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NPY modulates miR-30a-5p and BDNF in opposite direction in an in vitro model of Alzheimer disease: a possible role in neuroprotection?

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Abstract Using in vitro models of Alzheimer's disease (AD), we found that the toxic effects of amyloid beta 25–35 ($A\beta_{25-35}$) on the neurotrophin brain-derived neurotrophic factor (BDNF) were counteracted by pre-incubation with neuropeptide Y (NPY), a neuropeptide expressed within the central nervous system. Nonetheless, the mechanism of action of NPY on BDNF neuronal production in the presence of Aβ is not known. BDNF expression might be directly regulated by microRNA (miRs), small non-coding DNA fragments that regulate the expression of target genes. Thus, there is the possibility that miRs alterations are present in AD-affected neurons and that NPY influences miR expression. To test this hypothesis, we exposed NPY-pretreated primary rat cortical neurons to $A\beta_{25-35}$ and measured miR-30a-5p (a member of the miR-30a family involved in BDNF tuning expression) and BDNF mRNA and protein expression after 24 and 48 h. Our results demonstrated that pre-treatment with NPY decreased miR-30a-5p expression and increased BDNF mRNA and protein expression at 24 and 48 h of incubation with Aβ. Therefore, this study demonstrates that NPY modulates BDNF and its regulating microRNA miR-30a-5p in opposite direction with a mechanism that possibly contributes to the neuroprotective effect of NPY in rat cortical neurons exposed to Aβ.

Keywords NPY · miR-30a-5p · BDNF · Primary cortical neurons · Alzheimer disease

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Introduction

Alzheimer's disease (AD) is a disease of aging, normally viewed as originating within the brain, leading to a slow inexorable decline in cognitive functions [1]. Pathophysiologically, AD is characterized by the accumulation of amyloid β-peptide (Aβ) in neurons in the form of senile plaques and neurofibrillary tangles, leading to synaptic degeneration [2].

Many studies have analyzed the involvement in AD of the neurotrophin brain-derived neurotrophic factor (BDNF), a critical factor for neuronal survival and neural plasticity associated with memory formation and consolidation [3]. BDNF promotes the survival of the major neuronal types affected in AD, like hippocampal and neocortical neurons, cholinergic septal, basal forebrain neurons, and nigral dopaminergic neurons [4]. In particular, post-mortem studies performed in hippocampus and temporal cortex from AD patients have shown a reduction of BDNF mRNA [5] and protein [6] expression compared to that of healthy subjects. Also, analysis of serum BDNF levels revealed a significant increase in early phases AD patients [7] and a decrease at more severe stages of AD [8] when compared to that of healthy subjects. Moreover, among AD patients, BDNF serum levels were lower in subjects characterized by rapid cognitive decline, suggesting that higher BDNF serum levels might be associated with slower disease progression [9].

Using an in vitro model of AD, a human neuroblastoma cell line incubated with the pathogenic fragment amyloid beta 25–35 ($A\beta_{25–35}$), we found that incubation with Aβ induced a reduction in cell viability and a decrease in BDNF protein levels [10], a result in line with the human studies mentioned before. In the same experiment, we also found that these effects were counteracted by a pre-incubation with neuropeptide Y (NPY) [10], a small neuropeptide involved in different functions, such as cardiovascular physiology and feeding behavior, but also able to provide neuroprotection against the damage caused by amyloid plaques and neurofibrillary tangles in animal models [11]. This neuroprotective effect against Aβ was also confirmed by our group in rat cortical primary neurons pre-incubated with NPY [12]. Nonetheless, although these data suggest that NPY may be a molecule of potential interest in AD therapy, the mechanism of action of NPY on BDNF neuronal production in the presence of Aβ is still not known.

Several studies demonstrated that BDNF expression might be directly regulated by microRNA (miRs) [13]. MiRs are small non-coding DNA fragments the transcripts of which act mostly as down-regulators of matching mRNAs. Accordingly, Mellios et al. [14] have reported that miR-30a-5p and miR-195 target the 3′UTR of BDNF, and in particular, the former down-regulates BDNF protein neuronal production in humans. Thus, there is the possibility that miR-30a-5p alterations are present in AD-affected neurons and that NPY, by influencing miR expression, may affect BDNF production and/or exert neuroprotective effects. However, to date, data on miR-30a-5p levels, BDNF, and NPY in AD pathophysiology are missing.

