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Neuropeptide Y can rescue neurons from cell death following the application of an excitotoxic insult with kainate in rat organotypic hippocampal slice cultures

S. Xapelli [a,1], A.P. Silva [a,b,1], R. Ferreira [a], J.O. Malva [a,c,*]

[a] Center for Neuroscience and Cell Biology of Coimbra, 3004-517 Coimbra, Portugal
[b] Institute of Pharmacology and Therapeutics, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal
[c] Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal

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ABSTRACT

In the present work we investigated the neuroprotective role of neuropeptide Y (NPY) after an excitotoxic insult in rat organotypic hippocampal slice cultures. Exposure of 2 week-old rat hippocampal slice cultures to 12 μM kainate (KA) for 24 h induced neuronal death in dentate gyrus (DG) granular cell layer, CA1 and CA3 pyramidal cell layers, as quantified by cellular propidium iodide (PI) uptake. The activation of Y_1 or Y_2 receptors 30 min after starting the exposure to the excitotoxic insult with kainate resulted in neuroprotection by reducing the PI uptake in DG, CA1 and CA3 cell layers. The use of Y_1 or Y_2 receptors antagonists, BIBP3226 (1 μM) or BIIE0246 (1 μM), resulted in the loss of the neuroprotection induced by the activation of Y_1 or Y_2 receptors, respectively, in all hippocampal subfields. Taken together these results suggest that activation of NPY Y_1 or Y_2 receptors activates neuroprotective pathways that are able to rescue neurons from excitotoxic cell death.

1. Introduction

NPY is one of the most abundant and widely distributed neuropeptides in the mammalian central nervous system and has been associated with a number of physiological and pathological conditions [47]. This peptide has been shown to modulate anxiety, pain, memory, eating behavior, and many other functions in the central as well as the peripheral nervous system [16,47]. Also, the NPY system has been suggested to be involved in excitation associated with limbic seizures [8].

Six G-protein-coupled NPY receptor subtypes have been identified (Y_1–Y_6) and mediate inhibition of cAMP synthesis [15,24,25]. In the central nervous system, and specifically in the hippocampus, Y_1 and Y_2 receptors are highly expressed [40]. Y_1 receptors are mainly located on dentate granule cells and hilar NPY-containing interneurons [31], whereas Y_2 receptors are expressed at relatively high concentrations in terminal regions of mossy fibers and Schaffer collaterals [18].

In rats, Y_1 receptor antagonists inhibit kainate-induced seizures and delay kindling epileptogenesis, while a Y_1 receptor agonist antagonizes these effects [3,13]. The activation of Y_2 receptors suppresses seizure activity in hippocampal slices *in vitro* [6,10] and *in vivo* models [10,45]. Also, NPY, acting via Y_2 receptor, inhibits impulse-dependent synaptic excitation of CA3 pyramidal cells of the rat hippocampus by presynaptic mechanisms [28]. Accordingly, the NPY system

* *Corresponding author at*: Institute of Biochemistry, Faculty of Medicine, University of Coimbra, 3004-504 Coimbra, Portugal. Tel.: +351 239 833369; fax: +351 239 822776.
E-mail address: jomalva@fmed.uc.pt (J.O. Malva).

[1] S. Xapelli and A.P. Silva contributed equally to this study.

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has been proposed to act as an endogenous anticonvulsant [40,43]. The role of endogenous NPY in regulating seizure activity [43] is supported by evidence of increased NPY levels under epileptic conditions in both inhibitory interneurons and in excitatory granule cells which in control conditions do not contain the peptide [44,45].

Several *in vitro* studies have demonstrated that, in the hippocampus, NPY selectively inhibits glutamatergic synaptic transmission in mossy fiber CA3 and Schaffer collaterals CA1 pyramidal cell synapses [9,14,19,28] by depressing glutamate release [14,37] through a mechanism involving the inhibition of the voltage-dependent Ca^{2+} influx in presynaptic nerve terminals [33,36,37]. The control in excess glutamate release, potentially relevant for hyperexcitability brain disorders, may contribute to the identified neuroprotection properties of NPY against excitotoxic injuries [39,40].

