

Neuropeptide Y Protects Rat Cortical Neurons against β -Amyloid Toxicity and Re-establishes Synthesis and Release of Nerve Growth Factor

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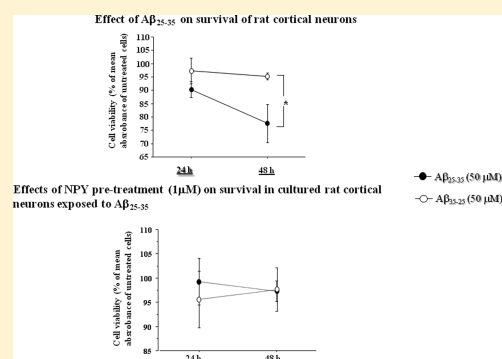
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ABSTRACT: Neuropeptide Y (NPY) is a 36 amino acid peptide, widely distributed within central nervous system neurons. More recently, it has been shown that NPY is involved in Alzheimer's disease (AD), a disorder characterized by accumulation of amyloid β -peptide ($A\beta$) in neurons. In a previous study, we investigated the effect of NPY on neuronal damage by exposing SH-SY5Y cells (an established human derived neuroblastoma cell line) to $A\beta$'s pathogenic fragment 25–35 ($A\beta_{25-35}$). We found a NPY-neuroprotective action associated with changes in intracellular production of nerve growth factor (NGF), a member of the neurotrophin family. Since our results were encouraging, we decided to replicate our data using primary cortical neurons cultured in presence of $A\beta_{25-35}$, and investigated whether NPY had similar neuroprotective action. Moreover, since cortical neurons are able to produce and release NGF, we investigated whether the synthesis and release of NGF were modified in such experimental conditions. Our results showed that a preincubation with NPY counteracted the toxic effect of $A\beta$, as measured by increased cell viability. Moreover, NPY pretreatment had an effect on NGF since its intracellular synthesis was increased, release was normalized, and mRNA expression was downregulated. Notably, these effects on NGF were in the opposite direction of those produced by incubating the cells with $A\beta$ alone. This study in primary cortical neurons supports the hypothesis that NPY may be a neuroprotective agent against β -amyloid neurotoxicity. These data also suggest that NPY may influence the synthesis and the release of NGF by cortical neurons.

KEYWORDS: NPY, β -amyloid, primary cells, cerebral cortex, NGF, cortical neurons



Neuropeptide Y (NPY) is a 36 amino acid peptide, widely distributed within neurons of the central and peripheral nervous systems, and it is found in mammalian brain in higher concentrations than all other peptides studied to date.¹ It acts as a neurotransmitter and/or a modulator of several neuroendocrine functions including cardiovascular physiology and feeding behavior.² Studies performed in laboratory animals have shown that, in the central nervous system (CNS), NPY produces behavioral effects that are close to those induced by anxiolytics,^{3–5} antiepileptics,⁶ and antidepressants.^{7–9}

More recently, it has been shown that NPY is involved in Alzheimer's disease (AD), a disorder characterized by the accumulation of amyloid plaques, built up mainly of the amyloid β -peptide ($A\beta$), and neurofibrillary tangles, made by the microtubule-binding protein tau.¹⁰ A significant NPY reduction in the hippocampal regions of AD brains has been shown¹¹ in conjunction with an altered number of NPY receptors.¹² Also, reduced plasma¹³ and cerebrospinal fluid NPY levels¹⁴ have been observed during AD progression, thus confirming a close correlation between NPY and AD pathogenesis and/or pathophysiology.

Interestingly, a neuroprotective effect of NPY against AD neuronal damages in the CNS has been reported in AD transgenic mouse models,¹⁵ although the mechanism of action of this NPY-mediated neuroprotection is still under investigation. In the attempt to elucidate this mechanism, in a previous study, we investigated the effect of NPY on neuronal damage by exposing SH-SY5Y cells (a well established human derived neuroblastoma cell line) to $A\beta$'s pathogenic fragment 25–35 ($A\beta_{25-35}$).¹⁶ We found a NPY-neuroprotective action on these cells associated with changes in intracellular production of nerve growth factor (NGF), a member of the neurotrophin family. NGF is relevant to AD because it supports survival and function of cholinergic neurons of the basal forebrain (BFCNs)¹⁷ which undergo degeneration early and contribute significantly to cognitive decline in AD.¹⁸ Accordingly, it has been shown that decreased supply of NGF at the level of BFCN cell bodies leads to loss of neuronal markers and

