Journal of Neurochemistry, 2005, **93**, 163–170 doi:10.1111/j.1471-4159.2004.03005.x

Up-regulation of neuropeptide Y levels and modulation of glutamate release through neuropeptide Y receptors in the hippocampus of kainate-induced epileptic rats

Ana P. Silva,*,[1] Sara Xapelli,*,[1] Paulo S. Pinheiro,* Raquel Ferreira,* Joana Lourenço,* Armando Cristóvão,* Eric Grouzmann,† Cláudia Cavadas,*,‡ Catarina R. Oliveira,*,§ and João O. Malva*,§

*Center for Neuroscience and Cell Biology, University of Coimbra, Portugal

†Centre Hospitalier Universitaire Vaudois, Division de Pharmacologie et Toxicologie Clinique, Lausanne, Switzerland

‡Laboratory Pharmacology, Faculty of Pharmacy, University of Coimbra, Portugal

§Institute of Biochemistry, Faculty of Medicine, University of Coimbra, Portugal

Abstract

Kainate-induced epilepsy has been shown to be associated with increased levels of neuropeptide Y (NPY) in the rat hippocampus. However, there is no information on how increased levels of this peptide might modulate excitation in kainate-induced epilepsy. In this work, we investigated the modulation of glutamate release by NPY receptors in hippocampal synaptosomes isolated from epileptic rats. In the acute phase of epilepsy, a transient decrease in the efficiency of NPY and selective NPY receptor agonists in inhibiting glutamate release was observed. Moreover, in the chronic epileptic hippocampus, a decrease in the efficiency of NPY and the Y_2 receptor agonist, NPY13-36, was also found. Simultaneously, we observed that the epileptic hippocampus expresses higher levels of NPY, which may account for an increased basal inhibition of glutamate release. Consistently, the blockade of Y_2 receptors increased KCl-evoked glutamate release, and there was an increase in Y_2 receptor mRNA levels 30 days after kainic acid injection, suggesting a basal effect of NPY through Y_2 receptors. Taken together, these results indicate that an increased function of the NPY modulatory system in the epileptic hippocampus may contribute to basal inhibition of glutamate release and control hyperexcitability.

Keywords: epilepsy, glutamate, hippocampus, neuropeptide Y, synaptosomes.

J. Neurochem. (2005) **93,** 163–170.

Changes in the expression of various neuropeptides have been observed in different models of temporal lobe epilepsy (Bellmann *et al*. 1991; Schwarzer *et al*. 1995) and the hippocampus has attracted particular interest due to its central role in limbic epilepsy (Ben-Ari 1985). Neuropeptide Y (NPY), a 36 amino acid peptide, is one of the most abundant and widely distributed neuropeptides in the central and peripheral nervous systems. It modulates numerous physiological processes including memory, anxiety, seizures and feeding behaviour (Michel *et al*. 1998). Central administration of NPY decreases pharmacologically- and electrically-evoked seizures (Smialowska *et al*. 1996; Woldbye *et al*. 1996, 1997), and transgenic mice lacking endogenous NPY exhibit increased sensitivity to seizure induction (Baraban *et al*. 1997). Moreover, the role of endogenous NPY in regulating seizure activity is supported by evidence of increased NPY levels under epileptic conditions in both inhibitory interneurones and in excitatory granule cells that, in control conditions, do not contain the peptide (Vezzani *et al*. 1999, 2002). Park and collaborators (2001) also observed an increase in the number of NPY-positive neurones, and that these neurones are resistant to kainate-induced seizures in the whole hippocampus. More recently,

Received October 12, 2004; revised manuscript received November 24, 2004; accepted November 24, 2004.

Address correspondence and reprint requests to João O. Malva, PhD, Center for Neuroscience and Cell Biology, Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004–504 Coimbra, Portugal. E-mail: jomalva@cnc.uc.pt

[1]Ana P. Silva and Sara Xapelli contributed equally to this study.

Abbreviations used: AMV, avian myeloblastosis virus; BSA, bovine serum albumin; DEPC, diethylpyrocarbonate; DG, dentate gyrus; EEG, electroencephalogram; EPSPs, excitatory postsynaptic potentials; KA, kainic acid; NPY, neuropeptide Y; OD, optical density.

others (Richichi *et al*. 2004) showed that long-lasting NPY expression in the rat hippocampus, using a recombinant adeno-associated viral vector, reduced electroencephalogram (EEG) seizures and delayed seizure onset markedly.

Seizure-related increase in NPY expression is accompanied by modified levels of NPY receptor subtypes in the hippocampus (Kofler *et al*. 1997; Gobbi *et al*. 1998; Schwarzer *et al*. 1998; Vezzani *et al*. 2000), and it seems that more than one receptor subtype could be responsible for mediating the anti-epileptic effects of NPY. This peptide inhibits excitatory neurotransmission and reduces glutamate release by acting mainly on presynaptic Y_2 receptors (Greber *et al*. 1994; Silva *et al*. 2001, 2003a). Indeed, electrophysiological and pharmacological studies have revealed that NPY acts predominantly through Y_2 receptors (Weiser *et al*. 2000; Silva *et al*. 2003a) and inhibits glutamate release by reducing Ca^{2+} influx (Schwarzer *et al*. 1998; Silva *et al*. 2001, 2003a). Moreover, the increase in Y_2 receptor binding in the hilus of the dentate gyrus is associated with enhanced release of NPY after kainate injection (Röder *et al*. 1996). The involvement of the Y_1 receptor subtype is not so clear, but previous studies have shown that these receptors also inhibit Ca^{2+} influx and glutamate release in the dentate gyrus (McQuiston *et al*. 1996) and CA3 hippocampal subregions, as well as in total hippocampus (Silva *et al*. 2001, 2003a). The pharmacological profile of centrally administered NPY analogues capable of inhibiting kainate-induced seizures and hippocampal excitability in rats also suggests the involvement, at least in part, of Y_5 receptors (Woldbye *et al*. 1997; Reibel *et al*. 2001). Indeed, using Y_5R-deficient mice, Marsh *et al.* (1999) demonstrated that these mice do not exhibit spontaneous seizure-like activity.

