NPY reduced bursting frequency by about 40% in saline (P < 0.03, n = 6) or in the presence of 1 μM Novartis 1 (P < 0.02, n = 6) but its actions were blocked by the Y2 antagonist (P < 0.003). By contrast, the Y5 agonist showed no measurable effect under any of these conditions (Fig. 2).

We next assessed the response to NPY and selective agonists and antagonists in STIB induced in area CA3 of hippocampal slices from rat, WT (C57BL/6 and C57 × 129) and Y2–/– mice. In slices from rats and WT (C57BL/6 and C57 × 129) mice, application of only 300 nM NPY induced a profound, prolonged but reversible suppression of the afterdischarges (Fig. 3a). Similar though less prolonged effects were observed with application of the Y2-preferring agonist [Ahx5–24]NPY (1 μM). However, consistent with previous results (El Bahh et al., 2002), neither the Y1- nor the Y2-preferring agonists (applied at 1 μM) reduced the afterdischarges in either of the WT mouse strains used in these experiments. Consistent with the results obtained on evoked synaptic responses, pharmacological blockade of Y2 receptors with 100 nM BIIE2046 also completely eliminated the actions of NPY and the Y2-preferring agonist on STIB in slices from either WT strain (Fig. 3a). As expected, the Y2-specific antagonist did not affect the suppression of STIB by baclofen or 2-CA (data not shown). Pharmacological blockade of Y5 receptors with Novartis 1 (1 μM) reduced the afterdischarges in either of the WT mouse strains used in these experiments.

![Fig. 2. NPY inhibition of spontaneous (0-Mg2+) bursting in slices from C57 × 129 WT mice is mediated by Y2 receptors. (a) Recordings of spontaneous activity in a slice from a C57 × 129 WT mouse, each showing a 60-s segment of 0-Mg2+ bursting recorded in control, during drug application and after washout. Upper panel: effect of NPY alone. Middle panel: effect of NPY (1 μM) in the continuing presence of BIIE2046 (100 nM). Lower panel: effect of the Y2-preferring agonist AlaAibNPY. (b) Effects of NPY and AlaAib NPY on 0-Mg2+ bursting in slices from C57 × 129 hippocampus in control, in the presence of the Y5 antagonist Novartis 1 (1 μM) or in the presence of BIIE0246 (100 nM). n = 6 for all conditions. Paired-t-test was used to compare responses as in Fig. 1. *P < 0.05, **P < 0.01.](image-url)
NPY anti-epileptic effect is via \( Y_2 \) not \( Y_5 \) receptors

Fig. 3. NPY inhibition of STIB epileptiform discharges in hippocampus requires the \( Y_2 \) receptor. (a and b) Representative traces showing the effects of NPY on primary afterdischarges (1\(^{st}\)AD) induced in vitro by high-frequency stimulation (arrowheads) in area CA3 of the hippocampus. (a) NPY (300 nM) completely blocked the 1\(^{st}\)AD in slices from rat, C57Bl/6 and C57 x 129 WT mice; pretreatment of the same slice with the \( Y_2 \) antagonist BIIE0246 (100 nM) completely blocked the effect of NPY. Identical results were obtained in slices from 3–6 animals per group. (b) In hippocampal slices from \( Y_2^{-/-} \) mice, none of the NPY agonists tested affected the 1\(^{st}\)AD, although the GABA\(_B\) agonist baclofen was effective (lower right panel).

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did not alter the effects of NPY or the Y$_2$-preferring agonist in slices from WT (129/SvJ) mice. Neither the Y$_2$ nor the Y$_5$ antagonists by themselves had significant effects on STIB.

