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Treatment with the neurotoxic Aβ (25–35) peptide modulates the expression of neuroprotective factors Pin1, Sirtuin 1, and Brain-derived neurotrophic factor in SH-SY5Y human neuroblastoma cells

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Abbreviations: Alzheimer's disease (AD); Amyloid β (Aβ); Amyloid precursor protein (APP); Brain-derived neurotrophic factor (Bdnf); glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Phosphate-buffered saline (PBS); Sirtuin 1 (Sirt1).

Abstract

The deposition of Amyloid β peptide plaques is a pathological hallmark of Alzheimer's disease (AD). The Aβ (25-35) peptide is regarded as the toxic fragment of full-length Aβ (1-42). The mechanism of its toxicity is not completely understood, along with its contribution to AD pathological processes. The aim of this study was to investigate the effect of the neurotoxic Aβ (25-35) peptide on the expression of the neuroprotective factors Pin1, Sirtuin1, and Bdnf in human neuroblastoma cells.

Levels of Pin1, Sirtuin 1, and Bdnf were compared by real-time PCR and Western blotting in SH-SY5Y cells treated with Aβ (25–35) or administration vehicle. The level of Pin1 gene and protein expression was significantly decreased in cells exposed to 25 μM Aβ (25-35) compared to vehicle-treated controls. Similarly, Sirtuin1 expression was significantly reduced by Aβ (25-35) exposure. In contrast, both Bdnf mRNA and protein levels were significantly increased by Aβ (25-35) treatment, suggesting the activation of a compensatory response to the insult. Both Pin1 and Sirtuin 1 exert a protective role by reducing the probability of plaque deposition, since they promote amyloid precursor protein processing through non-amyloidogenic pathways. The present results show that Aβ (25-35) peptide reduced the production of these neuroprotective proteins, thus further increasing Aβ generation.

Keywords: Amyloid β; Pin1; Sirtuin 1; Brain-derived neurotrophic factor; SH-SY5Y cells

1. Introduction

The pathological hallmarks of Alzheimer's disease (AD) are abundant extracellular senile plaques of amyloid β (Aβ) peptides in cerebral blood vessels and brain parenchyma, along with intracellular neurofibrillary tangles of aggregated phosphorylated tau protein (Selkoe, 2011). Aβ peptides derived from the proteolytic cleavage of the large transmembrane polypeptide amyloid precursor protein (APP) are able to form β-sheets structures and fibrillary aggregates (Selkoe, 2011). The accumulation of Aβ plaques in the brain is considered a central event in the etiology of AD, but it is still debated if the plaques themselves represent a primary cause of AD or if they are a by-product of underlying pathological processes (Drachman, 2014). The Aβ (25-35) peptide is regarded as the biologically active fragment of full-length Aβ (1-42), since it is the shortest Aβ peptide retaining full toxicity (Millucci et al., 2010). This peptide displays rapid aggregation properties forming stable fibrils and it is neurotoxic immediately upon dissolution. Moreover, immunohistochemical analyses demonstrated the presence of the Aβ (25-35) peptide in AD brains, suggesting that soluble Aβ (1-40) is released from plaques and converted to the toxic Aβ (25-35) fragment (Millucci et al., 2010).

It is conceivable that the modulation of neuroprotective pathways may contribute to Aβ neurotoxicity by suppressing basal restorative responses. Among neuroprotective proteins, the peptidyl-prolyl cis/trans isomerase Pin1 plays a role in the alteration of protein phosphorylation state by regulating protein conformation. Pin1 has been implicated in the pathophysiology of AD through a dual mechanism. Since Pin1 binds to phosphorylated tau and promotes its dephosphorylation, reduced protein amount is associated to increased accumulation of phosphorylated tau and formation of neurofibrillary tangles. In addition,

Pin1 catalyses the isomerization of APP, favoring its processing in the direction of non-amyloidogenic pathways and reducing Aβ production (Driver et al., 2014).