Accumulating evidence clearly suggests that seizure activity increases the expression of NPY and NPY receptors. In the present work, we have identified a role for NPY receptors in modulating glutamate release and the variation of NPY levels under epileptic conditions. Moreover, we also suggest that endogenous NPY contributes to basal inhibition of glutamate release in synaptosomes isolated from the epileptic rat hippocampus.

Materials and methods

Kainate treatment

Male 6-week-old Wistar rats (150–200 g) were injected intraperitoneally with kainic acid (KA) (Sigma, St Louis, MO, USA; 10 mg/kg body weight) dissolved in a maximum volume of 500 μL sterile 0.9% NaCl, and a control group of animals were injected with the same volume of saline. Rats were observed for at least 3 h and their behaviour was rated as described previously (Sperk *et al*. 1983). All the animals used in the present study achieved status epilepticus and were killed 6 h, 24 h, 72 h and 30 days after KA injection.

All procedures involving experimental animals were performed in accordance with European Community guidelines. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Isolation of rat hippocampal synaptosomes

A partially purified synaptosomal fraction (P_2) was isolated from hippocampi or from hippocampal subregions (CA1, CA3 and dentate gyrus-DG) of male Wistar control or epileptic rats (killed 6 h, 24 h, 72 h and 30 days after KA injection), essentially as described previously for brain cortex (McMahon *et al*. 1992) with some modifications (Malva *et al*. 1996; Silva *et al*. 2001). The hippocampi were homogenized in 0.32 M sucrose, 10 mM HEPES-Na, pH 7.4, using a Thomas B-Potter homogenizer (Thomas Scientific, Swedesboro, NJ, USA), and centrifuged at 3000 *g* for 2 min. The pellet obtained was resuspended, followed by sedimentation at the same speed. The combined supernatant fluids were spun for 12 min at 14 600 *g* and a P_2 pellet was obtained. The upper, whiter layer of the pellet, containing synaptosomes (Whittaker 1984), was removed with a small spoon and resuspended in the same sucrose medium as that used before.

Coronal slices of hippocampus (800 μm thick) were prepared for the isolation of synaptosomes from hippocampal subregions (CA1, CA3 and DG) of non-epileptic and epileptic rats (24 h and 30 days after KA injection). In each slice, the fimbria and the subiculum were separated from the rest of the slice under stereomicroscopic observation. CA3 subslices were obtained by separation from CA1 and dentate gyrus, and the last separation (CA1 from DG) was performed through the hippocampal sulcus (Silva *et al*. 2001). The pooled subslices were homogenized in the sucrose medium using a Thomas AA-Potter homogenizer, transferred to Eppendorf tubes and centrifuged as described for the isolation of whole hippocampal synaptosomes. The protein concentration was determined by the Biuret method (Layne 1957) for glutamate release experiments, and the synaptosomes were stored as drained pellets containing 1 mg protein. For assessing the NPY content, protein concentration was determined by the bicinchoninic acid (BCA) method (Smith *et al*. 1985).

Isolation of total RNA from rat hippocampus

Total RNA was isolated from rat hippocampus using TRI REAGENT (Sigma) according to manufacturer's instructions. Briefly, tissue was homogenized in guanidium thiocyanate and phenol. After adding chloroform and centrifuging, the RNA was isolated in the aqueous phase. The RNA was precipitated with isopropanol, the RNA pellet washed with 75% ethanol and the pellet redissolved in diethylpyrocarbonate (DEPC)-treated water.

The total amount of RNA was quantified by optical density (OD) measurements at 260 nm, and the purity was evaluated by measuring the ratio of OD at 260 and 280 nm (RNA/DNA calculator GeneQuant II, Pharmacia Biotech Amersham Biosciences AB, Uppsala, Sweden).

Measurement of glutamate release

The release of endogenous glutamate was followed using a continuous fluorimetric assay as previously described (Nicholls *et al*. 1987) with some modifications (Malva *et al*. 1996; Silva *et al*. 2001). Synaptosomes (1 mg protein) were pre-incubated for 20 min at 37°C in the following medium (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 0.1 $CaCl_2$, 10 glucose, 10 HEPES-Na, pH 7.4,

with 0.1% fatty acid-free bovine serum albumin (BSA). After this period, NPY or Y_1, Y_2 or Y_5 receptor agonists {[Leu^{31}, Pro^{34}]NPY, NPY13-36 or NPY (19–23)-(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})-PP, respectively; Bachem, Bubendorf, Switzerland} or antagonist [BIBP3226 (Peninsula Laboratories, Belmont, CA, USA), BIIE0246 (provided by Dr Henri Doods, Boehringer Ingelheim Pharma KG, Germany) and L-152 804 (Tocris, Bristol, UK), respectively] were added to the incubation medium for an additional 10 min. Synaptosomes were then centrifuged at 15 800 *g* and resuspended in 1 mL of the same medium but without BSA and with 1 mM $CaCl_2$. The suspension was transferred to a stirred acrylic cuvette maintained at 37°C, followed by the addition of 1 mM $NADP^+$, 50 U purified glutamate dehydrogenase and again, NPY receptor agonists or antagonists. Fluorescence was measured using a Perkin Elmer model LS-5B luminescence spectrometer (Perkin-Elmer Ltd, Beaconfield, Buckinghamshire, UK) at the excitation and emission wavelengths of 340 and 460 nm, respectively, with excitation and emission slits of 5 and 10 nm, respectively. The data were collected at 0.5 s intervals and at the end of each experiment, 2.5 nM L-glutamate were added as a callibration to allow the quantification of released glutamate.

Glutamate release was monitored for 11 min and synaptosomes were stimulated 4 min after the beginning of each experiment with 15 mM KCl.