In slices from Y$_2^{-/-}$ mice, application of NPY or any of the Y$_1$, Y$_2$, and Y$_5$ receptor-preferring agonists (all at 1 µM) had no effect on the occurrence, latency or duration of the STIB afterdischarges (Fig. 3b).
Fig. 5. Effects of Y<sub>2</sub>- and Y<sub>5</sub>-preferring agonists on kainic acid-induced EEG seizures in mouse hippocampus in vivo. (a) Intrahippocampal injection of the Y<sub>2</sub>-preferring agonist [ahx<sup>5-24</sup>]NPY 5 min before KA injection induced a significant inhibition of seizure number, increase in latency of seizure onset and reduction in time spent in seizures in C57BL/6 mice compared with animals pretreated with ‘scrambled’ peptide. (b) Effects similar to those seen with the Y<sub>2</sub> agonist were observed when the mice were pre-injected with AlaAibNPY. However, pretreatment of the animals with a Y<sub>5</sub> antagonist (GW438014A) 30 min before intrahippocampal KA injection did not block the AlaAibNPY effects on seizures. (c) Effects of intrahippocampal injection of KA in WT (C57·129) and Y<sub>2</sub>−/− mice. Pre-injection of either [ahx<sup>5-24</sup>]NPY or AlaAibNPY before KA injection had no effect on seizures in Y<sub>2</sub>−/− mice. Each bar represents the mean ± SEM for 8–25 animals per group. Comparisons in (a) were made using Student’s t-test, comparisons in (b) were made using a one-way ANOVA followed by Tukey’s test and comparisons in (c) were made using a two-way ANOVA followed by Tukey’s test. **P < 0.01 vs. control.

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$^{125}$I-hPYY$_{3-36}$ $^{125}$I-Pro$_{34}^3$hPYY

$\text{WT}$  
$\text{WT}$

$\text{+ Novartis 1}$  
$\text{+ BIBO3304}$

$\text{+ NPY (1μM)}$

$\text{Y1$^+$}$  
$\text{Y1$^+$}$

$\text{Y2$^+$}$  
$\text{Y2$^+$}$

$\text{Y5$^+$}$  
$\text{Y5$^+$}$

$\text{Y5$^+$}$  
$\text{Y5$^+$}$

+ BIBO3304
although they remained sensitive to 10 μM baclofen (Fig. 3b) and 1 μM 2-CA (data not shown).

The results from these two in vitro models of epileptiform activity are consistent with the idea that the anti-epileptic effect of NPY in vitro requires the activation only of Y2 receptors.

In vivo seizures

Here we tested the anticonvulsant action of the intrahippocampal injection of Y2 and Y5 receptor-prefering agonists on limbic motor seizures induced by local injection of KA in WT and Y2 knockout mice (Sperk, 1994). Seizures were monitored behaviorally and with EEG recordings (Vezzani et al., 2000).

Although most Y5−/− mice did not exhibit any behavioral phenotypes, a small minority of mice demonstrated spontaneous seizures after handling, and in 2/10 mice spontaneous interictal spiking was recorded in the absence of any manipulation. Because of its rarity, we did not study this effect in detail. WT (C57BL/6 or C57 × 129) mice were never observed to display spontaneous or handling-induced seizures.

As described previously (e.g. Vezzani et al., 2000), intrahippocampal injection of 33 pmol KA results in epileptic-like discharges (ictal activity) and interictal spiking in both ipsilateral and contralateral hippocampus (Fig. 4). No generalized behavioral convulsions were observed; however, staring and immobility often preceded and accompanied the occurrence of EEG ictal episodes. Prior injection of 24 nmol of the Y2-prefering agonist [ahx5–24]NPY into the ipsilateral hippocampus of WT (C57BL/6) mice (n = 8–25) significantly increased the latency to onset of KA-induced seizures by 134% (P < 0.01), reduced the number of seizure episodes by 50% (P < 0.01) and shortened the duration of epileptic activity by 64% (P < 0.01), compared with control animals injected with scrambled peptide (Figs 4 and 5a). Injection of 6.8 nmol of the Y5-prefering agonist AlaAib NPY, a dose that induced food intake in rats (Cabrele et al., 2000), also reduced the number of seizure episodes by 44% (P < 0.01) and the time spent in seizure activity by 38% (P < 0.01) in KA-treated mice (n = 7–12) (Figs 4 and 5b). However, pretreatment with intraperitoneal injection of 10 mg/kg of the Y5 receptor-specific antagonist GW438014A, a treatment that blocked feeding induced by centrally administered NPY (Daniels et al., 2002), did not alter the ability of the Y5-prefering agonist to reduce seizure activity in WT (C57BL/6) mice (Fig. 5b). Because of its local toxicity, we could not test the effects of BIIE0246 in these in vivo experiments.