Another protein exerting a protective activity against neurodegeneration by reducing APP amyloidogenic processing and Aβ deposition is the histone deacetylase Sirtuin 1 (Sirt1) (Bonda et al., 2011). Sirt1 beneficial effects are mediated through an increased transcription of ADAM10, which activates the non-amyloidogenic α secretase pathway and the notch neurogenetic pathway (Bonda et al., 2011). Moreover, Sirt1 exerts a further neuroprotective activity by promoting the deacetylation and subsequent degradation of tau pathogenic forms (Min et al., 2010).

Growing evidence suggests that reduced brain-derived neurotrophic factor (Bdnf) expression in brain is a common feature of AD and other cognitive dysfunctions (Tapia-Arancibia et al., 2008) and restore of its function has been suggested as a possible therapeutic approach in neurodegenerative diseases (Lu et al., 2013). Neuroprotective effects against Aβ toxicity exerted by Bdnf have been described in cellular and animal models (Tapia-Arancibia et al., 2008; F. Zhang et al., 2012). The neuroprotective activity against Aβ toxicity involves several components, including the shifting of APP processing towards the α-secretase pathway (Holback et al., 2005) and the rescue of Aβ-induced inhibition of hippocampal long-term potentiation by enhanced CaMKII autophosphorylation (Zeng et al., 2010). Moreover, available data support the notion that Aβ peptides are able to interfere with Bdnf signal transduction pathways involved in neuronal survival and synaptic plasticity, hampering the transmission of neurotrophic responses (Tong et al., 2004).

The aim of the present study was to investigate the effect of the neurotoxic Aβ (25-35) peptide on the expression of the neuroprotective factors Pin1, Sirt1, and Bdnf in human neuroblastoma cells to help elucidating their role in Aβ toxicity.

2. Materials and Methods

2.1 Cell culture and treatments

Human neuroblastoma SH-SY5Y cells, purchased from ICLC-IST (Genoa, Italy), were cultured in Dulbecco's modified Eagle medium, supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine (Lonza, Italy) at 37°C in a humidified atmosphere containing 5% CO_2. Experiments were performed at 80% confluence.

Aβ (25-35) peptide (Sigma-Aldrich, St. Louis, MO, USA) was dissolved at 1 mM in sterile distilled water. The unaggregated peptide was incubated at 37°C for 72h, and gently mixed to promote aggregation (Millucci et al., 2009). Treatments were performed with a working solution at 25 μM in cell medium supplemented with 2% serum. The dose was selected based on previous studies reporting toxic effects in neuroblastoma cells and in primary neurons (Croce et al., 2011; Resende et al., 2007; Sultana et al., 2006; Wang et al., 2014; Xi et al., 2012)

2.2 MTT cell viability assay

Cell viability was assessed with a modified MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich) assay. Briefly, cells were plated in 24-well plates at 3 x 10^4 cells/. After 24h, the medium was removed; the cells were rinsed with phosphate-buffered saline (PBS) and treated with Aβ (25-35) for 5, 24 or 48 h. At the end-point cells were rinsed with PBS and treated with the MTT solution (0.5 mg/mL in PBS) in

medium without phenol red and serum. The cells were incubated for 3h in the dark at 37°C and 5% CO_2. The supernatant was removed and dimethyl sulfoxide : ethanol (4:1) was added. Absorbance at 590 nm was read in a microplate spectrophotometer (GENios Tecan). Results were expressed as percentage of vehicle-treated controls.