NPY assay

To determine NPY levels in nerve terminals from hippocampal subregions (CA1, CA3 and DG) obtained from control and epileptic rats (killed 24 h and 30 days after KA injection), synaptosomal P_2 pellets were resuspended in 350 μL (CA1) or 200 μL (DG, CA3) of the following medium (in mM): 132 NaCl, 1 KCl, 1 $MgCl_2$, 1.2 H_3PO_4, 1 $CaCl_2$, 10 glucose, 10 HEPES-Na, pH 7.4. The suspension of synaptosomes was then centrifuged at 14 600 *g* for 12 min at 4°C, and the pellets were frozen at −20°C. Each pellet was resuspended in 250 μL Krebs buffer (50 mM EDTA, 0.08% Tween 20) and 10 μL of this preparation were used to quantify NPY levels. An ELISA system and two antibodies (NPY02 and NPY05) recognizing distinct epitopes of NPY were used as described previously (Grouzmann *et al*. 1992) with some modifications (Cavadas *et al*. 2001). This assay measures amidated biologically-active NPY. A 96-well plate (Polysorp; Nunc, Naperville, IL, USA) was coated with 200 ng NPY02 diluted in Tris buffer 50 mM, pH 7.5, overnight at 4°C. The plate was washed three times with Tris-Tween buffer (Tris 50 mM, pH 7.5, with 0.08% Tween 20), and 200 μL 5% low-fat milk in Tris-Tween buffer were added for 30 min. The plate was washed four times with Tris-Tween buffer and the NPY standards and samples (100 μL of final volume) were then added overnight at room temperature with slow agitation. Then, after four washes with Tris-Tween buffer, the second antibody, NPY05, conjugated with alkaline phosphatase diluted (1 : 2500) in 5% low-fat milk in Tris-Tween buffer (7 h at room temperature, with slow agitation), was added. After washing twice with Tris buffer containing 0.25% Tween 20 and twice with Tris buffer containing 0.88% NaCl (w/v), 50 μL of substrate (NADPH solution from the ELISA amplification kit; Invitrogen Life Technologies, Carlsbad, CA, USA) was added. After 40 min of incubation, 50 μL of amplifier (ethanol dehydrogenase mixed with diaphorase from the ELISA amplification kit) was added and the OD measured at 492 nm using an ELISA render (spectra II, SLT lab instruments, Salzburg, Austria). The detection limit was 0.25 pM.

RT-PCR analyses

NPY Y_2 mRNA expression was determined by reverse transcription-PCR (RT-PCR). First, cDNA was transcribed from 2 μg RNA using avian microblastosis virus (AMV) and an Oligo-p(dT)$_{15}$ primer. PCR was performed in a 100 μL reaction system (Roche Molecular Biochemicals, Indianapolis, IN, USA) containing 3 μL template cDNA, 2 μL deoxynucleotide mix, 10 μL 10 × buffer, 2 μL upstream and 2 μL downstream primer, a variable volume of water and 0.5 μL Taq DNA polymerase (35 cycles: at 95°C for 60 s, at 55°C for 60 s and at 72°C for 60 s) (Amersham Biosciences, Amersham, UK). Primers used in PCR reactions were as follows: NPY Y_2, forward primer 5′-CTCCAAGCAAATCAGCTTCC-3′ and the reverse primer 5′-GTTTTGTGCCTTCGCTGATGG-3′; β-actin, upstream primer 5′-GACTACCTCATGAAGATCCT-3′ and the downstream primer 5′-ATCTTGATCATGGTGCTG-3′ (MWG-Biotech AG, Ebersberg, Germany).

PCR products of each sample were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. Densitometrical analysis was performed on Versa-Doc Imaging System (Model 3000, Bio-Rad Laboratories, Hercules, CA, USA) and mRNA expression was evaluated by the band-intensity ratio of control versus epileptic rats (% of control).

Statistical analysis

The data are expressed as means ± SEM. Statistics were performed using one-way ANOVA, followed by Dunnett's or Bonferroni's post-tests, as indicated in the figure legends.

Results

Characterization of the modulation of glutamate release by NPY receptors in hippocampal synaptosomes obtained from control versus epileptic rats

Recently, we showed that NPY (1 μM) inhibited 15 mM KCl-evoked glutamate release from non-epileptic rat hippocampal synaptosomes (48.2 ± 2.2% of control); total glutamate release in control conditions was 1.1 ± 0.1 nM glutamate/mg protein/min while in the presence of 1 μM NPY, it was 0.60 ± 0.1 nM glutamate/mg protein/min (Silva *et al*. 2003a) (Fig. 1a). Also, the selective activation of Y_1, Y_2 or Y_5 receptors using 1 μM [Leu^{31}, Pro^{34}]NPY, 300 nM NPY13-36 or 1 μM NPY (19–23)-(Gly^1, Ser^3, Gln^4, Thr^6, Ala^{31}, Aib^{32}, Gln^{34})-PP, respectively, inhibited glutamate release to 71.6 ± 1.8% (Fig. 1b), 60.1 ± 2.1% (Fig. 1c) or 68.2 ± 2.5% (Fig. 1d), respectively; these inhibitory effects were prevented by selective NPY receptor antagonists (Silva *et al*. 2001, 2003a). In the present work, we showed that the efficiency of the NPY receptor-mediated inhibition of KCl-induced release of glutamate from hippocampal synaptosomes isolated from epileptic rats depends on the stage of the epileptogenic process.