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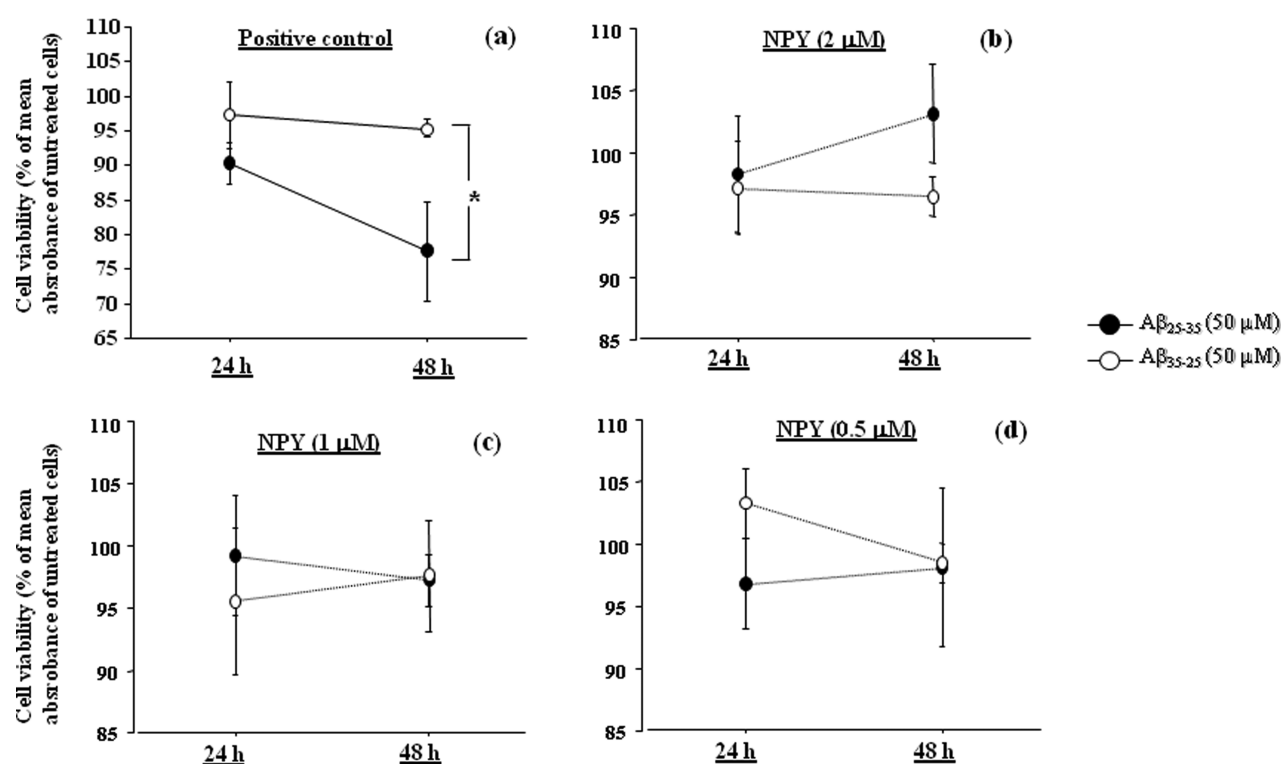
NPY effects on cell viability in cultured rat cortical neurons exposed to $A\beta_{25-35}$ 

Figure 1. NPY effects on cell viability in cultured rat cortical neurons exposed to $A\beta_{25-35}$. Cortical neurons were preincubated alone (a) or with three concentrations of NPY [(b) 2 μ M, (c) 1 μ M, and (d) 0.5 μ M] for 24 h and then exposed to $A\beta_{25-35}$ (50 μ M) or $A\beta_{35-25}$ (50 μ M) for 48 h (number of experiments performed = 4). After 24 and 48 h, cells were tested with MTS assay. NPY treatment restored normal cell survival. Cell viability is expressed as percent of the mean absorbance value of untreated cells at each time point. Data represent means \pm SEM

shrinkage, mimicking what is observed in AD.¹⁹ In addition, NPY shares with NGF the ability to counteract excitotoxicity²⁰ and modulate neurogenesis.^{21–23}