2.3 Real-time RT-PCR

After 5, 24, or 48h exposure to 25 μM Aβ (25-35), the medium was removed and cells were washed twice with ice-cold PBS. RNA extraction, cDNA synthesis, and real-time PCR were performed as previously reported (Lattanzio et al., 2014). All data were normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The following primers were used for qRT-PCR amplifications: Pin1: forward: 5' GACGAGGAGAAGCTGCCGCC 3'; reverse: 5' CAGGCTCCCCCTGCCCGTTT 3'; Sirt1: forward: 5' GCGATTGGGTACCGAGATAA 3'; reverse: 5' GTTCGAGGATCTGTGCCAAT 3'; Bdnf: forward: 5' AGAAGGCAGCCCTAGGAAAC 3', reverse: 5' GCATCGATGTCGAAAAACCT 3'; GAPDH: forward: 5' ATTCCACCCATGGCAAATTC 3'; reverse: 5' TGGGATTTCCATTGATGACAAG 3'.

2.4 *Western blot analysis*

After Aβ (25-35) treatments, cells were washed twice with ice-cold PBS, harvested, and centrifuged at 4°C (2000g 5 min). Cells were lysed on ice in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate with a protease and phosphatase inhibitor cocktail (Sigma-Aldrich), incubated 30 min on ice and centrifuged at 4°C (13600×g, 10 min). Lysates were stored at -80°C. Protein concentration was determined using the BCA protein assay kit (Thermo Scientific, Milan,

Italy). 60 µg protein samples were separated in 4-20% Precise Tris-Glycine Gels (Thermo Scientific, Milan, Italy) and immunoblotting experiments were carried out as previously reported (Lattanzio et al., 2014). The following antibody concentrations were used: polyclonal anti-Pin1 (Millipore, USA): 1:1000; polyclonal anti-Sirt1 (Millipore, USA): 1:1000; polyclonal anti-BDNF (Santa Cruz, USA): 1:200; monoclonal anti-GAPDH (Millipore, USA): 1:1000; horseradish peroxidase-linked secondary antibodies (Amersham Biosciences, Little Chalfont, UK): 1:2000. Representative Western blot images are shown in Supplementary Fig. S1.

Optical density values were normalized to GAPDH levels. Results were expressed as percentage of vehicle-treated controls.

2.5 Statistical analysis

Data were analyzed by two-way ANOVA with time and treatment as factors, followed by LSD post-hoc tests with InVivoStat software (Clark et al., 2012). Data are reported as the mean values ± SEM of at least three independent experiments. A value of $p<0.05$ was considered statistically significant.

3. Results

The exposure of SH-SY5Y neuroblastoma cells to 25 µM Aβ (25-35) induced a significant decrease of the viability in a time-dependent manner: at 5h: 88.05 ± 2.12 vs. control 100 ± 1.52, $p<0.0001$; at 24h: 81.27 ± 2.44 vs. 100 ± 1.92, $p<0.0001$; at 48h: 79.80 ± 1.98 vs. 100 ± 2.54.

Pin1 gene expression was significantly decreased in cells exposed to 25 µM Aβ (25-35) for 5 and 24h compared to vehicle-treated controls (Fig. 1A) (at 5h: 0.80 ± 0.04 vs. control 1.00 ± 0.02, $p<0.001$; at 24h: 0.79 ± 0.06 vs. control 1.00 ± 0.03, $p<0.01$). No changes of

gene expression were observed after Aβ (25-35) exposure for 48h (Fig. 1A). Similarly, Pin1 protein levels were significantly decreased in cells exposed to Aβ (25-35) for 5h (81.36 ± 3.49 vs. control 100 ± 4.67, $p<0.01$, Fig. 1B) and 48h (85.51 ± 1.46 vs. control 100 ± 2.85, Fig. 1B).

Sirt1 gene expression was significantly decreased in cells exposed to 25 μM Aβ (25-35) for 5h (0.78 ± 0.07 vs. control 1.00 ± 0.04, $p<0.05$) and 24h (0.74 ± 0.09 vs. control 1.00 ± 0.07, $p<0.05$) (Fig. 2A). No gene expression modifications were observed at 48h (Fig. 2A). Sirt1 protein levels did not change in cells exposed to Aβ (25-35) for 5 or 24h (Fig. 2B). Interestingly, Sirt1 protein significantly decreased at 48h (73.82 ± 3.47 vs. control 100 ± 2.92, $p<0.05$, Fig. 2B).