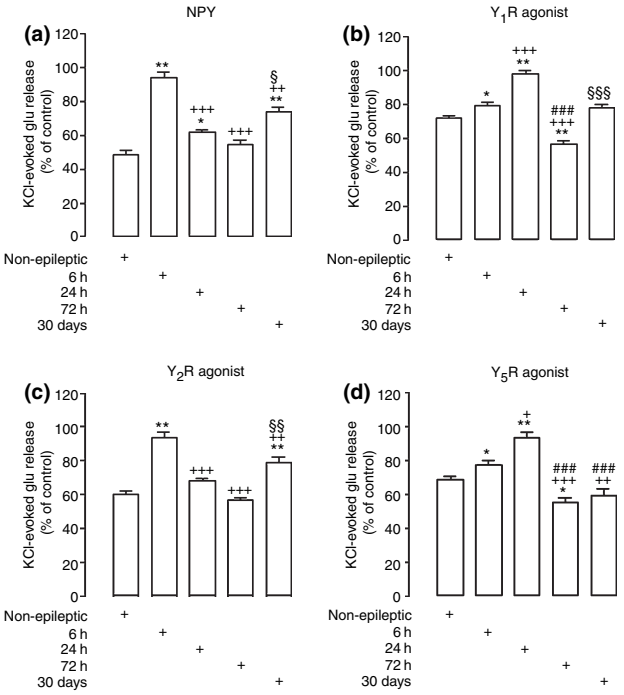

Fig. 1 Quantitative analysis of the effect of (a) 1 μM NPY, (b) 1 μM [Leu^{31}, Pro^{34}]NPY, (c) 300 nM NPY13-36 or (d) 1 μM NPY (19–23)-(Gly^{1}, Ser^{3}, Gln^{4}, Thr^{6}, Ala^{31}, Aib^{32}, Gln^{34})-PP on glutamate release evoked by 15 mM KCl depolarization in hippocampal synaptosomes obtained from control and epileptic rats. To induce epilepsy, the rats were injected with KA (10 mg/kg) and killed after 6 h, 24 h, 72 h or 30 days. The results represent the mean ± SEM of three to 14 independent experiments, in different synaptosomal preparations. $^{*}p < 0.05$, $^{**}p < 0.01$: Dunnett's post-test, statistical significance when compared with non-epileptic animals; $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$: Bonferroni's post-test, statistical significance when compared with 6 h; $###p < 0.001$: Bonferroni's post-test, statistical significance when compared with 24 h; $§p < 0.05$, $§§§p < 0.001$: Bonferroni's post-test, statistical significance when compared with 72 h.

In hippocampal synaptosomes prepared from epileptic rats 6 h after KA injection, the activation of Y_1 receptors with 1 μM [Leu^{31}, Pro^{34}]NPY inhibited the release of glutamate (79.3 ± 1.9% of control) (Fig. 1b). However, Y_1 receptor-mediated inhibition of glutamate release was lost 24 h post-injection (97.8 ± 1.8% of control) and at 72 h or 30 days, the inhibitory effect have been recovered (56.9 ± 2.5% and 79.0 ± 2.5% of control) (Fig. 1b).

The activation of Y_2 receptors with 300 nM NPY 13–36 in synaptosomes obtained from the hippocampus of epileptic rats led to a transient loss of Y_2 receptor-mediated inhibition of glutamate release 6 h post-injection, followed by recovery at 24 h and 72 h post-injection, and again, a loss of Y_2 receptor-mediated inhibition of glutamate release 30 days post-injection. In the presence of 300 nM NPY13-36, KCl-induced release of glutamate was 60.1 ± 2.1% in non-epileptic animals and 93.4 ± 3.3%, 68.6 ± 1.4%, 57.2 ± 1.0% or 79.0 ± 3.3% in hippocampal synaptosomes prepared 6 h, 24 h, 72 h or 30 days post-injection with KA (Fig. 1c).

The activation of Y_5 receptors in epileptic rats 6 h after KA injection with 1 μM NPY (19–23)-(Gly^{1}, Ser^{3}, Gln^{4}, Thr^{6}, Ala^{31}, Aib^{32}, Gln^{34})-PP inhibited glutamate release to 77.4 ± 2.2% of control (Fig. 1d). However, 24 h after KA injection the efficiency of the Y_5 receptors in inhibiting the release of glutamate decreased (93.0 ± 3.6% of control). The inhibitory modulation of glutamate release in the presence of Y_5 receptor agonist was recovered 72 h and 30 days after KA injection, decreasing to levels above control (55.3 ± 2.3% and 53.0 ± 2.0% of control, respectively) (Fig. 1d).

Interestingly, the use of the endogenous agonist, NPY (1 μM) (Fig. 1a), revealed a pharmacological profile very similar to that mediated by the activation of Y_2 receptors alone (Fig. 1c). In agreement, in rats killed 6 h after KA injection, the inhibitory effect of NPY on glutamate release was abolished (94.1 ± 3.3% of control) but recovered 24 h and 72 h after KA injection (62.0 ± 1.4% and 54.7 ± 2.5% of control) (Fig. 1a). Thirty days after KA injection the inhibition of glutamate release was again less efficient (71.5 ± 2.5% of control) (Fig. 1a). All the absolute values of KCl-evoked glutamate release in the presence of the Y_1, Y_2, Y_5 receptor agonists or NPY obtained in the previously described experiment are summarized in table 1.

Since NPY and NPY receptors seem to be involved in other factors unrelated to epilepsy, such as stress, we performed some further control experiments. Modulation of glutamate release by NPY13-36 and NPY was observed in synaptosomes obtained from rats injected with saline (0.9% NaCl) and killed 6 h or 24 h after injection. Like in the

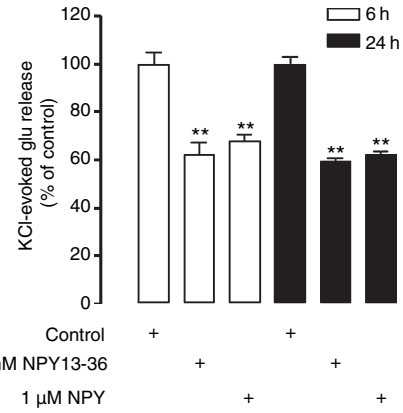

Fig. 2 Quantitative analysis of the effect of 300 nM NPY13-36 or 1 μM NPY on glutamate release evoked by 15 mM KCl depolarization in hippocampal synaptosomes obtained from rats injected with saline (0.9% NaCl) and killed after 6 h or 24 h. The results represent the mean ± SEM of three to nine independent experiments, in different synaptosomal preparations. $^{**}p < 0.01$: Bonferroni's post-test, statistical significance when compared with 6 h or 24 h control.

control non-injected rats, we observed that in the presence of 300 nM NPY13-36 (Fig. 2), glutamate release was inhibited after 6 h or 24 h (62.0 ± 5.9% or 59.2 ± 1.4% of control, respectively), as well as in the presence of 1 μM NPY (Fig. 2) (67.7 ± 2.9% and 62.1 ± 1.7% control, respectively). These results suggest that the loss of inhibition mediated by NPY13-36 or NPY in rats injected with kainate and killed after 6 h is due to the epileptic stage and not to possible stress induced by the injection itself.