Nevertheless, although our results were encouraging, the neuroblastoma cells used in that study were tumor cells with protein expression patterns rather different from nontumor cells. Thus, being aware of these issues, we decided to replicate our data using primary cells which are more close to normal physiologic conditions. We used the rat primary cortical neurons cultured in presence of $A\beta_{25-35}$, and investigated whether NPY had similar neuroprotective action. Moreover, since cortical neurons are able to produce and release NGF, which is in turn retrogradely transported to BFCNs,²⁴ we investigated whether the synthesis and release of NGF would be modified in such experimental conditions.

RESULTS AND DISCUSSION

In this study, we investigated the effect of preincubation with NPY on primary cortical neurons of fetal rats exposed to amyloid beta peptide fragment 25–35. Our aim was to verify the possibility that NPY may exert neuroprotective effects on cortical neurons and regulate the expression of nerve growth factor by these cells.

$A\beta_{25-35}$ Reduces Rat Cortical Neuron Viability and Pretreatment with NPY Protects Neurons from $A\beta_{25-35}$ Toxicity. The neuroprotective effect of NPY on neuronal survival was investigated. Primary cortical neurons were preincubated either alone (positive control) or with three concentrations of NPY (0.5, 1, and 2 μ M) for 24 h and then

exposed to $A\beta_{25-35}$ (50 μ M) or $A\beta_{35-25}$ (50 μ M) for 48 h (Figure 1). At 24 and 48 h, neuronal survival was evaluated with MTS assay.

As shown in Figure 1a, ANOVA showed that there was a significant effect of the treatment in positive control ($p < 0.05$). Posthoc comparisons revealed that after 48 h of incubation $A\beta_{25-35}$ significantly reduced cell viability as compared to $A\beta_{35-25}$ (50 μ M) ($p < 0.05$).

The results also showed that NPY was able to protect cortical neurons from $A\beta_{25-35}$ toxicity. We found that 2 μ M NPY abolished the toxic effects of $A\beta_{25-35}$ ($p = 0.27$ for the effect of the treatment) at 24 and 48 h (Figure 1b). The same effect on neuronal survival was observed in neurons exposed to 1 μ M (Figure 1c) ($p = 0.71$; treatment effect) and 0.5 μ M (Figure 1d) ($p = 0.27$; treatment effect) NPY pretreatments.

$A\beta_{25-35}$ Reduces NGF Synthesis and Release and Increases NGF mRNA. In order to evaluate the effect of $A\beta$ treatment on NGF synthesis and release, we incubated for 24 h cortical neurons with $A\beta_{25-35}$ (50 μ M) or $A\beta_{35-25}$ (50 μ M) and measured NGF protein levels in neurons and the release in culture medium (Figure 2a,b). We found an effect of the treatment on NGF synthesis ($p < 0.05$) and an interaction between treatment and time ($p < 0.05$) (Figure 2a). Post hoc analysis showed that $A\beta_{25-35}$ reduced NGF synthesis at 24 h ($p < 0.01$) and 48 h ($p < 0.05$). The release of NGF was also affected by $A\beta_{25-35}$ treatment ($p < 0.01$) and reduced NGF was observed in the culture medium of $A\beta_{25-35}$ exposed cortical neurons at 24 h ($p < 0.05$) and 48 h ($p < 0.05$) (Figure 2b).