Bdnf gene expression did not change after 25 μM Aβ (25-35) exposure for 5h, whereas a significant increase was detected at 24h (1.76 ± 0.15 vs. control 1.00 ± 0.14, $p<0.001$) and at 48h (1.52 ± 0.20 vs. control 1.00 ± 0.13, $p<0.05$) (Fig. 3A). A parallel increase of Bdnf protein levels was detected at all time-points: at 5h: 121.55 ± 7.67 vs. control 100 ± 2.58, $p<0.01$; at 2h: 115.72 ± 5.03 vs. 100 ± 4.23, $p<0.05$; at 24h: 129.40 ± 2.71 vs. 100 ± 5.75, $p<0.001$, Fig. 3B).

4. Discussion

The objective of the present study was to investigate the effects of the neurotoxic peptide Aβ (25-35) on the expression of neuroprotective proteins that are putatively involved in the mechanism of Aβ toxicity.

The analysis of the in vitro neurotoxicity of 25 μM Aβ (25-35) aggregates showed a time-dependent reduction of cell viability, reaching a maximum mortality rate of 20%. These results demonstrated that a significant neurotoxicity was induced, allowing the

investigation of the associated mechanisms. Nevertheless, the level of neurotoxicity was not so extreme as to produce a broad damage to cellular processes that would hamper the investigation of specific mechanisms.

The present study showed that in SH-SY5Y cells the exposure to Aβ (25-35) induced a significant decrease of Pin1 gene expression, paralleled by a decrease of Pin1 protein levels. Compelling evidence supports a protective role for Pin1 in preventing the formation of neurofibrillary tangles by increased tau dephosphorylation. Moreover, Pin1 binds to phosphorylated APP, facilitating its processing in non-amyloidogenic pathways and thus reducing the probability of plaque deposition (Balastik et al., 2007; Driver et al., 2014). This protective role is validated by the observation that Pin1 deletion causes premature aging and multiple dysfunctions related to the inability to correct protein misfolding (Liou et al., 2003). In human brain studies, contrasting findings contribute to the notion of a crucial role of Pin1 in AD, although suggesting that further investigations are required to completely elucidate the mechanism. Indeed, in AD hippocampus, Pin1 down-regulation and increased oxidation of has been reported (Sultana et al., 2006), in agreement with a previously reported inverse association between tangles and Pin1 expression (Liou et al., 2003). In contrast, Dakson et al. reported that Pin1 inclusions were enriched in cortical and hippocampal regions of AD patients (Dakson et al., 2011). Our findings provide a contribution to the clarification of Pin1 role in AD showing that when the generation of toxic Aβ peptides has started, a down-regulation of this protective protein is induced. Therefore, it is likely that a further shift is produced in the direction of neuronal damage due to increased phosphorylated tau and Aβ peptides synthesis. The present results thus suggest that a positive feedback mechanism can be started involving Pin1 expression.

Moreover, this study showed that Aβ (25-35) exposure induced a significant down-regulation of Sirt1 gene expression at 5 and 24 hours. A parallel reduction of Sirt1 protein levels was observed at a later time-point (48h). Available data suggest that decreased Sirt1 levels are brought about by Aβ (25-35) treatments, at least in in vitro models. Indeed, in line with the present findings, Feng et al. (Feng et al., 2013) revealed that Aβ (25-35) decreased Sirt1 mRNA and protein levels in PC12 cells. Corresponding results were also obtained in primary cultures of rat cortical neurons (Sun et al., 2014). Compelling evidence supports the notion that Sirt1 exerts protective action against plaque formation, pathology and cognitive decline in AD models by directing APP processing toward the α-secretase pathway, thus reducing the accumulation of the toxic Aβ species resulting from β-secretase activity (Bonda et al., 2011). Interestingly, the present results combine with the findings obtained by Feng et al. (Feng et al., 2013) and Sun et al. (Sun et al., 2014) to propose a model in which the generation of Aβ peptides causes a reduction in Sirt1 levels which in turn further promotes the synthesis of neurotoxic Aβ products, similarly to what observed with Aβ-induced Pin1 down-regulation.