Variation of NPY levels in the epileptic hippocampus

The NPY content of synaptosomes prepared from dentate gyrus, CA3 and CA1 hippocampal synaptosomes from control and epileptic rats (killed 24 h and 30 days after KA injection) was determined, as described in Materials and methods. We observed that in non-epileptic animals the NPY content in synaptosomes from the dentate gyrus was higher (271.5 ± 20.4 fM NPY/mg protein) compared with the CA1 and CA3 subregions (144.8 ± 10.9 and 129.3 ± 26.6 fM NPY/mg protein, respectively) (Fig. 3a). Moreover, we observed that the NPY content in dentate gyrus obtained from epileptic rats killed 24 h after kainate injection increased significantly (444.2 ± 55.1 fM NPY/mg protein) (Fig. 3a). In the CA1 subregion, the NPY content decreased in epileptic rats (68.4 ± 8.7 fM NPY/mg protein) whereas in the CA3 subregion, the NPY levels remained similar to those observed in the non-epileptic condition (124.4 ± 24.0 fM NPY/mg protein) (Fig. 3a). However, in synaptosomes obtained from rats killed 30 days after KA injection, we observed increased levels of NPY in all hippocampal subregions (dentate gyrus, 427.1 ± 15.0; CA3, 210.0 ± 10.9 and CA1, 235.3 ± 20.6 fM NPY/mg protein) when compared with the non-epileptic values of the same subregions (138.6 ± 15.0, 76.1 ± 12.4 and 79.3 ± 8.1 fM NPY/mg protein, respectively) (Fig. 3b).

With regard to the results described above, we observed an increased level of NPY (Fig. 3b) in the chronic epileptic hippocampus but simultaneously, a diminished functional effect of NPY in inhibiting the release of glutamate (Fig. 1d). In an attempt to understand these apparent contradictory findings, we decided to investigate a putative basal effect of NPY in inhibiting the KCl-evoked release of glutamate from synaptosomes isolated from the epileptic rat hippocampus. We therefore measured the KCl-evoked release of glutamate in the presence of the Y_1, Y_2 or Y_5 receptor antagonists (1 μM BIBP3226, 1 μM BIIE0246 or 1 μM L-152 804, respectively). A potentiation of glutamate release was observed only in the presence of the Y_2 receptor antagonist (133.0 ± 3.5% of control) (Fig. 4). We also performed the same experiments in non-epileptic rats and found that none of the antagonists had an effect by themselves on KCl-evoked glutamate release (data not shown). Moreover, in agreement with the effect of BIIE0246 (Fig. 4), we also

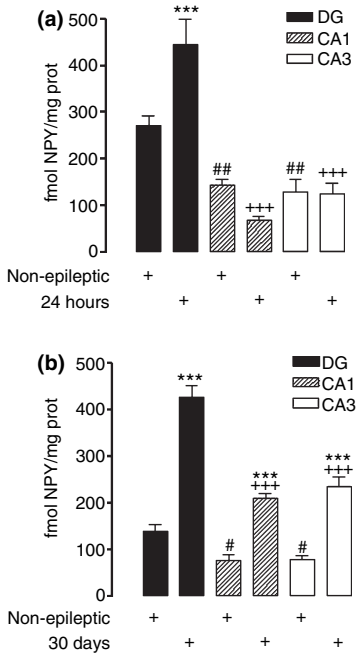

Fig. 3 NPY levels in synaptosomes isolated from the dentate gyrus (DG), CA1 and CA3 hippocampal subregions obtained from both non-epileptic and epileptic rats killed (a) 24 h or (b) 30 days after KA injection. The results are expressed as mean fM NPY/mg protein ± SEM of two to four independent experiments performed in triplicate. $^{***}p < 0.001$: Bonferroni's post-test, statistical significance when compared with non-epileptic rats in the same subregion; $\#p < 0.05$, $\#\#p < 0.01$: Bonferroni's post-test, statistical significance when compared with DG from non-epileptic rats; $^{+++}p < 0.001$: Bonferroni's post-test, statistical significance when compared with DG from epileptic rats.

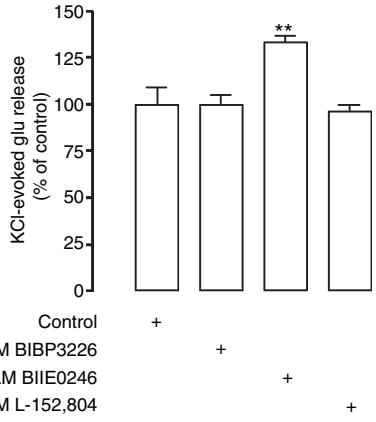

Fig. 4 Quantitative analysis of the effect of 1 μM BIBP3226, 1 μM BIIE0246 and 1 μM L-152 804 (Y1, Y_2 and Y_5 receptor antagonist, respectively) on glutamate release evoked by 15 mM KCl depolarization in hippocampal synaptosomes of epileptic rats injected with KA (10 mg/kg) and killed after 30 days. The results represent the mean ± SEM of five to 10 independent experiments, in different synaptosomal preparations.

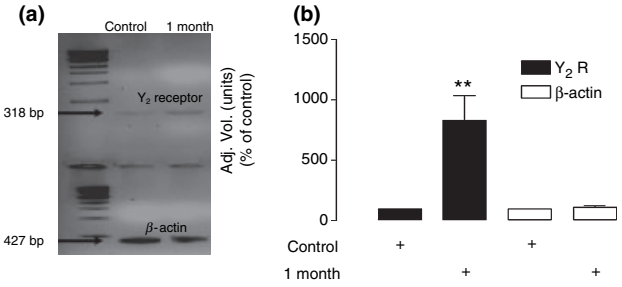

Fig. 5 (a) Total hippocampal RNA was subjected to RT-PCR analysis for determination of NPY Y_2 (318 bp) and actin (427 bp) mRNA levels. (b) Quantification of hippocampal NPY Y_2 mRNA levels in control versus epileptic rats injected with 10 mg/kg kainate and killed after 30 days. $^{**}p < 0.01$: Dunnett's post-test, statistical significance when compared with control (non-epileptic rats).

observed an increase in Y_2 receptor mRNA levels in the hippocampus of epileptic rats compared with control non-injected animals (827.8% of control) (Figs 5a and b).