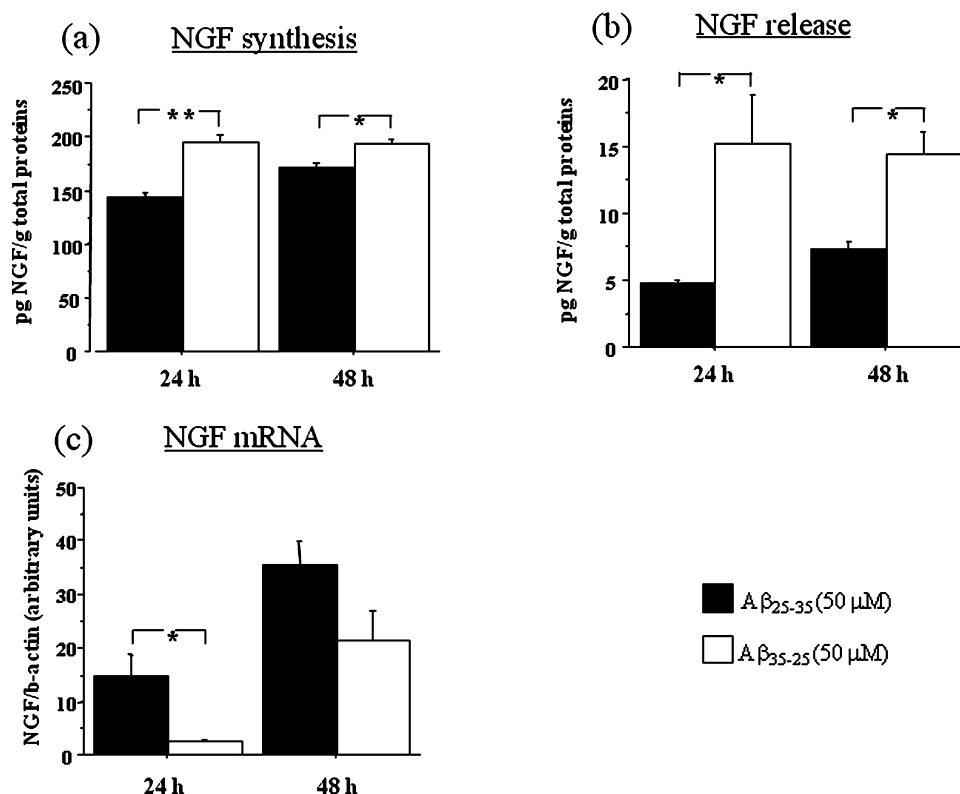
NGF protein and mRNA in cortical neurons exposed to $A\beta_{25-35}$ 

Figure 2. NGF protein [(a) synthesis, (b) release] and mRNA (c) in cortical neurons incubated with $A\beta_{25-35}$ (50 μ M) or its inactive control $A\beta_{35-25}$ (50 μ M). Cell pellets were collected at 24 and 48 h. NGF protein levels are expressed in pg/g total proteins. NGF mRNA was measured by real time PCR and expressed as amount of target gene normalized to an endogenous reference (β -actin) and relative to a calibrator ($\Delta\Delta$ Ct) Data represent means \pm SEM. Asterisks indicate statistical level of significance (* $p < 0.05$; ** $p < 0.01$).

NGF mRNA was evaluated by real time PCR. An effect of $A\beta$ treatment was observed ($p < 0.05$). While NGF synthesis was reduced, we found that NGF mRNA was increased by $A\beta_{25-35}$ at 24 h ($p < 0.05$). It is possible that the increase in NGF mRNA at 24 h may represent an early attempt to counteract $A\beta_{25-35}$ neurotoxic insult. An effect of time was also noted ($p < 0.01$) because NGF mRNA increased with time in both groups (Figure 2c).

Pretreatment with NPY Increases NGF Synthesis, Reduces NGF mRNA, and Restores NGF Release in Cortical Neurons Exposed to $A\beta_{25-35}$. To investigate whether the effects of NPY on cell survival were associated with changes in NGF production, NPY (1 μ M) pretreated cortical neurons were exposed to $A\beta_{25-35}$ (50 μ M) or its inactive control $A\beta_{35-25}$ (50 μ M) (Figure 3).

For intracellular NGF production, we found an effect of the treatment ($p < 0.05$) and an interaction between treatment and time ($p < 0.01$). Post hoc analysis showed that NGF synthesis was increased in $A\beta_{25-35}$ exposed neurons ($p < 0.05$) at 24 h (Figure 3a). Release of NGF in the culture medium was normalized by NPY pretreatment (Figure 3b). No differences in NGF release between $A\beta_{25-35}$ and $A\beta_{35-25}$ were noted. An effect of time was present, since release of NGF significantly decreased at 48 h in both groups ($p < 0.01$).