We also report that the exposure of SH-SY5Y cells to Aβ (25-35) induced a significant increase of Bdnf mRNA expression at 24 and 48h, which was paralleled by increased protein levels. The present data are in agreement with a previous in vitro study showing increased Bdnf expression in astrocytes exposed to Aβ (1-42) (Kimura et al., 2006). However, other studies observed a decrease of Bdnf levels induced by Aβ peptides in differentiated SH-SY5Y and primary neuron cultures (Croce et al., 2013, 2011; Garzon and Fahnestock, 2007; Wang et al., 2009; Xiao et al., 2010; R. Zhang et al., 2012). Moreover, the time-course analysis of the effects of Aβ (25-35) treatments on Bdnf gene expression

both in rat cultured cortical neurons and in differentiated SH-SY5Y cells showed a dual response, with an increase at shorter end-points (3-5 h) followed by decrease at longer times (24 or 48 h) (Aliaga et al., 2010; R. Zhang et al., 2012). In addition, in vivo intracranial administration of Aβ (25-35) increased Bdnf gene expression in rat hippocampus (Tang et al., 2000). These data suggest that Aβ (25-35) may trigger distinct effects on Bdnf expression in different systems and conditions. We can speculate that the increase of Bdnf levels might act as a compensatory response against amyloid toxicity.

5. Conclusion

The molecular mechanisms underlying the pathogenesis of AD are largely unknown, probably due to its multifactorial and complex etiology. Among the different factors that contribute to AD pathogenesis, genetic evidence and experimental data suggest a relevant role of Aβ peptides toxicity. The present study revealed that Pin1, Sirt1, and Bdnf gene and protein expressions are differently modulated by Aβ (25-35) exposure, highlighting their involvement in the molecular mechanisms of amyloid toxicity.

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Conflicts of interest: none.

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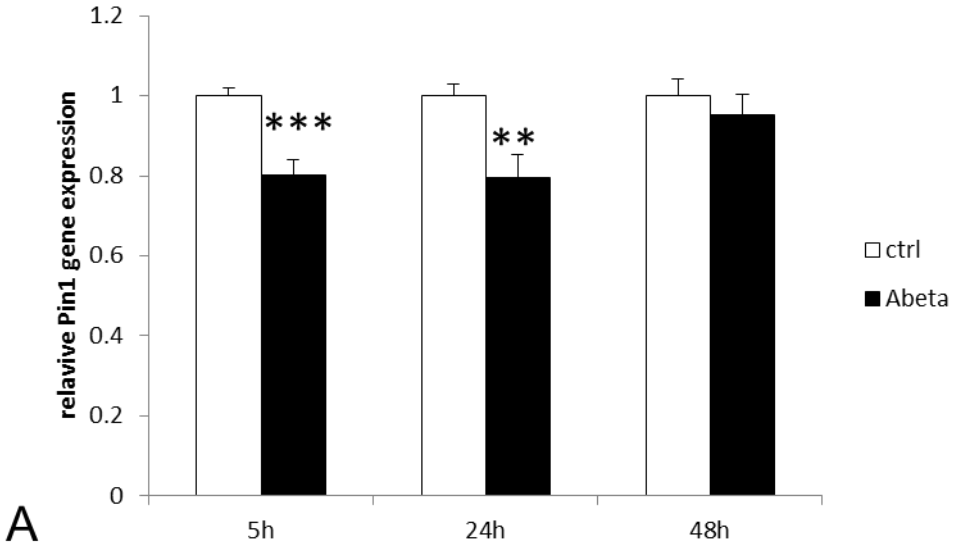
Figure legends