Discussion

It is known that NPY receptors modulate the release of glutamate in the hippocampus (Greber *et al*. 1994; Qian *et al*. 1997; Whittaker *et al*. 1999; Silva *et al*. 2001) and that the inhibition caused by NPY is partially mediated by the activation of the Y_2 receptor subtype, despite the simultaneous presence of Y_1 and Y_5 receptors in the hippocampus (Silva *et al*. 2003a). In this study, we demonstrate that in different stages of the epileptogenic process following intraperitoneal injection of rats with KA the ability to modulate the release of glutamate through the activation of NPY receptors is changed. We suggest that in the acute phase of epilepsy (6–24 h post-injection), a transient loss of NPY-mediated inhibition of glutamate release may be causally associated with hyperexcitability of the hippocampal neuronal network and seizure activity. Interestingly, following the acute phase (72 h post-injection), an adaptation of the NPYergic modulation of glutamate release was identified and the inhibitory properties of NPY on glutamate release were again close to control levels.

There is considerable evidence suggesting that the plastic changes in NPY receptors (Vezzani *et al*. 1999) and the functional and morphological alterations in peptidergic neurones found in epileptic brain tissue (Schwarzer *et al*. 1996) are mediated by glutamate release during seizures. Röder *et al.* (1996) showed that in the chronic period of epilepsy 7–30 days after KA treatment, NPY Y_2 receptor binding dropped below control except in the hilus of dentate gyrus where it remained elevated. Moreover, following seizures, changes in Y_1-R mRNA in the hippocampus are widespread but rapid and transient, whereas there is a major increase in Y_2-R mRNA expression and a rapid and transient increase in Y_5-R mRNA in the dentate granule cell layer (Kopp *et al*. 1999). However, others have suggested a down-regulation of Y_5 receptor in KA-treated rats and kindledrats (Bregola *et al*. 2000). The differences that we observed on the modulation of glutamate release by NPY receptor activation could also be due to a variation of protein expression in nerve terminals. We also show that the modulation of glutamate release following activation of the Y_2 receptor subtype is similar to that activated with the endogenous peptide, so it may be expected, as in non-epileptic rats (Silva *et al*. 2003a), that NPY inhibits glutamate release by preferentially activating Y_2 receptors rather than Y_1 and Y_5 receptors. Despite the strong evidence associating NPY receptor modulation of glutamate efflux to the epilepsy stage, we should also consider the neurodegenerative processes that occur under such conditions. Borges *et al.* (2004) recently showed that after pilocarpine-induced status epilepticus in mice, most hilar neurones died and NPY immunoreactivity appeared in the dentate inner molecular layer, indicative of mossy fibre sprouting. Moreover, we have previously shown (Silva *et al*. 2003b) that under excitotoxic conditions, the activation of NPY receptors has a neuroprotective effect.

The higher levels of NPY in the dentate gyrus compared with the CA1 and CA3 subregions may result in a higher presynaptic inhibition of glutamate release, probably through the activation of Y_2 receptors. Indeed, we have previously shown that selective Y1, Y_2 or Y_5 receptor activation significantly inhibits glutamate release in rat dentate gyrus (Silva *et al*. 2001, 2003a). Klapstein and Colmers (1993) showed that NPY inhibited excitatory postsynaptic potentials (EPSPs) evoked in CA1 pyramidal cells from either stratum

Table 1 Absolute values of KCl-evoked glutamate release (nM glutamate/mg protein/min) in the presence of the Y_1, Y_2, Y_5 receptor agonist or NPY, measured in synaptosomes obtained from non-epileptic rats or from epileptic rats injected with kainate (10 mg/kg) and killed after 6 h, 24 h, 72 h and 30 days. The results represent the mean ± SEM of three to 14 independent experiments, in different synaptosomal preparations

	Y_1 R agonist	Y_2 R agonist	Y_5 R agonist	NPY
Non-epileptic	0.67 ± 0.07	0.58 ± 0.05	0.76 ± 0.07	0.60 ± 0.1
6 h	0.79 ± 0.07	0.96 ± 0.05	0.69 ± 0.06	1.1 ± 0.09
24 h	1.02 ± 0.09	0.68 ± 0.03	0.88 ± 0.05	0.58 ± 0.01
72 h	0.47 ± 0.08	0.47 ± 0.09	0.46 ± 0.09	0.40 ± 0.03
30 days	0.90 ± 0.01	0.89 ± 0.03	0.70 ± 0.07	0.93 ± 0.07

radiatum or oriens, and also at mossy fibre-CA3, stratum oriens-CA3 and CA3-CA3. By contrast, no inhibitory effect was observed in EPSPs evoked in dentate granule cells from either perforant path or commissural inputs. However, neurochemical studies have already suggested that NPY is abundantly expressed in the dentate gyrus in rodent models of epilepsy and in the epileptic human (Greber *et al*. 1994; Schwarzer *et al*. 1998). Others have shown that NPY inhibits KCl-induced glutamate release in rat dentate gyrus synaptosomes (Whittaker *et al*. 1999) and in the epileptic human dentate gyrus (Patrylo *et al*. 1999). However, as in glutamate release studies, we have also observed different variations in NPY levels depending on the time elapsed after KA injection. At 30 days post-injection, there was an increase in NPY levels not only in the dentate gyrus but also in the CA1 and CA3 subregions, compared with non-epileptic animals. Moreover, Causing *et al.* (1996) showed an increase in NPY mRNA levels in neurones of the ipsilateral and contralateral dentate gyrus and Ammon's Horn after intermittent excitatory stimulation; NPY mRNA levels peaked at 4–24 h and had returned to baseline by 48 h post-stimulation.

Taken together, the results obtained in synaptosomes isolated 30 days after KA injection show that there is a marked decrease in the inhibition of glutamate release due to the activation of Y_2 receptors and a simultaneous increase in NPY levels. These apparently contradictory results were better understood following our observation of a robust increase in the expression of Y_2 receptor mRNA and an increase in KCl-evoked release of glutamate by blocking Y_2 receptors. These results suggest that endogenous NPY inhibits glutamate release through Y_2 receptors under chronic epileptic conditions. Again, a very relevant role for Y_2 receptors in mediating the effects of NPY was identified. In accordance, Röder *et al.* (1996) showed that the increase in Y_2 receptor binding in the hilus of the dentate gyrus is associated with enhanced NPY release after kainate injection.