Based on these evidences, the aim of this study was to investigate whether exposure to NPY may influence miR-30a-5p and BDNF expression in an AD in vitro model. We used primary rat cortical neurons pre-incubated with NPY and then exposed to $A\beta_{25–35}$ fragment in the same experimental conditions where NPY showed neuroprotective effects [10], and measured miR-30a-5p expression by these cells. In addition, we also measured BDNF mRNA and protein levels and compared them to those of miR-30a-5p.

Materials and methods

Primary cortical neurons

Primary cortical neuron cultures were taken from embryonic day 17–19 fetal Wistar rats. The brains were removed, and the meninges were taken out from cortices, washed with 6 ml of Earl's balanced salt solution and pelleted at 150 g for 2 min. The tissue was incubated at 37 °C for 30 min with 0.02 % trypsin; then, DNase I (80 μg/ml) and trypsin inhibitor (0.52 mg/ml) were added. Digested tissues were mechanically dissociated and centrifuged at 150 g for 10 min. The dissociated cells were seeded at a density of 2×10^5 cells/cm^2 on poly-L-lysine (Sigma-Aldrich) wells in Neurobasal medium (Gibco, cat. no. 21103-049) enriched with 2 % B27 (Gibco, cat. no. 17504-044) [15], 0.5 mM L-glutamine (Euroclone, cat. no. ECB3000D), and 50 U/ml penicillin/streptomycin solution (Euroclone, cat. no. ECB3001D). Cells were grown at 36 °C and 5 % CO_2, changing half the medium every 3 days from the plating to the beginning of the experiments. Cells from the 11th day on were used for experiments, adding a half of B-27 supplement to the medium.

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

Drugs

Aβ-amyloid (fragment 25–35; Bachem AG, Switzerland, cat. no. H-1192) and its inactive form (35–25; Bachem AG, cat. no. H-2964) were resuspended in sterile distilled water at a concentration of 1 mM, filtered/sterilized and stored at −20 °C. Aliquots were incubated just before the experiments at 37 °C for 72 h to induce aggregation into fibrils. Aggregation was confirmed by electron microscopy (not shown). $A\beta_{25–35}$ was used because this short fragment preserved the toxicity of the full-length protein $A\beta_{1–42}$ [16–19], but unlike this one, it forms fibrils immediately after solution [20].

NPY (human, rat, cat. no. H-6375, Bachem AG) powder was resuspended in sterile distilled water at 0.1 mM, filtered/sterilized and immediately stored at −20 °C.

Cell treatments

Based on previous experiments where the toxicity of Aβ and the neuroprotective effect of NPY were evaluated with an MTS survival assay [10, 12], the concentrations were fixed at 50 μM for $A\beta_{25–35}$ and 1 μM for NPY.

Neurons were preincubated with NPY (1 μM) for 24 h, then 50 μM of $A\beta_{25–35}$ or its inactive form $A\beta_{35–25}$was added to the medium for an additional 48 h. In these conditions, it is known that NPY possesses biological activity for at least 48 h in primary cell cultures [21–23]. Untreated cells, NPY- or Aβ-exposed cells were included as additional controls.

At the end of each experiment, cortical neurons were gathered with QIAzol Lysis Reagent (Qiagen), and stored at −80 °C for analysis using the RNA, while cells (gathered in RIPA buffer) were collected and stored at −80 °C for BDNF immunoassay (ELISA) and for quantification of total proteins with Bradford assay (BioRad Protein Assay, BioRad Laboratories GmbH). Cell collection was made

at 24 and 48 h. Three independent experiments were performed.

RNA isolation and reverse transcription

At the end of each experiment, the cells were gathered using QIAzol Lysis Reagent (Qiagen), then total and micro- RNA were extracted and purified together using the miRNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The quality and quantity of mRNA were assessed by the NanoDrop ND-1000 spectrophotometer (Thermo Scientific), with $A_{260/280} > 2.00$ and $A_{260/230} > 1.8$.

Total cDNA reverse transcription (RT) was carried out using reagents of the ImProm-II Reverse Transcription System (Promega). The final volume of 20 µl contained 500 ng RNA, 500 ng of random hexamers, 0.5 mM dNTPs mix, 1× ImProm-II Reaction Buffer, 5 mM $MgCl_2$, 20 U Rnasin, and 1 µl of ImProm-II reverse transcriptase.