NGF mRNA was also evaluated in cortical neurons pretreated with NPY (1 μ M) and exposed to $A\beta_{25-35}$ (50 μ M) or its inactive control $A\beta_{35-25}$ (50 μ M) (Figure 3c). There was a significant effect of the treatment ($p < 0.05$) and an

interaction between treatment and time ($p < 0.05$). We found that NGF mRNA level after $A\beta_{25-35}$ treatment was not different from that of $A\beta_{35-25}$ at 24 h and even decreased at 48 h suggesting that NPY pretreatment had a counteracting effect on NGF mRNA on neurons subsequently exposed to toxic levels of $A\beta_{25-35}$.

This study was the logical extension of a previous experiment performed in our laboratory using a human neuroblastoma cell line.¹⁶ In that study, we observed a neuroprotective effect of NPY against $A\beta$ neurotoxicity associated with increased synthesis of neurotrophins by neuroblastoma cells. Thus, in order to verify these data in primary cells (which are taken directly from living tissue) and further investigate the mechanism of action of NPY on neurotrophins, we exposed rat cortical neurons to the biologically active region of $A\beta$ (fragment $A\beta_{25-35}$),²⁵ pretreated them with NPY, and measured synthesis, release, and gene expression of nerve growth factor.

Our results showed that a preincubation with NPY counteracted the toxic effect of $A\beta$, as measured by increased cell viability. Moreover, NPY pretreatment also had an effect on NGF since its intracellular synthesis was increased, release was normalized, and mRNA expression was downregulated. Notably, these effects on NGF were in the opposite direction of those produced by incubating the cells with $A\beta$ alone.

This study represents an additional step to further investigate the possibility that NPY is a potential neuroprotective agent in Alzheimer's disease. The fact that NPY exerted neuroprotection against $A\beta$ toxicity in both neuroblastoma (an immortalized cell

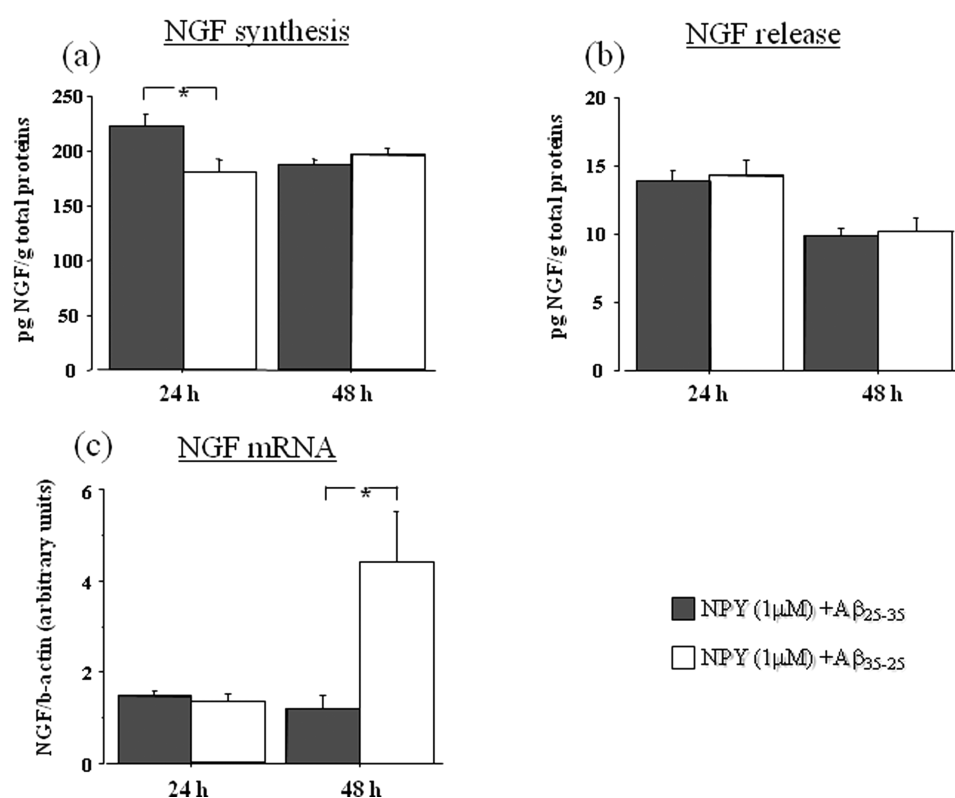
NGF protein and mRNA in NPY pre-treated neurons exposed to $A\beta_{25-35}$ 