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In response to intrahippocampal KA injection, both $Y_2^R$ and WT (C57 × 129) mice ($n = 15–18$) exhibited similar latency to onset, number of seizures and duration of ictal activity. The only difference observed was a greater duration of interictal activity seen in $Y_2^R$ mice ($P < 0.01$, Fig. 5c). However, in contrast to the WT mice, pre-injection of either the $Y_2$ or the $Y_5$ agonist in $Y_2^R$ animals did not significantly affect any of these indices of seizure activity (Fig. 5c).

These results strongly suggest that the $Y_2$ receptor is essential for NPY suppression of limbic seizures in vivo.

**Receptor autoradiography**

The $Y_2$- or $Y_5$-preferring agonist $[^{125}]$hPYY$_{3-36}$ demonstrated pronounced labeling of the strata radiatum and oriens in areas CA1 to CA3 of WT and $Y_2^R$ (Howell et al., 2003) but not $Y_2^R$ animals (Fig. 6a). This binding was not significantly reduced by the $Y_2$-preferring agonists AlaAibNPY (30 and 100 nm) and [D-Trp$^2$]NPY (30 nm) or the $Y_5$-specific antagonist Novartis 1 (30 and 100 nm), indicating that $[^{125}]$hPYY$_{3-36}$ specifically labeled $Y_2$ but not $Y_5$ receptors. The $Y_1$, $Y_2$ receptor agonist $[^{3}H]Phe^3$hPYY (Dumont et al., 1996; Marsh et al., 1999) bound intensely to receptors in the outer layer of the cerebral cortex, in the molecular layer of the dentate gyrus and in the strata oriens and radiatum of CA1 to CA3 of WT and $Y_2^R$ mice (Fig. 6b). Binding in the cortex and dentate gyrus molecular layer was abolished when slices were incubated with 100 nM of the potent $Y_1$, receptor-specific antagonist BIBO3304 (Wieland et al., 1998) and in $Y_2^R$ mice (Fig. 6b). Binding of $[^{125}]$hPYY in these regions was unaffected by Novartis 1 (30 or 100 nm), AlaAibNPY (30 or 100 nm) or 30 nm [D-Trp$^2$]NPY, consistent with its labeling only $Y_1$ receptors there. In strata oriens and radiatum of areas CA1 to CA3, binding of $[^{125}]$hPYY was not reduced either by 100 nM BIBO3304 or in $Y_2^R$ mice, consistent with the presence of $Y_2$ binding sites. Here, however, we observed slight but measurable reductions in $[^{125}]$hPYY binding in the presence of $Y_5$ ligands. Thus, AlaAibNPY (100 nm) reduced it by 21%, [D-Trp$^2$]NPY (30 nm) reduced it by 9% and Novartis 1 (100 nm) reduced it by 12%. These results are at best consistent with very low levels of $Y_5$ receptors; indeed, similar levels of $[^{125}]$hPYY binding were also seen in $Y_2^R$ mice (Fig. 6b). Quantitative measurements are shown in Fig. 6c.

**Discussion**

Various neurotransmitter and neuromodulatory systems have been found to contribute to inhibition in the brain. Thus, enhancing the activity of a given inhibitory system by either exogenous application of an agonist or increasing the endogenous release of the natural agonist not surprisingly results in increased inhibition. However, it does not necessarily follow from this that the primary biological role for that system is to act as an ‘endogenous anticonvulsant’. Thus, NPY potently suppresses seizure activity in vitro and in vivo. The present study provides broad pharmacological and genetic evidence both in vitro and in vivo that NPY and a wide variety of agonists modulate excitatory synaptic transmission and epileptiform activity in the hippocampus by activation of exclusively the $Y_2$ receptor subtype, while providing no evidence for a significant role of $Y_5$ or other $Y$ receptors, despite conditions biased in favor of finding such evidence. However, consistent with the caveat above, our results do not indicate that the ablation or pharmacological blockade of the $Y_2$ receptor significantly alters excitability in vitro nor does ablation of the hippocampal $Y_2$ receptor result in development of significant seizure activity in mice in vivo.