In conclusion, our data support the hypothesis that NPY can act as an endogenous neuromodulator that may limit hyperexcitability in a rodent model of epilepsy, since NPY levels and the expression of Y_2 receptor mRNA are up-regulated and the modulation of glutamate release through NPY receptors is modified in KA-induced epilepsy. These compensatory changes in the NPYergic system in the epileptic hippocampus may play an important role in the control of pathological excitability. Taken together, these findings may suggest that overexpression of NPY and Y_2 receptors may represent an important adaptation mechanism in the epileptogenic process.

Acknowledgements

We acknowledge Dr Henri Doods (Boehringer Ingelheim Pharma KG) for generously providing us with the BIIE0246 compound. This work was supported by Fundação para a Ciência e a Tecnologia, Portugal (Project POCTI/NSE/46848/2002 and Grant BPD/11484/2002), FEDER, and by a grant from Fundação Calouste Gulbenkian, Portugal.

References

Baraban S. C., Hollopeter G., Erickson J. C., Schwartzkroin P. A. and Palmiter R. D. (1997) Knock-out mice reveal a critical antiepileptic role for neuropeptide Y. *J. Neurosci.* **17**, 8927–8936.

Bellmann R., Humpel C., Krause J. E., Marksteiner J., Saria A. and Sperk G. (1991) Differential changes in mRNAs encoding for preprotachykinin A and B after kainic acid-induced seizures in the rat.*Synapse* **8**, 71–73.

Ben-Ari Y. (1985) Limbic seizure and brain damage produced by kainic acid: mechanisms and relevance to human temporal lobe epilepsy. *Neuroscience* **14**, 375–403.

Borges K., McDermott D. L. and Dingledine R. (2004) Reciprocal changes of CD44 and GAP-43 expression in the dentate gyrus inner molecular layer after status epilepticus in mice. *Exp. Neurol.* **188**, 1–10.

Bregola G., Dumont Y., Fournier A., Zucchini S., Quirion R. and Simonato M. (2000) Decreased levels of neuropeptide Y(5) receptor binding sites in two experimental models of epilepsy. *Neuroscience* **98**, 697–703.

Causing C. G., Makus K. D., Ma Y., Miller F. D. and Colmers W. F. (1996) Selective upregulation of T alpha 1 alpha-tubulin and neuropeptide Y mRNAs after intermittent excitatory stimulation in adult rat hippocampus *in vivo*. *J. Comp. Neurol.* **367**, 132–146.

Cavadas C., Silva A. P., Mosimann F., Cotrim M. D., Ribeiro C. A., Brunner H. R. and Grouzmann E. (2001) NPY regulates catecholamine secretion from human adrenal chromaffin cells. *J. Clin. Endocrinol. Metab.* **86**, 5956–5963.

Gobbi M., Gariboldi M., Piwko C., Hoyer D., Sperk G. and Vezzani A. (1998) Distinct changes in peptide YY binding to, and mRNA levels of, Y1 and Y2 receptors in the rat hippocampus associated with kindling epileptogenesis. *J. Neurochem.* **70**, 1615–1622.

Greber S., Schwarzer C. and Sperk G. (1994) Neuropeptide Y inhibits potassium-stimulated glutamate release through Y_2 receptors in rat hippomcampal slices *in vitro*. *Br. J. Pharmacol.* **113**, 737–740.

Grouzmann E., Aubert J. F., Waeber B. and Brunner H. R. A. (1992) Sensitive and specific two-site, sandwich-amplified enzyme immunoassay for neuropeptide Y. *Peptides* **13**, 1049–1054.

Klapstein G. J. and Colmers W. F. (1993) On the sites of presynaptic inhibition by neuropeptide Y in rat hippocampus *in vitro*. *Hippocampus* **3**, 103–112.

Kofler N., Kirchmair E., Schwarzer C. and Sperk G. (1997) Altered expression of NPY-Y1 receptors in kainic acid induced epilepsy in rats. *Neurosci. Lett.* **230**, 129–132.

Kopp J., Nanobashvili A., Kokaia Z., Lindvall O. and Hökfelt T. (1999) Differential regulation of mRNAs for neuropeptide Y and its receptor subtypes in widespread areas of the rat limbic system during kindling epileptogenesis. *Mol. Brain Res.* **72**, 17–29.

Layne E. (1957) Spectophotometric and turbidimetric methods for measuring proteins, in *Methods in Enzymology* (Colowick S. P. and Kaplan N. O., eds), Vol. 3, pp. 447–451. Academic Press, New York.

Malva J. O., Carvalho A. P. and Carvalho C. M. (1996) Domoic acid induces the release of glutamate in the rat hippocampal CA3 subregion. *Neuroreport* **7**, 1330–1344.

Marsh D. J., Baraban S. C., Hollopeter G. and Palmiter R. D. (1999) Role of the Y5 neuropeptide Y receptor in limbic seizures. *Proc. Natl Acad. Sci. USA* **96**, 13518–13523.

McMahon T. H., Foran P., Dolly J. O., Verhage M., Wiegant V. M. and Nicholls D. G. (1992) Tetanus toxin and botulinum toxins type A and B inhibit glutamate, γ-aminobutyric acid, aspartate, and Met-en-kephalin release from synaptosomes. *J. Biol. Chem.* **267**, 21338–21343.

McQuiston A. R., Petrozzino J. J., Connor J. A. and Colmers W. F. (1996) Neuropeptide Y_1 receptors inhibit N-type calcium currents and reduce transient calcium increases in rat dentate granule cells. *J. Neurosci.* **16**, 1422–1429.

Michel M. C., Beck-Sickinger A., Cox H., Doods H. N., Herzog H., Larhammar D., Quirion R., Schwartz T. and Westfall T. (1998) International Union of Pharmacology recommendations for the nomenclature of neuropeptide Y, peptide YY, and pancreatic polypeptide receptors. *Pharmacol. Rev.* **50**, 143–150.