For retrotranscription of miR-30a-5p, TaqMan® MicroRNA Reverse Transcription kit (Applied Biosystems, cat. no. 4366596) was used on 10 ng of RNA, following the manufacturer's protocol. The RT primers were taken from the TaqMan® MiRNA Assay (ABI) ID 417 for hsa-30a-5p and ID 1712 for U87 control miR (sequence ACAAUGAU GACUUAUGUUUUUGCCGUUUACCCAGCUGAGGG UUUCUUUGAAGAGAGAAUCUUAAGACUGAGC).

Real time PCR for miR-30a-5p microRNA

Real-time PCR for miR quantification was carried out with TaqMan miRNA kits (Applied Biosystems) following the manufacturer's protocol, using 1 ng of cDNA in a total mix of 20 µl containing 10 µl of TaqMan® Universal Master Mix II (Applied Biosystems, cat. no. 4440040) and 1 µl of TaqMan probes taken from the TaqMan® miRNA Assay (ABI) ID 000417 for hsa-30a-5p and ID 001712 for U87. The samples were run in triplicate, and the thermal standard profile (according to ABI protocol) for miR RT-PCR was used. The 2 ^-DDCt method was used to calculate relative changes in gene expression determined from real-time quantitative PCR experiments [24].

Real time PCR for BDNF mRNA

Real-time PCR for BDNF quantification was fulfilled in the Mx3005P Stratagene (La Jolla, Calif., USA) instrument, using the DNA binding dye SYBR Green (SYBR GREEN PCR Master Mix; Applied Biosystems). Twenty microliters of PCR mix contained 10 µl SYBR Green mix, forward and reverse primers, and 2 µl of cDNA obtained from the RT. Three hundred nanomolar of forward and reverse primers for BDNF amplification and 150 nM of both primers for the housekeeping gene β-actin were used.

Table 1 Real-time BDNF and β-actin primer sequences

BDNF forward	5′-GCG GAT ATT GCA AAG GGT TA-3′	Product length (bp): 367
BDNF reverse	5′-TCG TCA GAC CTC TCG AAC CT -3′	
β-actin forward	5′-CTT CCT GGG TAT GGA ATC CTG T-3′	Product length (bp): 364
β-actin reverse	5′-GGT GTA AAA CGC AGC TCA GTA AC-3′	

Primers were designed by Primer Blast (NCBI) and synthesized by Primm srl (Italy). The sequences of each primer are listed in Table 1.

Negative controls with no cDNA sample were run to exclude PCR mix contamination. Samples for BDNF and β-actin transcripts were run in triplicate. The thermal profile used an annealing temperature of 59 °C for 45 cycles. After PCR, a dissociation curve was added to confirm that no secondary products were formed. Standard curves for BDNF and β-actin were also performed to check that the amplification efficiencies of both the target and control genes were comparable within a range of 5 % from each other. As for miR-30a-5p, 2 ^-DDCt method was used to measure changes in gene expression [24].

Protein extraction

For BDNF extraction, cell pellets were resuspended and homogenized in 0.3 ml ice-cold lysis buffer, containing 137 mM NaCl, 20 mM Tris–HCl (pH 8.0), 1 % NP40, 10 % glycerol, 1 mM PMSF, 10 µg/ml aprotinin, 1 µg/ml leupetin, and 0.5 mM sodium vanadate. The tissue homogenate solutions were centrifuged at 14,000×*g* for 5 min at 4 °C. The supernatants were collected and used for quantification of BDNF and total proteins.

BDNF determination with immunoassay (ELISA)

Brain-derived neurotrophic factor protein concentrations in cell pellet were determined using sandwich ELISAs according to the manufacturer's instructions (Promega, USA). The assay was performed on F-bottom 96-well plates (Nunc, Wiesbaden, Germany). Tertiary antibodies were conjugated to horseradish peroxidase. Wells were developed with tetramethylbenzidine, and optical density was measured at 450/570 nm. BDNF concentrations were determined from the regression line for the standard (ranging from 3.9 to 500 pg/ml-purified mouse BDNF) incubated under similar conditions in each assay. The detection limit was <4 pg/ml. BDNF concentration was expressed as pg BDNF/g total proteins. Cross-reactivity to other related neurotrophins was less than 3 %. All assays were performed in duplicate.