We have previously shown in vitro that activation of presynaptic NPY receptors reduces glutamate release from presynaptic terminals by suppressing voltage-dependent Ca$^{2+}$ currents (Toth et al., 1993; Qian et al., 1997) and that this action was sufficient to suppress epileptiform activity in vitro (Klapstein & Colmers, 1997). Here, we demonstrate that the $Y_2$ receptor is both necessary and sufficient to mediate this effect in hippocampus in vitro and in vivo. Genetic ablation of $Y_2$ receptors totally abolishes the anti-epileptic actions of NPY and all agonists tested in vitro and in vivo, in contrast to previous studies (Woldbye et al., 1997; Marsh et al., 1999; Baraban, 2002) which had suggested that $Y_2$ receptors are essential for NPY’s actions. In a spectrum of WT animals, even micromolar concentrations of the potent, $Y_2$ receptor-specific antagonist Novartis 1 affected neither the modest effects of $Y_2$-preferring agonists in vitro nor their more significant effects in WT mice in vivo. By contrast, the $Y_2$ antagonist B19E0246, at concentrations of only 30–100 nm, sharply reduced or fully blocked the in vitro effects of the $Y_2$ receptor-preferring agonists tested in these same WT strains, including the 129/SvJ strain used as the WT for the $Y_5$ receptor-knockout mouse (Marsh et al., 1999). Neither the knockout nor the pharmacological blockade of the $Y_2$ receptor affected the ability of the neuromodulatory GABA$\beta$ and adenosine A$_1$ receptors, which also use the same intracellular signaling pathway as NPY to suppress glutamate release (Klapstein & Colmers, 1992; Qian et al., 1997). Despite an experimental approach that favored the identification of $Y_2$ receptor-mediated effects, we found no evidence to support a role of $Y_2$ receptors in any of the present experiments. It is also important to note that even agonists which were designed to have both high affinity and specificity for the $Y_5$ receptor subtypes nevertheless still had measurable effects on $Y_2$ receptors, as these effects were blocked by the $Y_2$ receptor antagonist (but not by the $Y_2$ antagonist) or were absent in $Y_2^R$ mice. Thus, any conclusions about NPY receptor subtypes based on agonist responses alone must be interpreted with caution until properly verified with receptor-specific antagonists or knockout animals.

Whereas knockout of $Y_2$ receptors in these mice completely prevented the effects of NPY on synaptic activity and in vitro seizure models, the $Y_2$ knockout mice showed remarkably little difference from the WT in their responses to intrahippocampal application of KA. Indeed, the only difference in the response between $Y_2^R$ and WT mice to kainate was a slightly increased duration of interictal activity. Previous studies with the NPY knockout (Erickson et al., 1996; Baraban et al., 1997) or the $Y_5$ receptor knockout mouse (Marsh et al., 1999) reported the dramatic, highly lethal consequences for genetic alterations to the NPY system in the responses to kainate. The difference with the present results may arise from the in vivo experimental approaches. Here we injected KA and receptor-selective agonists directly into the hippocampus, while in previous reports KA was applied either peripherally or i.c.v., both of which would cause a much greater volume of the brain to be affected by KA than in our experiments. In addition, the genetic background of the animals appears to influence the seizure susceptibility in $Y_2$ knockout mice (Marsh et al., 1999). In the present experiments, the in vitro pharmacological results were remarkably consistent in rats and a broad spectrum of WT mice, including the 129/SvJ (Marsh et al., 1999) and C57BL/6 parent strains and the C57 × 129 animals which had the same backcrossed background as the $Y_2^R$ (Sainsbury et al., 2002). The in vivo results were furthermore in complete agreement with the results from the in vitro experiments. Our findings also highlight that some receptor-preferring agonists that appear very selective at the low concentrations of radioligand employed in binding experiments can lose their selectivity when used at elevated concentrations, which frequently occurs in in vivo experiments. One...
important caveat in comparing the present results is that the in vitro electrophysiological experiments were performed using younger animals (3–5 weeks) than were used for the other experiments (2–3 months). However, earlier results on the potent inhibition of glutamate release by NPY and Y2 agonists (Greber et al., 1994), which were conducted in hippocampal slices from 2- to 3-month-old rats, support and are entirely consistent with the present in vitro and in vivo findings.