Figure 1: A: Pin1 mRNA expression after 25 μM Aβ (25-35) treatment for 5, 24, or 48h. Gene expression was measured by real-time PCR. Data represent 2^{-DDCt} values normalized to GAPDH levels. Data are expressed as mean ± SEM of controls of three independent experiments. **p<0.01; ***p<0.001. B: Pin1 protein levels. Protein levels were evaluated by Western blot analysis. Data normalized to GAPDH levels are expressed as percentage of controls (mean ± SEM) of three independent experiments. *p<0.05; ***p<0.001.

Figure 2: A: Sirt1 mRNA expression after 25 μM Aβ (25-35) treatment for 5, 24, or 48h. Gene expression was measured by real-time PCR. Data represent 2^{-DDCt} values normalized to GAPDH levels. Data are expressed as mean ± SEM of controls of three independent experiments. *p<0.05. B: Sirt1 protein levels . Protein levels were evaluated by Western blot analysis. Data normalized to GAPDH levels are expressed as percentage of controls (mean ± SEM) of three independent experiments. *p<0.05.

Figure 3: A: Bdnf mRNA expression after 25 μM Aβ (25-35) treatment for 5, 24, or 48h. Gene expression was measured by real-time PCR. Data represent 2^{-DDCt} values normalized to GAPDH levels. Data are expressed as mean ± SEM of controls of three independent experiments. *p<0.05; ***p<0.001. Bdnf protein levels. Protein levels were evaluated by Western blot analysis. A: Data normalized to GAPDH levels are expressed as percentage of controls (mean ± SEM) of three independent experiments. *p<0.05; **p<0.01; ***p<0.001.

Fig. 1


A
relavive Pin1 gene expression
1.2
1
0.8
0.6
0.4
0.2
0
***
**
5h
24h
48h
ctrl
Abeta

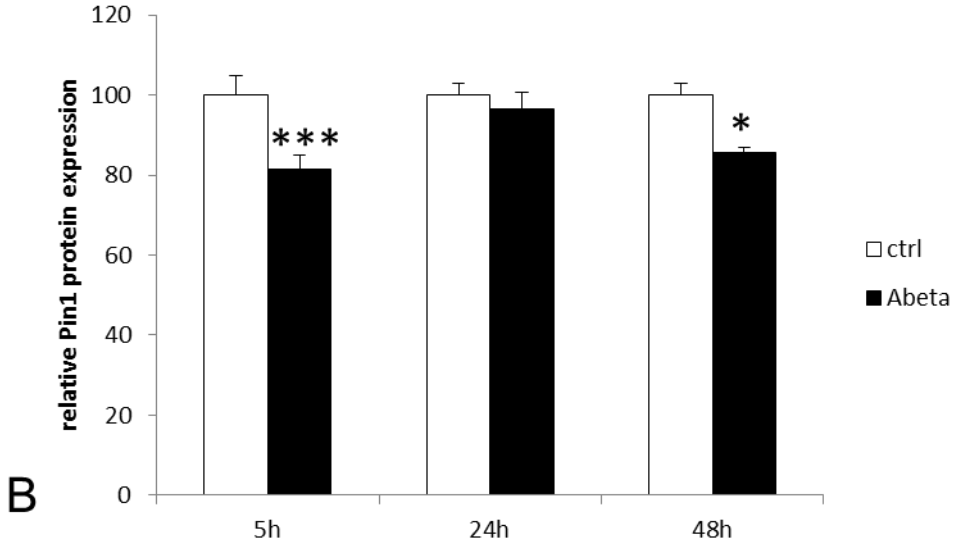
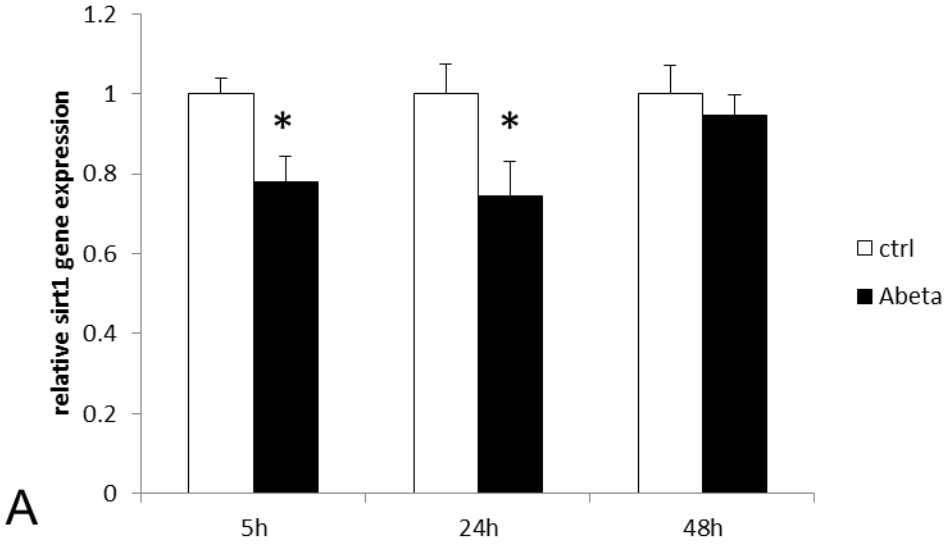