Figure 3. NGF protein [(a) synthesis, (b) release] and mRNA (c) in cortical neurons pretreated with NPY (1 mM) for 24 h and then exposed to toxic concentration (50 μM) of $A\beta_{25-35}$ or its inactive control $A\beta_{35-25}$ (50 μM). Cell pellets were collected at 24 and 48 h. NGF protein levels are expressed in pg/g total proteins. NGF mRNA was measured by real time PCR and expressed as amount of target gene normalized to an endogenous reference (β -actin) and relative to a calibrator ($\Delta\Delta\text{Ct}$). Data represent means \pm SEM. Asterisks indicate statistical level of significance ($*p < 0.05$).

line) and primary cells is encouraging. However, the mechanism of this NPY-induced neuroprotection is not completely clear. In our previous paper,¹⁶ we hypothesized that NPY exerts its neuroprotective action by inhibiting neuronal excitability through a mechanism involving the inhibition of the voltage-dependent Ca^{2+} influx in presynaptic nerve terminals,^{26–28} an effect probably mediated by presynaptic activation of NPY Y2 receptors.^{20,29,30} This idea is supported by some studies showing that NPY is able to inhibit neuronal excitability in *in vivo* and *in vitro* models.³¹ In addition, transgenic mice for human amyloid precursor protein (hAPP) have spontaneous nonconvulsive seizure activity in cortical and hippocampal networks, associated with GABAergic sprouting, enhanced synaptic inhibition, and synaptic plasticity deficits in the dentate gyrus supporting the idea that agents that prevent neuronal excitability may be neuroprotective to $A\beta$ -induced neurological deficits.³²

Further pharmacological characterization of NPY-receptor subtypes will assess the possibility that NPY-receptor agonists or antagonists can be considered as valuable compounds to prevent neurodegeneration. In addition, it is of interest that NPY may cross the blood-brain barrier (BBB) by passive diffusion,³³ while other proteins of potential therapeutic interest in AD, including NGF, possess poor permeability to the BBB.

Another important finding is the effect of NPY on synthesis and release of NGF. It is well documented that NGF is a critical survival factor for cholinergic neurons which undergo neuro-

degeneration in late stage Alzheimer's disease.^{18,19} We found that $A\beta$ reduced the synthesis and release of this neurotrophins. Since NGF is synthesized in the cortex and retrogradely transported to basal forebrain cholinergic neurons,¹⁹ it is possible that basal forebrain neurons may be damaged not only by the accumulation of $A\beta$ and neurofibrillary tangles but also by reduced NGF support from cerebral cortex neurons. Thus, there is the possibility that NPY by increasing NGF synthesis and release in cortical neurons may have beneficial effects to the basal forebrain. However, this hypothesis needs to be further investigated. A necessary step to validate this hypothesis could be to administer NPY in appropriate AD animal models and verify its potential neuroprotective effects to the cholinergic system. This idea is also supported by an immunocytochemistry study demonstrating that loss of cholinergic neurons in the basal forebrain of AD patients is selective and, notably, NPY-positive neurons are preserved.³⁴ Another issue to be studied is the potential side effects of NPY *in vivo*, consisting in peripheral hemodynamic effects which may limit its use in human therapy.

In conclusion, this study in primary cortical neurons supports the hypothesis that NPY may be a neuroprotective agent against beta amyloid neurotoxicity. These data also suggest that NPY may influence the synthesis and the release of NGF by cortical neurons. The possible implications of these findings in AD pathophysiology need to be explored.