Results from binding experiments were also fully consistent with the functional observations. Thus, prominent populations of Y2 (strata oriens and radiatum of areas CA1 to CA3) and Y1 receptors (dentate gyrus molecular layer) were observed. Ligands at these binding sites were selectively displaced by Y2- and Y1-preferring ligands and were abolished in Y2- and Y1-mice, respectively, but were neither displaced by Y3 ligands nor abolished in Y3 mice, and thus represent unambiguous Y2 and Y1 receptor sites. Thus, we found no evidence in these experiments for Y5 receptors, despite previous reports (e.g. Bregola et al., 2000), however, the nature of some of the binding sites labeled by [125I]Pro4-hPYY in strata radiatum and oriens is not clear. A significant population of these sites, seen in WT mice, which was not displaced by the Y2-preferring agonists AlaAibNPY and [d-Trp5]NPY, or the specific Y2 and Y1 receptor antagonists Novartis 1 and BIBO3304, respectively, also remained in all three knock-out lines, and were only reduced by only about 15–20% by the Y2/Y1 ligand rPp, and by about 40% with the Y2-preferring agonist [a,x5-24]NPY (data not shown). Such [125I]Pro4-hPYY-labeled sites were not seen in the human hippocampus (Fürtinger et al., 2001) and could represent y6 sites in the mouse (Michel et al., 1998), or potentially another, as yet uncharacterized, receptor (Lin et al., 2005).

Regardless, the present binding data do not provide any evidence for the presence of detectable Y5 receptors in mouse hippocampus.

Interestingly, the absence of significant Y5 receptor binding in the rat is at odds with the high levels of Y5 mRNA observed in all hippocampal subfields (e.g. Kopp et al., 1999). Thus, Dumont et al. (1998) reported Y5-specific binding only in area CA3 of the ventral extension of rat hippocampus. Our present results also indicate the presence of a small proportion of binding sites that are labeled by the Y1/Y5-preferring ligand [125I]Pro4-hPYY (50–90%) and that are displaced by the Y2-preferring agonist AlaAibNPY and the antagonist Novartis 1. Provided that the [125I]Pro4-hPYY at a concentration of 50 pm sufficiently labels Y5 sites in this tissue, these data argue for the presence of very low, but demonstrable, levels of Y5 binding sites in the hippocampus. By contrast, the binding of [125I]Pro3-hPYY that was not displaced by the Y1 antagonist BIBO3304 in the Y5-mice may represent an additional class of Y-receptors, as recently suggested (Dumont et al., 2005; Lin et al., 2005).

Although the present results make it certain that the Y2 receptor is essential for NPY regulation of limbic seizures originating in the hippocampus, different NPY receptor subtypes are known to regulate excitability in other regions of the brain, including Y5 receptors (e.g. Sun et al., 2001). Recent evidence in rats in vivo, using the same Y5 antagonist used here, indicates that the threshold for hippocampal rapid kindling can be reduced by Y5 agonists, and that this effect is blocked by the Y3 antagonist (Benmaamar et al., 2005).

The site of this action is most likely extrahippocampal (Benmaamar et al., 2005). The effect of an acute functional inactivation of the Y2 receptor in adult animals remains unknown, but may be answered with the advent of Y2 antagonists with lower toxicity than that used here. This would also permit a test of the interesting question of the function of the Y2 receptor in the normal physiology of the hippocampus. Given the present results however, it is most unlikely that NPY receptors other than Y2 significantly regulate excitability within the hippocampal formation, making this the most promising Y receptor as a target for potential new treatments for some focal epilepsies.

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Abbreviations

EEG, electroencephalography; KA, kainic acid; NPY, neuropeptide Y; pEPSPs, population excitatory postsynaptic potentials; SBs, spontaneous bursts; STIB, stimulus train-induced bursting; WT, wild type.

References