B
relative Pin1 protein expression
120
100
80
60
40
20
0
***
*
5h
24h
48h
ctrl
Abeta

Fig. 2


1.2
1
0.8
0.6
0.4
0.2
0
relative sirt1 gene expression
*
*
5h
24h
48h
ctrl
Abeta
A

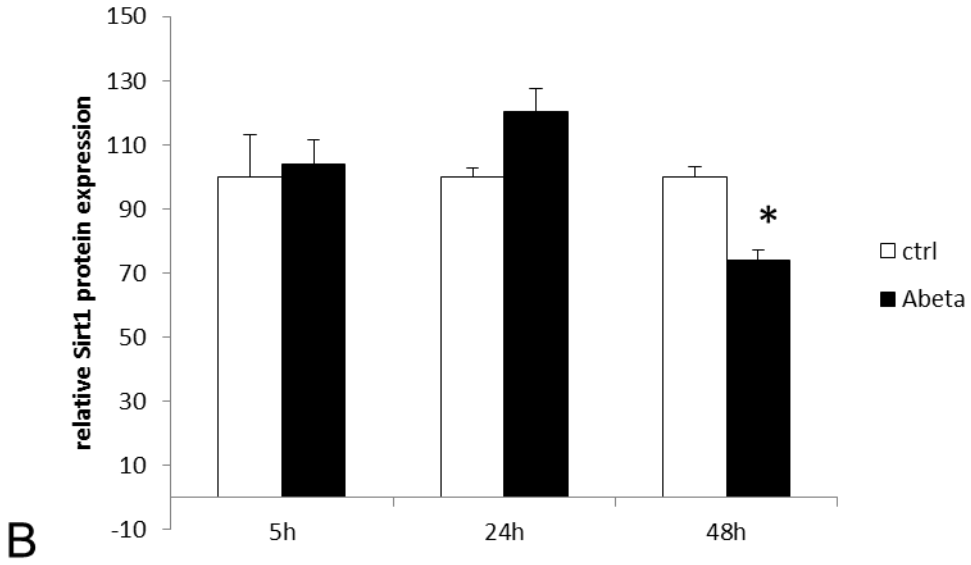
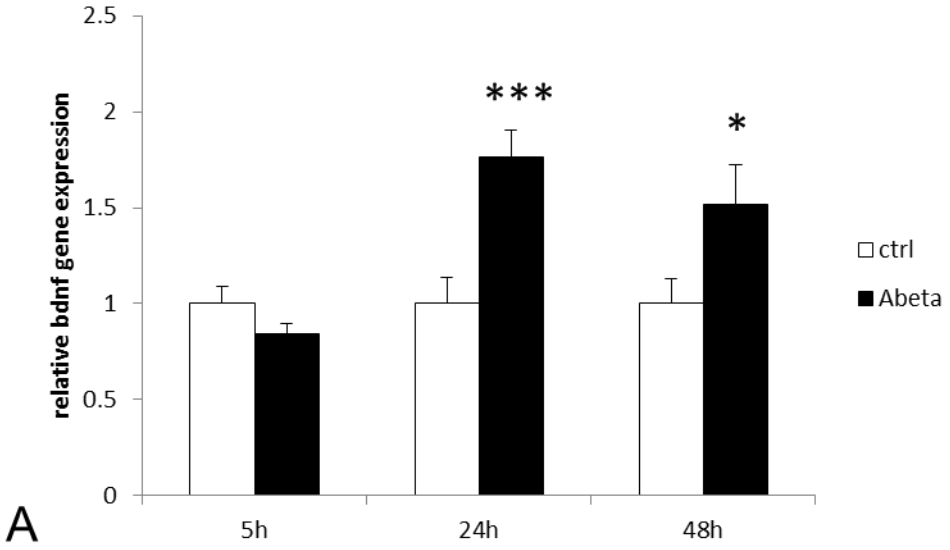
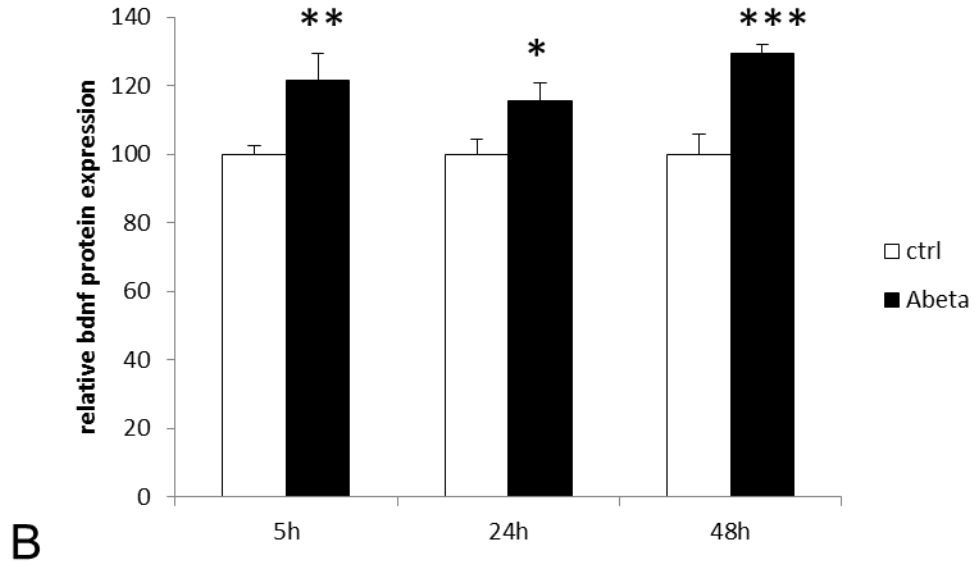

150
130
110
90
70
50
30
10
-10
relative Sirt1 protein expression
*
5h
24h
48h
ctrl
Abeta
B

Fig. 3


2.5
2
1.5
1
0.5
0
relative bdnf gene expression
***
*
ctrl
Abeta
5h
24h
48h
A


140
120
100
80
60
40
20
0
relative bdnf protein expression
**
*
***
ctrl
Abeta
5h
24h
48h
B