In the present work, we identified a post-injury (excitotoxic) neuroprotective role for NPY that contributes to rescue injured neurons from cell death.

2. Materials and methods

2.1. Rat organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared using the interface culture method [41] modified by Kristensen et al. and Noraberg et al. [21,29]. The 6–8-day-old Wistar rats were quickly decapitated in accordance with ethically approved standard procedures (European Community guidelines for the use of animals in the laboratory), the brains removed under sterile conditions, and the hippocampi isolated and sectioned into 350 μm thick slices using a McIlwain tissue chopper. Slices were placed in cold Gey's balanced salt solution (GBSS; Biological Industries, Kibbutz Beit Haemek, Israel) with 25 mM D-glucose (Merck, Darmstadt, Germany), and all excess tissue was gently trimmed out. Six individual slices were then transferred to each porous (0.4 μm) membrane insert (Millipore Corp., Bedford, MA, USA) and inserts placed in a six-well culture tray (Costar, Corning, NY, USA). Each well contained 1 ml of culture medium, with 50% OPTI-MEM, 25% heat-inactivated horse serum and 25% Hank's BSS (HBSS) (all from Invitrogen, Paisley, UK) and supplemented with D-glucose to a final concentration of 25 mM. The trays were kept in an incubator with 5% CO_2 and 95% atmospheric air at 36 °C. On the third day and thereafter the medium was changed to a chemically defined medium composed of serum-free Neurobasal medium (Invitrogen, Paisley, UK) supplemented with 1 mM L-glutamine (Sigma, St Louis, MO) and B-27 supplement (Invitrogen, Paisley, UK). The medium was changed twice a week for the next 2 weeks before starting the experiments. No antimitotic drugs or antibiotics were used at any stage.

2.2. Monitoring propidium iodide uptake

For detection of neuronal cell death, cellular uptake of the fluorescent dye propidium iodide (PI, 3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phenanthridinium diiodide; Sigma, St. Louis, MO) was recorded and quantified according to previously described procedures [21,29]. PI is a polar compound that only enters cells with damaged cell membranes (dead or dying cells), but once inside the cells, it binds to DNA, emitting a bright red fluorescence (630 nm) when exposed to blue-green light (493 nm). PI is basically non-toxic to neurons [17,22] and has been used as an indicator of neuronal membrane integrity [17,32] and cell damage [32,46]. Basal level of PI uptake was determined after adding 2 μM PI to the medium, and digital fluorescent micrographs were taken at least 3 h later. The same concentration was also used in all subsequent medium changes. PI uptake was recorded by fluorescence microscopy (Axioskop 2 plus, Carl Zeiss, Göttingen, Germany) using a standard rhodamine filter and digital camera (AxioCam HRc, Carl Zeiss, Göttingen, Germany) with 1000 ms exposure time. After exposing the cultures to the drugs, digital fluorescent micrographs of the cultures were taken at different time points during the experiments, for use in densitometric measurements of the PI uptake in CA1, CA3 pyramidal cell layers and also dentate gyrus (DG), and were performed by delineating the different subfields using NIH Image 1.62 analysis software (National Institutes of Health, Bethesda, MD). The densitometric data were expressed as the mean value ± standard error of mean (S.E.M.). Statistical analysis was performed using one-way ANOVA followed by Dunnett's multiple comparison test. Differences were considered significant when $p < 0.05$.

2.3. Exposure to neuropeptide Y receptor agonists/antagonists

To test the role of NPY receptors in kainate-mediated neuronal death, cultures were first pre-incubated for 30 or 60 min with 12 μM kainate. This concentration was chosen based on a concentration-response curve in which the EC50 for kainate in CA1, CA3 and DG was close to 12 μM (data not shown). After this period, cultures were exposed to the Y_1 or Y_2 receptor agonists ([Leu^{31},Pro^{34}] NPY (1 μM) or NPY13–36 (300 nM), respectively) (Bachem Bubendorf, Switzerland) for 24 h. The same experimental protocol was used to study the effect of 1 μM BIBP3226 (Peninsula Laboratories, Belmont, CA) or 1 μM BIIE0246 (generous gift from Dr. Henri Doods, Boehringer Ingelheim Pharma, Germany), Y_1 and Y_2 receptor antagonists, respectively. These concentrations were chosen based on previous studies [20,39]. Control cultures were not exposed to any drug. Digital images of PI uptake were obtained before any exposure (day 0) and 24 h after exposure to the NPY receptor agonists/antagonists (day 1). Basal death (day 0) was subtracted from the values at day 1.