Nicholls D. G., Sihra T. S. and Sanchez-Prieto J. (1987) Calcium-dependent and -independent release of glutamate from synaptosomes monitored by continuous fluorometry. *J. Neurochem.* **49**, 50–57.

Park C., Kang M., Kang K., Lee J., Kim J., Yoo J., Ahn H. and Huh Y. (2001) Differential changes in neuropeptide Y and nicotinamide adenine dinucleotide phosphate-diaphorase-positive neurons in rat hippocampus after kainic acid-induced seizure. *Neurosci. Lett.* **298**, 49–52.

Patrylo P. R., Van Den Pol A. N., Spencer D. D. and Williamson A. (1999) NPY inhibits glutamatergic excitation in the epileptic human dentate gyrus. *J. Neurophysiol.* **82**, 478–483.

Qian J., Colmers W. and Saggau P. (1997) Inhibition of synaptic transmission by neuropeptide Y in rat hippocampal area CA1: Modulation of presynaptic Ca^{2+} entry. *J. Neurosci.* **17**, 8169–8177.

Reibel S., Nadi S., Benmaamar R., Larmet Y., Carnahan J., Marescaux C. and Depaulis A. (2001) Neuropeptide Y and epilepsy: varying effects according to seizure type and receptor activation. *Peptides* **22**, 529–539.

Richichi C., Lin E. J., Stefanin D., Colella D., Ravizza T., Grignaschi G., Veglianese P., Sperk G., During M. J. and Vezzani A. (2004) Anticonvulsant and antiepileptogenic effects mediated by adeno-associated virus vector neuropeptide Y expression in the rat hippocampus. *J. Neurosci.* **24**, 3051–3059.

Röder C., Schwarzer C., Vezzani A., Gobbi M., Minnini T. and Sperk G. (1996) Autoradiographic analysis of neuropeptide Y receptor binding sites in the rat hippocampus after kainic acid-induced limbic seizures. *Neuroscience* **70**, 47–55.

Schwarzer C., Williamson J. M., Lothman E. W., Vezzani A. and Sperk G. (1995) Somatostatin, neuropeptide Y, neurokinin B and cholecystokinin immunoreactivity in two chronic models of temporal lobe epilepsy. *Neuroscience* **69**, 831–845.

Schwarzer C., Sperk G., Rizzi M., Gariboldi M. and Vezzani A. (1996) Neuropeptides-immunochemistry and their mRNA expression in kindling: functional implications for limbic epileptogenesis. *Brain Res. Rev.* **22**, 27–50.

Schwarzer C., Kofler N. and Sperk G. (1998) Up-regulation of neuropeptide Y-Y2 receptors in an animal model of temporal lobe epilepsy. *Mol. Pharmacol.* **53**, 6–13.

Silva A. P., Carvalho A. P., Carvalho C. M. and Malva J. O. (2001) Modulation of intracellular calcium changes and glutamate release by neuropeptide Y1 and Y2 receptors in the rat hippocampus: differential effects in CA1, CA3 and dentate gyrus. *J. Neurochem.* **79**, 286–296.

Silva A. P., Carvalho A. P., Carvalho C. M. and Malva J. O. (2003a) Functional interaction between neuropeptide Y receptors and modulation of calcium channels in the rat hippocampus. *Neuropharmacology* **42**, 282–292.

Silva A. P., Pinheiro P. S., Carvalho A. P., Carvalho C. M., Jakobsen B., Zimmer J. and Malva J. O. (2003b) Activation of neuropeptide Y receptors is neuroprotective against excitotoxicity in organotypic hippocampal slice cultures. *FASEB J.* **17**, 1118–1120.

Smialowska M., Bijak M., Sopala M. and Tokarski K. (1996) Inhibitory effect of NPY on the picrotoxin-induced activity in the hippocampus: a behavioural and electrophysiological study. *Neuropeptides* **30**, 7–12.

Smith P. K., Krohn R. I., Hermanson G. T., Mallia A. K., Gartner F. H., Provenzano M. D., Fujimoto E. K., Goeke N. M., Olson B. J. and Klenk D. C. (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85.

Sperk G., Lassmann H., Baran H., Kish S. J., Seitelberger F. and Hornykiewicz O. (1983) Kainic acid induced seizures: neurochemical and histopathological changes. *Neuroscience* **10**, 1301–1315.

Vezzani A., Sperk G. and Colmers F. (1999) Neuropeptide Y: emerging evidence for a functional role in seizure modulation. *Trends Neurosci.* **22**, 25–30.

Vezzani A., Rizzi M., Conti M. and Samanin R. (2000) Modulatory role of neuropeptide in seizures induced in rats by stimulation of glutamate receptors. *Am. Soc. Nutri. Sci.* **7402**, 1046S–1048S.

Vezzani A., Michalkiewicz M., Michalkiewicz T. *et al.* (2002) Seizure susceptibility and epileptogenesis are decreased in transgenic rats overexpressing neuropeptide Y. *Neuroscience* **110**, 237–243.

Weiser T., Wieland H. A. and Doods H. N. (2000) Effects of the neuropeptide Y Y(2) receptor antagonist BIIE0246 on presynaptic inhibition by neuropeptide Y in rat hippocampal slices. *Eur. J. Pharmacol.* **404**, 133–136.

Whittaker E., Vereker E. and Lynch M. A. (1999) Neuropeptide Y inhibits glutamate release and long-term potentiation in rat dentate gyrus. *Brain Res.* **827**, 229–233.

Whittaker V. P. (1984) The synaptosome, in *Handbook of Neurochemistry* (Lajtha A., ed.), Vol. 7, pp. 1–39. Plenum Press, New York.

Woldbye D. P. D., Madsen T. M., Larsen P. J., Mikkelsen J. D. and Bolwig T. G. (1996) Neuropeptide Y inhibits hippocampal seizures and wet dog shakes. *Brain Res.* **737**, 162–168.

Woldbye D. P. D., Larsen P. J., Mikkelsen J. D., Klemp K., Madsen T. M. and Bolwig T. G. (1997) Powerful inhibition of kainic acid seizures by neuropeptide Y via Y5-like receptors. *Nature Med.* **3**, 761–764.