Bradford assay

A Bradford assay on pellets used for ELISA assays was performed to determine the total protein amount. Bradford solution was purchased from Bio-Rad (USA), and BSA 1 mg/ml (Sigma, USA) was used to make 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) post hoc test. Interaction between treatment and time was evaluated by two-way ANOVA. A p value <0.05 was considered statistically significant. Statistical analysis was performed using the Statview software from SAS Institute.

Results

Pre-treatment with NPY decreases miR-30a-5p mRNA in cortical neurons exposed to $A\beta_{25–35}$

To investigate the effect of NPY on miR-30a-5p, NPY (1 μM) pre-treated cortical neurons were exposed to $A\beta_{25–35}$ (50 μM) or its inactive control $A\beta_{35–25}$ (50 μM) (Fig. 1). Untreated cells, NPY- or Aβ-exposed cells were included as additional controls. Intracellular miR-30a-5p mRNA expression was measured at 24 and 48 h. We found a significant effect of the treatment ($p < 0.05$). Post hoc analysis showed that NPY alone induced a decrease in miR-30a-5p mRNA expression as compared with untreated cells (ps < 0.05), and $A\beta_{25–35}$ increased miR-30a-5p expression as compared with the inactive control, $A\beta_{35–25}$ (ps < 0.05). When cells were pre-treated with NPY and exposed to $A\beta_{25–35}$, we found that miR-30a-5p mRNA expression was reduced in NPY-pretreated $A\beta_{25–35}$ exposed neurons at 24 h ($p < 0.05$) and 48 h ($p < 0.05$) as compared with the respective inactive control $A\beta_{35–25}$ (Fig. 1).

Pre-treatment with NPY increases BDNF mRNA in cortical neurons exposed to $A\beta_{25–35}$

Brain-derived neurotrophic factor mRNA was evaluated in cortical neurons pre-treated with NPY (1 μM) and exposed to $A\beta_{25–35}$ (50 μM) or $A\beta_{35–25}$ (50 μM) (Fig. 2). BDNF mRNA was also evaluated in the same additional controls. There was a significant effect of the treatment ($p < 0.01$), and an interaction between treatment and time ($p < 0.01$). We found that BDNF mRNA levels were increased at 24 h ($p < 0.001$) and 48 h ($p < 0.05$) in NPY-pretreated

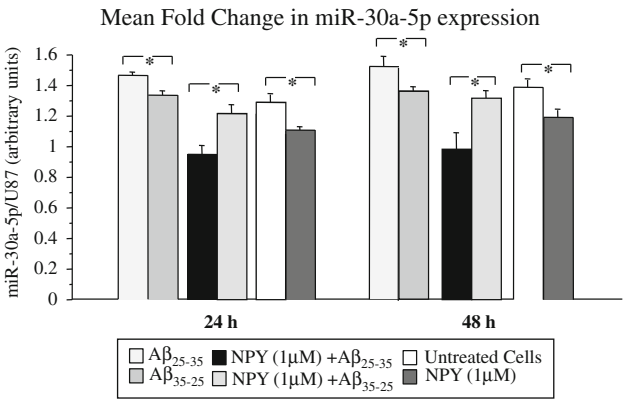

Fig. 1 Mean fold change in miR-30a-5p expression in cortical neurons pre-treated with NPY (1 mM) for 24 h and then exposed to toxic concentration (50 μM) of $A\beta_{25–35}$ or its inactive control $A\beta_{35–25}$ (50 μM). Untreated cells, NPY- or Aβ-exposed cells were included as additional controls. Cell pellets were collected at 24 and 48 h. MiR-30a-5p mRNA was measured by real time PCR and expressed as amount of target gene normalized to an endogenous reference (U87) and relative to a null treatment as calibrator (ΔΔCt). Data represent mean ± SEM. *Asterisks* indicate statistical level of significance between groups (*$p < 0.05$)