2.4. Evaluation of cell morphology and toluidine blue staining

Organotypic hippocampal slice cultures were prepared and stained as described by Noraberg et al. [29]. At the end of each experiment the cultures were fixed in 4% phosphate buffered paraformaldehyde (PFA) overnight. Then, cultures were washed for 1 h in 0.15 M phosphate buffer (25 mM KH_2PO_4, 125 mM $Na_2HPO_4{\cdot}2H_2O$), and then washed in distilled water for 1 h. After this, the slices were stained with toluidine blue solution (77 mM $Na_2HPO_4{\cdot}2H_2O$; 67.2 mM citric acid; 3.3 mM toluidine blue) for 5 min. The slices were washed three times

in distilled water, dehydrated in 70%, 96% and 99% and again 96% ethanol (2 min each), washed with distilled water (three times) and stained once more with toluidine blue solution. They were again washed with distilled water (three times) and dehydrated in 70%, 96% and 99% ethanol (5 min each). After this, the cultures were detached from the membrane, and each culture was placed on gelatin-coated glass slides (cultures from one insert on one glass slide). Finally the preparations were mounted with Entellan (Merck, Darmstadt, Germany).

3. Results

3.1. *Exogenous activation of Y_1 and Y_2 receptors can rescue neurons from cell death following the application of kainate*

The putative neuroprotective role of Y_1 and Y_2 receptors against kainate receptor-mediated excitotoxicity in CA1 and CA3 pyramidal cell layers and in DG was investigated. In these experiments, the mean value of PI uptake in all subfields, after 24 h exposure to 12 μM kainate ($n = 37$), was set to 100%, representing maximal PI uptake. Basal PI uptake in control conditions was 47.41 ± 3.94% ($n = 49$), 40.13 ± 3.93% ($n = 49$) and 46.52 ± 3.45% ($n = 49$) in CA1, CA3 pyramidal cell layers and DG, respectively.

The activation of Y_1 or Y_2 receptors for 24 h, by the selective Y_1 or Y_2 receptor agonists ([Leu^{31},Pro^{34}] NPY (1 μM) or NPY13–36 (300 nM), respectively) starting at 60 min after the excitotoxic insult with 12 μM kainate, did not protect neurons from cell death, resulting in PI uptake similar to that obtained with kainate alone (Fig. 1).

However, activation (for 24 h) of Y_1 receptors with the Y_1 receptor agonist [Leu^{31},Pro^{34}] NPY (1 μM), 30 min after starting the excitotoxic insult with 12 μM kainate, prevented neuronal death and reduced the PI uptake to 61.50 ± 6.14% ($n = 36$), 53.49 ± 6.37% ($n = 36$) and 60.20 ± 7.42% ($n = 36$) in CA1, CA3 and DG, respectively (Fig. 2). Also, Y_2 receptor activation with the Y_2 receptor agonist, NPY13–36 (300 nM), for 24 h, starting at 30 min after the excitotoxic insult was neuroprotective and decreased PI uptake to 51.27 ± 5.66%