METHODS

Primary Cell Cultures. Cortical neuron cultures were prepared from embryonic day 17–19 fetal Wistar rats. The brains were removed and cortices were freed of meninges, washed with 6 mL of Earl's balanced salt solution, and centrifuged for 2 min at 150g. The tissue was resuspended and incubated for 30 min at 37 °C with 0.02% trypsin followed by addition of DNase I (80 µg/mL final) and trypsin inhibitor (0.52 mg/mL). Digested tissues were mechanically dissociated twice and centrifuged at 150g for 10 min. The dissociated cells were plated at a density of 2×10^5 cells/cm² on poly-L-lysine (Sigma-Aldrich) wells in Neurobasal media (Gibco, cat. no. 21103-049) supplemented with 2% B27 (Gibco, cat. no. 17504-044),³⁵ 0.5 mM L-glutamine (Euroclone, cat. no. ECB3000D), and 50 U/mL penicillin/streptomycin solution (Euroclone, cat. no. ECB3001D). Cells were grown at 37 °C and 5% CO₂, changing half medium every 3 days from the seeding to the execution of the experiments, when we halved the concentration of B-27 supplement. Cells from the 11th day on were used for experiments.

All animal experiments were performed in accordance to the Italian law on the use and care of laboratory animals (DL 116/92).

Drugs. NPY (human, rat; cat. no. H-6375, Bachem AG, Switzerland) was resuspended in distilled H₂O at a stock concentration of 0.1 mM, filtered/sterilized, and stored at –20 °C.

A β protein (fragment 25–35; cat. no. H-1192, Bachem AG, Switzerland) and its inactive form (A β protein 35–25; cat. no. H-2964, Bachem AG, Switzerland) were both reconstituted in sterile distilled H₂O at a concentration of 1 mM, filtered/sterilized, incubated for 72 h at 37 °C to induce aggregation, and immediately stored at –20 °C. Aggregation was confirmed by electron microscope. The reason why we used A β _{25–35} is that this short fragment retains the toxicity of A β _{1–42}^{36–39} but, unlike the full-length peptide, it forms fibrils immediately after solution.⁴⁰

Cell Treatments for Survival Study and NGF Analysis. To evaluate the neuroprotective effects of NPY, neurons were preincubated alone or with different NPY concentrations (0.5, 1, and 2 µM) for 24 h and then the same concentration of active/inactive A β -peptide (50 µM) was added to the medium for 48 h. In such conditions, we have evidence in primary cell cultures that NPY may possess biological activity at least for 48 h.^{41–43} After incubation, neuronal survival was evaluated with MTS assay. Three independent experiments were performed for each condition.

Based on the data obtained from survival study, for NGF studies, peptide concentrations were fixed at 50 µM for A β _{25–35} and at 1 µM for NPY pretreatment. Neurons were first exposed to A β _{25–35} or its inactive control A β _{35–25} for 48 h. In the second experiment, the cells were pretreated with NPY (1 µM) and then exposed to A β peptides at the same conditions. At the end of each experiment, cortical neurons were gathered with QIAzol Lysis Reagent (Qiagen) and stored at –80 °C for analyses using the RNA, while cells (gathered in RIPA buffer) and media were collected and stored at –80 °C for NGF immunoassay (ELISA) and for quantification of total proteins with Bradford assay (BioRad Protein Assay, BioRad Laboratories GmbH). The suitable times of cell collection were fixed at 24 and 48 h. Three independent experiments were performed for each experimental treatment.

MTS Assay. At 24 and 48 h, we performed a MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay, Promega) in order to study cell survival and select the concentration of β -amyloid and NPY to be used in successive experiments. MTS assay was performed according to manufacturer instructions. Briefly, cortical neurons were cultured in a 96-well microtiter plate. After treatment, 20 µL of MTS solution was added to each well. Then, after an incubation of 1–2 h, the absorbance was measured at 492 nm by a multiplate reader (Benchmark Plus, Biorad).