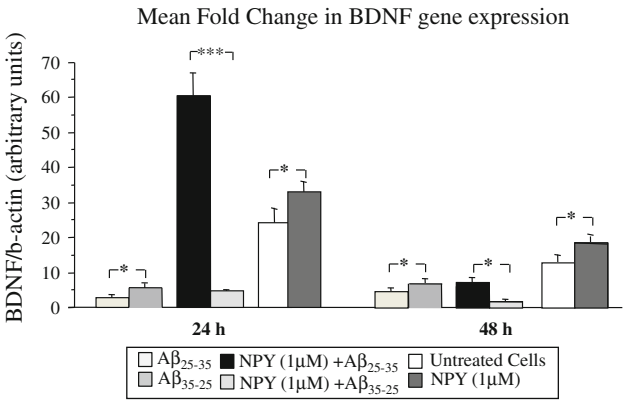

Fig. 2 Mean fold change in BDNF gene expression in cortical neurons pre-treated with NPY (1 mM) for 24 h and then exposed to toxic concentration (50 μM) of $A\beta_{25–35}$ or its inactive control $A\beta_{35–25}$ (50 μM). Untreated cells, NPY- or Aβ-exposed cells were included as additional controls. Cell pellets were collected at 24 and 48 h. BDNF 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 null treatment as calibrator (ΔΔCt). Data represent mean ± SEM. *Asterisks* indicate statistical level of significance between groups (*$p < 0.05$;***$p < 0.001$)

$A\beta_{25–35}$ as compared to those of $A\beta_{35–25}$ exposed neurons. NPY alone also induced a significant increase in BDNF mRNA level at 24 and 48 h ($p < 0.05$) as compared with untreated cells. On the contrary, $A\beta_{25–35}$ decreased BDNF mRNA as compared with the inactive control $A\beta_{35–25}$ (ps < 0.05) at 24 and 48 h. As a whole, BDNF mRNA expression decreased with time ($p < 0.05$) (Fig. 2).

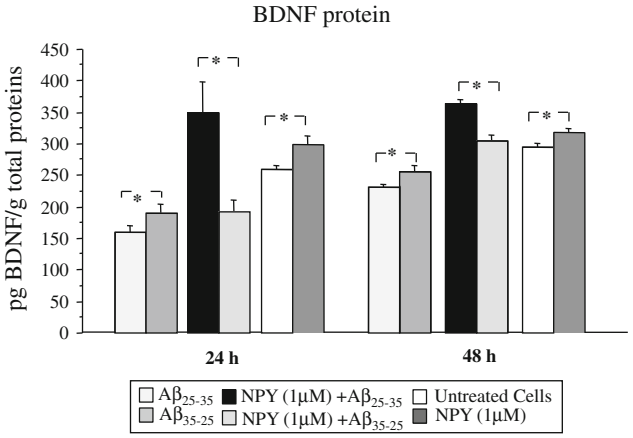

Fig. 3 BDNF protein levels in cortical neurons pre-treated with NPY (1 mM) for 24 h and then exposed to toxic concentration (50 μM) of $A\beta_{25–35}$ or its inactive control $A\beta_{35–25}$ (50 μM). Untreated cells, NPY- or Aβ-exposed cells were included as additional controls. Cell pellets were collected at 24 and 48 h. BDNF protein was measured by ELISA and expressed in pg/g total proteins. Data represent mean ± SEM. *Asterisks* indicate statistical level of significance between groups (*p <0.05)

Pre-treatment with NPY increases BDNF protein levels

In order to evaluate the effect of NPY treatment on BDNF protein levels, NPY (1 μM) pre-treated cortical neurons were incubated for 48 h with $A\beta_{25–35}$ (50 μM) or $A\beta_{35–25}$ (50 μM), and intracellular BDNF protein levels were measured at 24 and 48 h (Fig. 3). An effect of the treatment was observed ($p < 0.01$). As for BDNF mRNA, BDNF production was increased in NPY-pretreated $A\beta_{25–35}$-exposed neurons versus the respective inactive control at 24 h ($p < 0.05$) and 48 h ($p < 0.05$) (Fig. 3). Also, when incubated alone, NPY increased (ps < 0.05) and $A\beta_{25–35}$ decreased (ps < 0.05) the BDNF protein levels at 24 and 48 h. An overall effect of time was also present as BDNF protein levels were lower at 24 h as compared to those at 48 h ($p < 0.05$).

Discussion

This study was performed to investigate whether NPY modulates microRNA miR-30a-5p and/or BDNF expression [14] in an AD in vitro model. With this aim, we exposed NPY-pretreated rat cortical neurons to the AD pathogenic fragment $A\beta_{25–35}$ (or its inactive control) and measured miR-30a-5p and BDNF mRNA and protein expression after 24 and 48 h. Our results demonstrated that pre-treatment with NPY decreased miR-30a-5p expression, while it increased BDNF mRNA and protein levels at 24 and 48 h of incubation with Aβ.