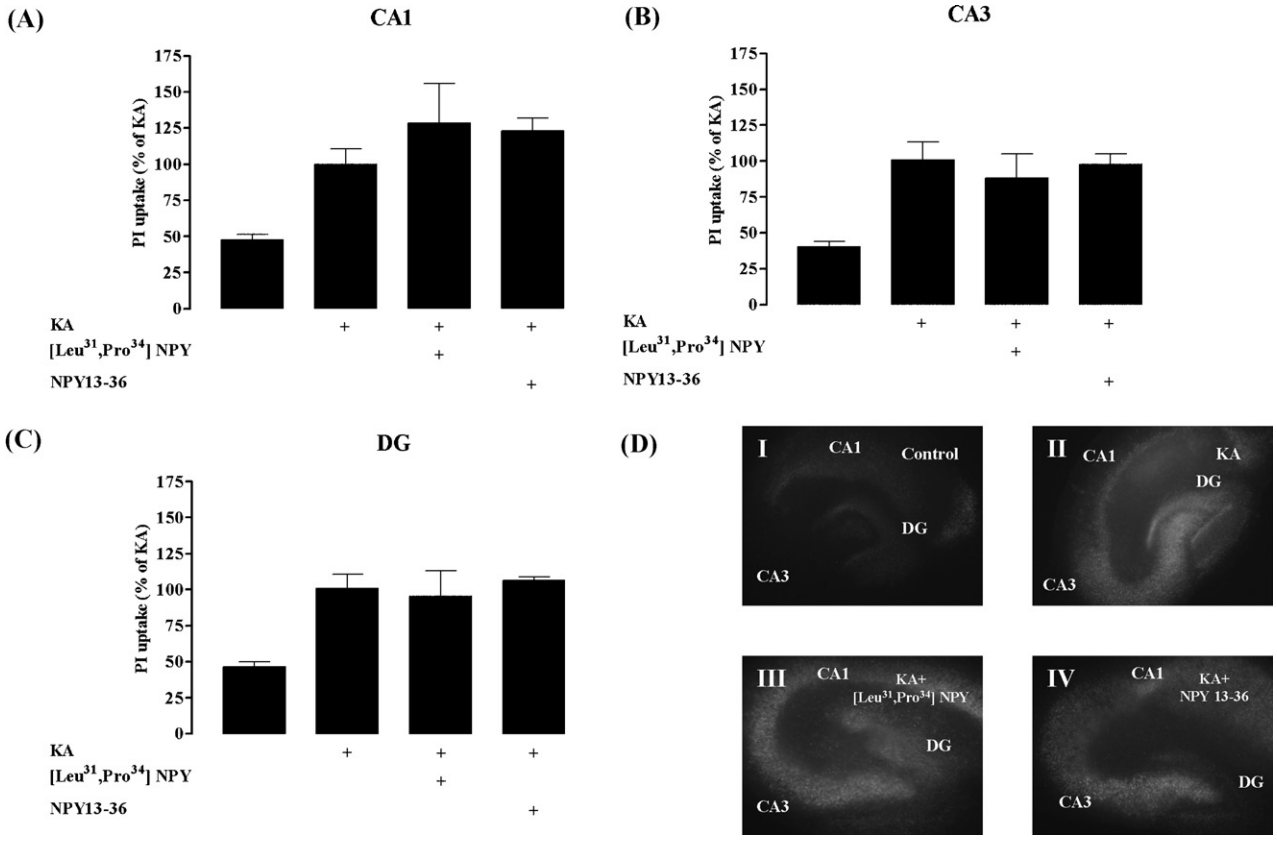

Fig. 1 – The activation of NPY Y_1 and NPY Y_2 receptor 60 min after starting the insult with kainate (KA) did not prevent the neuronal death in CA1 and CA3 pyramidal cell layers and in DG. Densitometric measurements of PI uptake induced by 12 μM kainate in CA1 (A) and CA3 (B) pyramidal cell layers and in DG (C) showing the effects of Y_1 and Y_2 receptor agonists ([Leu^{31},Pro^{34}] NPY (1 μM), NPY13–36 (300 nM), respectively) on the PI uptake induced by 12 μM kainate. The PI uptake induced by 12 μM kainate was set to 100%. Data are shown as means ± S.E.M., with $n = 12$–49. Fluorescent micrographs of PI uptake of organotypic hippocampal slice cultures showing the typical regional pattern of neuronal death induced by the experimental situation indicated next to each photograph (D): (I) control culture with low basal PI uptake, (II) culture exposed to 12 μM kainate, (III) culture exposed to 1 μM [Leu^{31},Pro^{34}] NPY 60 min after the exposure to 12 μM kainate and (IV) culture exposed to 300 nM NPY13–36 60 min after starting the exposure to 12 μM kainate.