Real-Time PCR. Total RNA was extracted and purified using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The quality and quantity of total RNA was assessed by using the NanoDrop ND-1000 spectrophotometer ($A_{260/280} > 2.00$; $A_{260/230} > 1.9$) and its integrity was checked by 1% agarose gel

electrophoresis. For total cDNA reverse transcription (RT), we used the protocol and reagents of the ImProm-II Reverse Transcription System (Promega) kit. The final volume of 20 µL contained 500 ng RNA, 500 ng of random examers, 0.5 mM dNTPs mix, 1× ImProm-II Reaction Buffer, 5 mM MgCl₂, 20 U Rnasin, and 1 µL of ImProm-II reverse transcriptase.

Real-time PCR for NGF quantification was performed in the Mx3000P Stratagene (La Jolla, CA) instrument, using the DNA binding dye SYBR Green (SYBR Green PCR Master Mix; Applied Biosystems). Twenty microliters of PCR mix containing 10 µL of SYBR Green mix, forward and reverse primers, and 2 µL of cDNA obtained from the RT procedure were used for comparative quantification. We used 300 nM of forward and reverse primers for NGF and 150 nM of both primers for the housekeeping gene β -actin. Primers were designed using Primer Blast (NCBI) and synthesized by Primm srl (Italy). All primers were designed to be specific for a unique sequence within the total rat genome as well as to be located on different exons to avoid DNA contamination. The sequences of each primer are listed in table 1.

Table 1. Real-Time PCR Primer Sequences

NGF forward	5'-GGACGCAGCTTCTATCCTG-3'	product length (bp): 368
NGF reverse	5'-CATGGGCTGGAAGTCTAAA-3'	
β -actin forward	5'-CTTCCTGGGTATGGAATCCTGT-3'	product length (bp): 364
β -actin reverse	5'-GGTGTAACACGCAGCTCAGTAAC-3'	

Negative controls with no cDNA sample were run to exclude PCR mix contamination. Samples for NGF and β -actin transcripts were run in triplicate.

The thermal profile was as follows: 5 min at 95 °C followed by 50 cycles of 95 °C for 15 s, 59 °C for 30 s, and 72 °C for 30 s. The amplification specificity was confirmed by melting-curve analysis of the PCR products. Standard curves for NGF and β -actin were also performed to verify that the amplification efficiencies of both the target and control genes were comparable within a range of 5% from each other. The 2-DDCt method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments.⁴⁴

Protein Extraction. Cell pellets were resuspended and homogenized in 0.3 mL of ice-cold RIPA buffer, containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% NP40, 0.1% SDS, 1 mM AEBBSF (freshly prepared), 2 mM DTT (freshly prepared), and 0.5% deoxycholate. The tissue homogenate solutions were centrifuged at 14 000g for 5 min at 4 °C. The supernatants were collected and used for quantification of NGF and total proteins.

NGF Determination with Immunoassay (ELISA). NGF concentrations in cell pellet and culture medium were determined in sandwich ELISAs according to the manufacturer's instructions (Promega). NGF concentrations were determined from the regression line for the standard (ranging from 3.9 to 500 pg/mL, purified mouse NGF) incubated under similar conditions in each assay. The detection limit was <4 pg/mL. NGF concentration was expressed as pg NGF/g total proteins. Cross-reactivity to other related neurotrophins, such as neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), was less than 3%. All assays were performed in duplicate.

Bradford Assay. We performed a Bradford assay on pellets and media used for ELISA assays to quantify the total protein amount. We used Bradford solution (Biorad) and BSA 1 mg/mL (Sigma) to do the standard curve in a Varian spectrophotometer.

Statistical Analysis. Differences between groups of treatment were analyzed by analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) posthoc test. Interaction between treatment and time was evaluated by two-way ANOVA. A *p*

value of <0.05 was considered statistically significant. Statistical analysis was performed using the Statview software from SAS Institute.

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Author Contributions

F.A., N.C., and F.G. designed the study and wrote the protocol. T.C. provided primary rat cortical neurons and protocols. N.C., F.G., and S.C. performed experimental procedures. G.F., C.C., and S.B. managed the literature searches and analyses. F.A. undertook the statistical analysis, and F.A. and N.C. wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

NPY, neuropeptide Y; AD, Alzheimer's disease; CNS, central nervous system; A β , amyloid β -peptide; NGF, nerve growth factor; BBB, blood-brain barrier; BFCNs, cholinergic neurons of the basal forebrain

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