We have previously found that NPY exerts a neuroprotective action in two AD in vitro models [10, 12]. Using SH-SY5Y neuroblastoma cell line differentiated with retinoic acid [10], we observed that pre-incubation with NPY prevented cell loss due to the toxic effects of $A\beta_{25–35}$ and restored the levels of the neurotrophins nerve growth factor (NGF) and BDNF previously reduced by Aβ. NPY neuroprotective action was also confirmed in primary rat cortical neurons using the same experimental procedure of the present experiment [12].

To the best of our knowledge in this study, we provide the first evidence that the action of NPY is not restricted to neurotrophins but is also mediated by regulation of miR-30a-5p expression. MiRs are endogenous non-coding small RNAs that regulate gene expression through repression of translational activity after binding to target mRNAs [25]. MiRs are involved in several cellular processes including differentiation, metabolism, apoptosis [26], and regulation of synaptic plasticity [27]. Recent evidence also suggests that a class of miRs could be involved in the development of sporadic AD form by regulating APP (amyloid precursor protein) gene expression [28]. Expanding the scenario of cross-relationship between miRs and AD, other miRs seem to be correlated with cell death [29] or seem to complement factor-H modification in AD [30].

The present data show that NPY modulates miR-30a-5p and BDNF in opposite direction and suggest that this mechanism may be part of NPY neuroprotective action at least in this in vitro model. This hypothesis is supported by data showing that BDNF has survival-promoting actions on a variety of CNS neurons [31] including those affected in AD, like hippocampal and neocortical neurons [4]. In addition, a reduced expression of BDNF in the brain of individuals with Alzheimer's disease has been reported [5]. Thus, the fact that NPY seems to induce an up-regulation of BDNF neuronal expression may influence neuronal survival in an AD-like condition. However, the mechanism of action of NPY on BDNF is not yet known. Other studies have previously shown that BDNF is able to induce NPY neuronal expression in the CNS [32, 33], while in our previous experiments [10, 12], we have found an opposite correlation, in which NPY does regulate the BDNF expression, suggesting the existence of a regulatory loop fine-tuning their pathways.

A possible involvement of miRs in regulating neuronal function through modulation of BDNF gene has been proposed. In particular, a subset of the miR-30a family is involved in the fine-tuning of BDNF expression via interaction with a conserved sequence located in the proximal portion of BDNF 3′-UTR, especially during late maturation and aging of human pre-frontal cortex [14]. Among miR-30a family, miR-30a-5p exerts a significant inhibitory interaction in functional assays with BDNF and decreases BDNF protein levels in neuronal cultures [14]. Thus, one possibility is that NPY alters BDNF expression by

inhibiting miR-30a-5p expression. However, the mechanism by which NPY can influence miR-30a-5p expression is at present not known. In a study on the prefrontal cortex of schizophrenic subjects, it was shown that deficits in NPY mRNA expression are positively correlated to BDNF protein levels which in turn are negatively correlated to the BDNF-regulating microRNA miR-195 [34]. These and our data suggest the existence of a complex interplay of protein coding and noncoding transcripts that regulates gene expression and that are possibly altered in brain disorders.

Nevertheless, some findings reported here also suggest that the identification of potential synergistic interactions between microRNAs, BDNF, and NPY expression warrants further investigation. We found that BDNF mRNA level in the presence of NPY is reduced at 48 h when compared to that observed at 24 h suggesting that the effects of NPY on BDNF are transient. In addition, magnitude of changes in BDNF mRNA is very different from 24 to 48 h and not adequately paralleled by BDNF protein. Since mRNA expression precedes protein synthesis, the difference observed may again indicate a short lasting effect on BDNF, even in the presence of downregulation of miR-30a-5p. Altogether these observations suggest that, although the neuroprotective effect of NPY in the presence of Aβ is documented, this is probably not the only mechanism involved in this loop.

In summary, this study demonstrates that NPY modulates BDNF and its regulating microRNA miR-30a-5p in opposite direction. This mechanism may contribute to the neuroprotective effect of NPY in rat primary cortical neurons exposed to the toxic effect of Aβ. The future challenge will be to better characterize these events in order to develop innovative therapeutic strategies aimed at boosting endogenous protective molecules, such as BDNF which represents a physiological reserve for healthy aging.

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