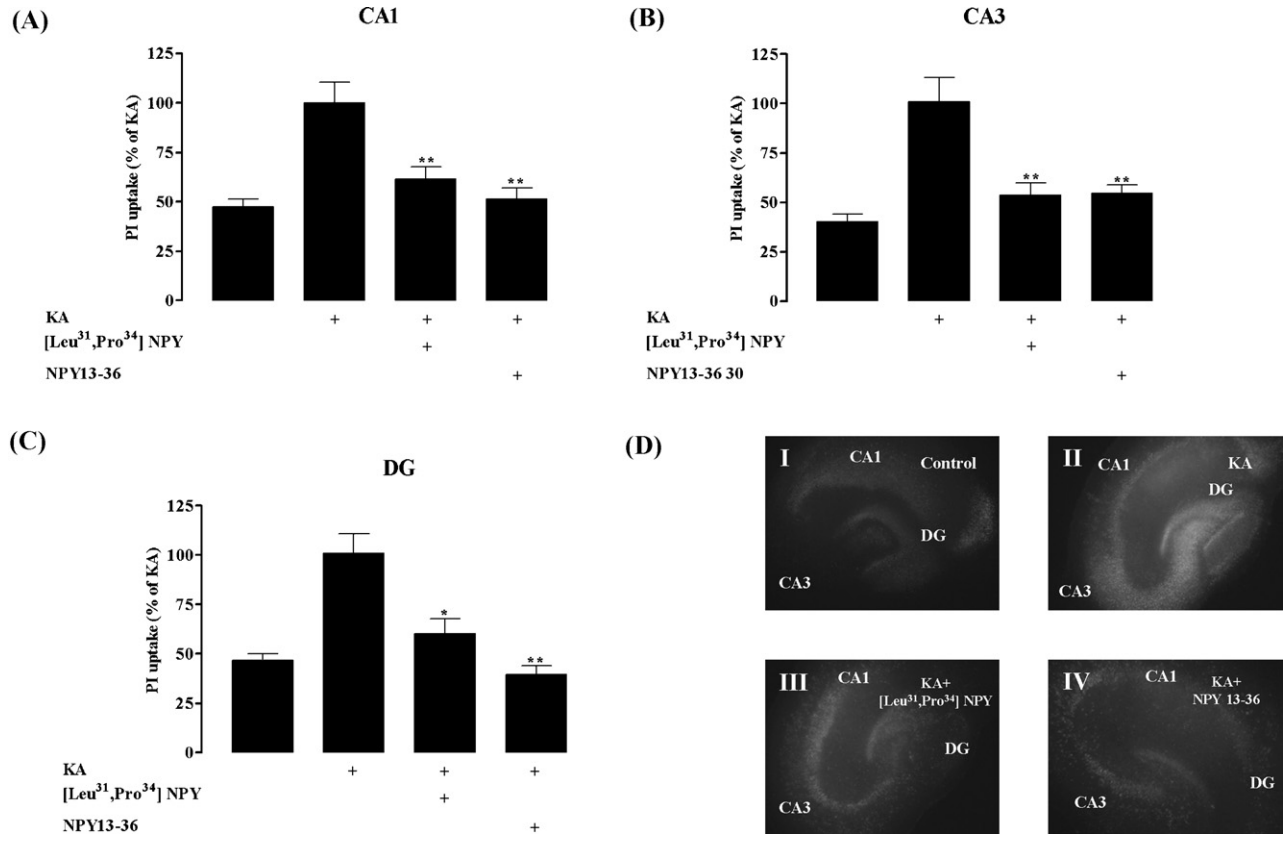

Fig. 2 – NPY Y_1 and NPY Y_2 receptor activation 30 min post-kainate (KA) insult prevented cell degeneration in CA1 and CA3 pyramidal cell layers and in DG. Densitometric measurements of PI uptake induced by 12 μM kainate in CA1 (A) and CA3 (B) pyramidal cell layers and in DG (C) showing the effects of Y_1 and Y_2 receptor agonists ([Leu^{31},Pro^{34}] NPY (1 μM), NPY13–36 (300 nM), respectively) on the PI uptake induced by 12 μM kainate. The PI uptake induced by 12 μM kainate was set to 100%. Data are shown as means ± S.E.M., with n = 12–49 and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to 12 μM kainate using one-way ANOVA followed by Dunnett's multiple comparison test. Fluorescent micrographs of PI uptake of organotypic hippocampal slice cultures showing the typical regional pattern of neuronal death induced by the experimental situation indicated next to each photograph (D): (I) control culture with low basal PI uptake, (II) culture exposed to 12 μM kainate, (III) culture exposed to 1 μM [Leu^{31},Pro^{34}] NPY 30 min after starting the exposure to 12 μM kainate and (IV) culture exposed to 300 nM NPY13–36 30 min after starting the exposure to 12 μM kainate.

(n = 30), 54.78 ± 4.10% (n = 30) and 39.40 ± 4.44% (n = 30) in CA1, CA3 and DG (Fig. 2).

Evaluation of morphology in toluidine blue stained cultures exposed to 12 μM kainate for 24 h indicated strong degenerative changes, especially in the CA3 pyramidal cell layer, where many swollen neurons with a necrotic-like morphology and condensed apoptotic-like nuclei were observed (Fig. 3B). These degenerative events were prevented following Y_1 and Y_2 receptor activation 30 min after starting the kainate exposure (Fig. 3C and D).

The selectivity of the effects observed in the presence of NPY Y_1 and Y_2 receptor agonists was further evaluated by using BIBP3226 or BIIE0246 (Y_1 or Y_2 receptor antagonist, respectively). The co-exposure to BIBP3226 (1 μM) and [Leu^{31},Pro^{34}] NPY (1 μM) 30 min after starting the exposure to 12 μM kainate resulted in the loss of the neuroprotection caused by the activation of Y_1 receptors: 84.29 ± 15.34% (n = 12) in CA1, 116.30 ± 16.75% (n = 12) in CA3 and 115.5 ± 21.19% (n = 12) in DG (Fig. 4). Similarly, co-exposure to BIIE0246 (1 μM) and NPY13-36 (300 nM) resulted in inhibition of the neuroprotective effects induced by activation of Y_2 receptors in all hippocampal subregions analyzed: 105.30 ± 14.85% (n = 12) in CA1, 96.87 ± 18.13% (n = 12) in CA3 and 102.10 ± 25.72% (n = 12) in DG (Fig. 4).

4. Discussion

We have previously shown that pre-incubation with NPY receptor agonists, before starting an excitotoxic insult with kainate, resulted in neuroprotection against kainate-induced excitotoxicity in rat organotypic hippocampal slice cultures [39]. In the present study we attempted to determine whether NPY receptor activation after starting the excitotoxic insult with kainate can still protect and rescue neurons from cell death. Here we show that, indeed, NPY Y_1 and Y_2 receptor activation at 30 min but not 60 min after starting kainate exposure prevents cell death

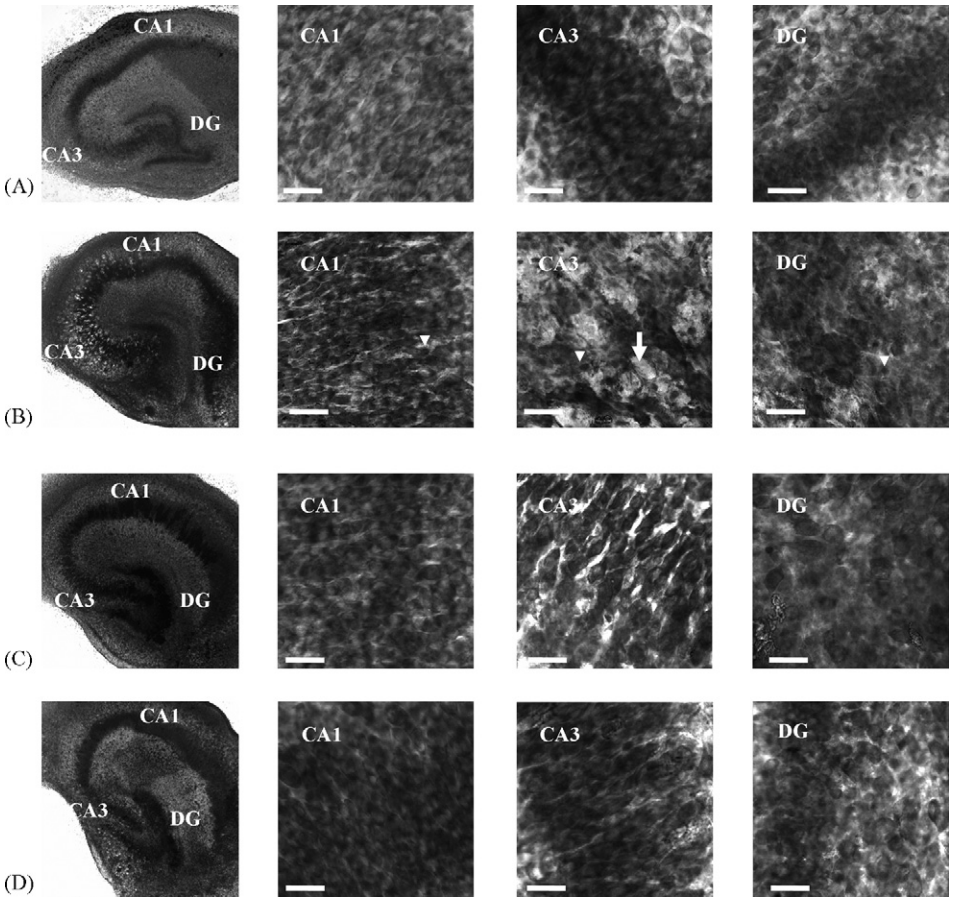

Fig. 3 – Morphological changes of cultured organotypic hippocampal slice cultures exposed to kainate (KA) for 24 h and neuroprotection afforded by NPY receptor agonists. (A) toluidine blue stained hippocampal slice cultures in control condition, (B) culture exposed to 12 μM kainate, (C) culture incubated with the Y_1 receptor agonist ([Leu^{31},Pro^{34}] NPY (1 μM) 30 min after starting the exposure to 12 μM kainate and (D) culture incubated with the Y_2 receptor agonist NPY13–36 (300 nM) after starting the exposure to 12 μM kainate. Arrow indicates swollen necrotic-like cells; arrow head indicate condensed apoptotic-like cell nuclei. Scale bar 40 μm.

in CA1 and CA3 pyramidal cell layer and in DG granular cells.

In accordance with published reports [12,39], kainate exposure results in both necrotic and apoptotic-like features. Necrosis is characterized by progressive loss of cytoplasmic integrity, rapid influx of Na^+, Ca^{2+}, and water, resulting in cytoplasmic swelling and nuclear pyknosis [2,4,27]. The latter feature leads to nuclear and cell fragmentation and the release of lysosomal and granular contents into surrounding extracellular space, causing subsequent inflammation [7,26,30,35]. Apoptosis can be induced by the ligation of plasma membrane death receptors (CD95, TNF-receptor, and TNF-related apoptosis-inducing ligand receptor), which stimulate the ''extrinsic pathway'', or by perturbation of intracellular environment homeostasis, the ''intrinsic pathway'' [5,11]. The intrinsic pathway involves mitochondrial dysfunction and the release of mitochondrial membrane proteins including cytochrome c, which stimulates cytosolic assembly of the apoptosome, the caspase activation complex [5,11,23]. Mitochondria participate in apoptotic and necrotic cell death, suggesting that death/toxic signals, through common mechanisms, can cause both apoptosis and necrosis depending on variables such as ATP content [5].

Since NPY receptors activate signaling pathways involved in the regulation of intracellular Ca^{2+} homeostasis [38], it is possible that NPY may exert its protective effects partially via its action on Ca^{2+} influx, as Thiriet et al. [42] suggested for NPY-mediated protection against methamphetamine-induced neuronal apoptosis. This idea is consistent with the demonstration that activation of both Y_1 and Y_2 receptors in hippocampal cells inhibits the increase in intracellular calcium concentration following KCl depolarization [36,38]. Also, by inhibiting Ca^{2+} influx NPY may inhibit the calcium-dependent death pathways involving caspase and calpain activation and mitochondrial dysfunction [1,34], and thus prevent cell death [39].

Taken together, the present data further support a putative role for NPY-dependent signaling mechanisms as potential neuroprotective targets against excitotoxic insults, able to rescue post-insult injured neurons.

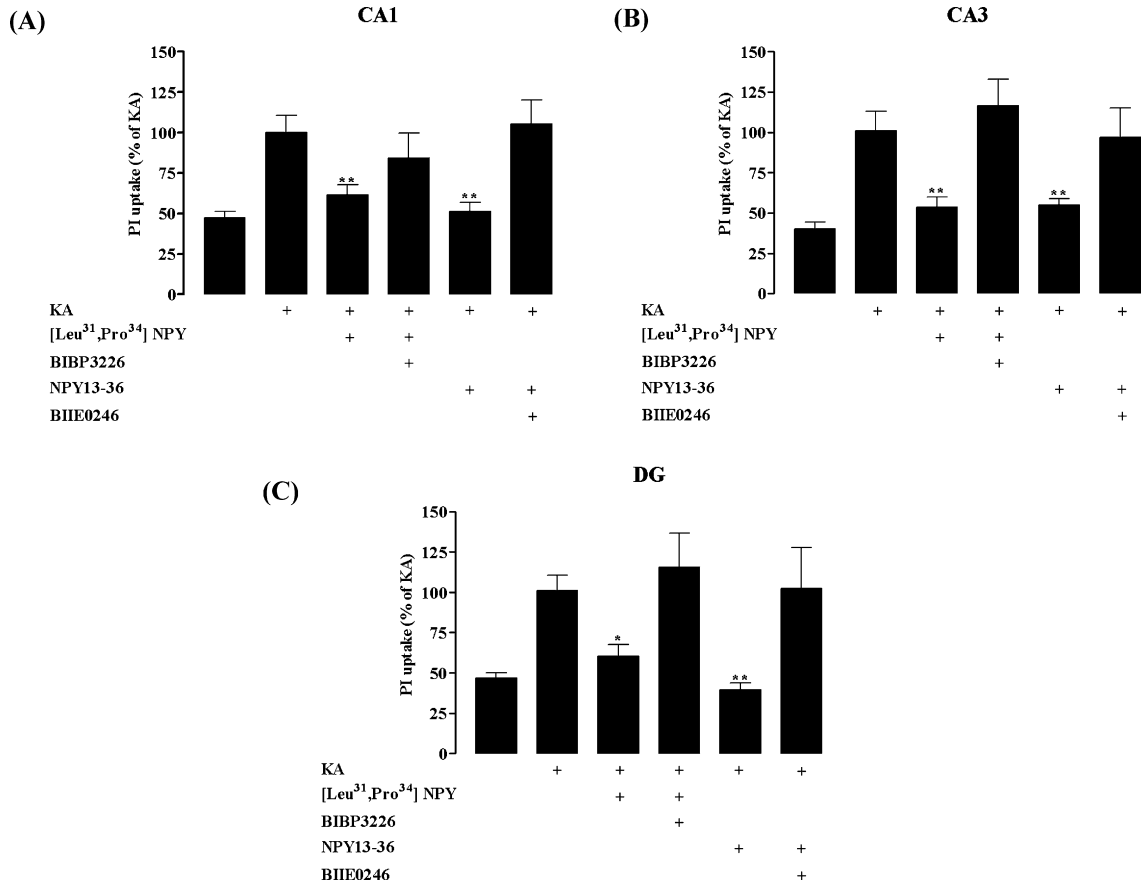

Fig. 4 – Blockade of Y_1 and Y_2 receptor-mediated neuroprotection by Y_1 and Y_2 selective antagonists. Effect of 1 μM BIBP3226 or 1 μM BIIE0246 (Y_1 and Y_2 receptor antagonists, respectively) on PI uptake induced by 12 μM kainate plus [Leu^{31},Pro^{34}] NPY (1 μM) or NPY13–36 (300 nM) (Y_1 and Y_2 receptor agonist, respectively) in the CA1 (A) and CA3 (B) pyramidal cell layers and in DG (C). Data are shown as means ± S.E.M., with n = 12–49 and $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the effect of 12 μM kainate, using one-way ANOVA followed by Dunnett's multiple comparison test.

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