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Pre-treatment with amitriptyline causes epigenetic up-regulation of neuroprotection-associated genes and has anti-apoptotic effects in mouse neuronal cells

(抗精神病薬アミトリプチリンはエピジェネティクス機構を介して神経細胞死を抑制する)

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Pre-treatment with amitriptyline causes epigenetic up-regulation of neuroprotection-associated genes and has anti-apoptotic effects in mouse neuronal cells

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ABSTRACT

Antidepressants, such as imipramine and fluoxetine, are known to alter gene expression patterns by inducing changes in the epigenetic status of neuronal cells. There is also some evidence for the anti-apoptotic effect of various groups of antidepressants; however, this effect is complicated and cell-type dependent. Antidepressants of the tricyclic group, in particular amitriptyline, have been suggested to be beneficial in the treatment of neurodegenerative disorders. We examined whether amitriptyline exerts an anti-apoptotic effect via epigenetic mechanisms. Using DNA microarray, we analyzed global gene expression in mouse primary cultured neocortical neurons after treatment with amitriptyline and imipramine. The neuroprotection-associated genes, activating transcription factor 3 (*Atf3*) and heme oxygenase 1 (*Hmox1*), were up-regulated at both mRNA and protein levels by treatment with amitriptyline. Quantitative chromatin immunoprecipitation assay revealed that amitriptyline increased enrichments of trimethylation of histone H3 lysine 4 in the promoter regions of *Atf3* and *Hmox1* and acetylation of histone H3 lysine 9 in the promoter regions of *Atf3*, which indicate an active epigenetic status. Amitriptyline pre-treatment attenuated 1-methyl-4-phenylpyridinium ion (MPP^+)- or amyloid β peptide 1–42 ($A\beta_{1-42}$)-induced neuronal cell death and inhibited the activation of extracellular signal-regulated kinase 1 and 2 (ERK1/2). We found that *Atf3* and *Hmox1* were also up-regulated after $A\beta_{1-42}$ treatment, and were further increased when pre-treated with amitriptyline. Interestingly, the highest up-regulation of *Atf3* and *Hmox1*, at least at mRNA level, was observed after co-treatment with $A\beta_{1-42}$ and amitriptyline, together with the loss of the neuroprotective effect. These findings suggest preconditioning and neuroprotective effects of amitriptyline; however, further investigations are needed for clarifying the contribution of epigenetic up-regulation of *Atf3* and *Hmox1* genes.

1. Introduction

Antidepressants have been used to treat depression for > 60 years, and were used originally because they increased serotonin and norepinephrine. Thus, the most frequently used antidepressants belong to groups of selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and tricyclic antidepressants (TCAs). Depressive disorders are commonly seen in patients with neurodegenerative diseases; hence, antidepressants have been widely used and have improved the quality of life of patients with Alzheimer's disease (AD) and Parkinson's disease (PD) (McDonald et al., 2003; Moretti et al., 2002). In addition, advanced studies of the pharmacology of antidepressants suggest that antidepressants may have the capability to modify epigenetic status. Imipramine, a TCA, restores the impaired epigenetic state in depression by inhibiting histone deacetylase 5 (HDAC5), which leads to an increase in histone acetylation and the subsequent expression of the brain-derived neurotrophic factor (*Bdnf*) gene in the hippocampal region in the mouse brain (Tsankova et al., 2006). Fluoxetine, an SSRI, is also known to have an epigenetic effect through a decrease in histone acetylation and an increase in histone H3

Abbreviations: AD, Alzheimer's disease; Aβ, amyloid β; BDNF, brain-derived neurotrophic factor; CaMKII, calmodulin-dependent protein kinase II alpha; CBP, CREB-binding protein; CREB, cAMP response element-binding protein; DNMT, DNA methyl transferase; ERK1/2, extracellular signal-regulated kinase 1 and 2; GEO, Gene Expression Omnibus; HDAC, histone deacetylase; JNK, c-Jun *N*-terminal kinase; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; MPP^+, 1-methyl-4-phenylpyridinium ion; mHTT, mutant huntingtin; NAc, nucleus accumbens; PD, Parkinson's disease; p-ERK1/2, phosphorylated ERK1/2; qChIP, quantitative chromatin immunoprecipitation; qRT-PCR, quantitative reverse transcription PCR; SNRI, serotonin-norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; TRK, tropomyosin receptor kinase

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lysine 9 dimethylation in the calmodulin-dependent protein kinase II alpha (*CaMKII*) gene in the nucleus accumbens (NAc), a brain reward region in the human and mouse brain (Robison et al., 2014). Epigenetic mechanisms are an important aspect of gene regulation that maintains normal brain function. Particularly, there are evidences that the pathology of AD and PD may relate to epigenetic mechanisms by alternating DNA methylation and histone acetylation (Chen et al., 2009; Kontopoulos et al., 2006). More recent studies have identified an abnormal epigenetic status in the post-mortem brains of patients with mental and neurodegenerative diseases, including AD and PD (Feng et al., 2015; Mastroeni et al., 2015). In addition to these findings, HDAC inhibitors have reduced cell death and improved outcomes in mouse models of AD and PD (Coppede, 2014), suggesting that epigenetic drugs are a potential treatment for these diseases. Therefore, the epigenetic function of antidepressants requires exploration to determine whether it is a general characteristic of antidepressants. Moreover, inhibition of apoptosis increases neural degeneration outcomes (Ghavami et al., 2014), an indication for the use of drugs with neuroprotective effects in the treatment of neurodegenerative disorders. Antidepressants have been shown to protect against hippocampal volume loss in patients with depression, suggesting their neuroprotective effect (Sheline et al., 2003). Antidepressants, particularly fluoxetine, tianeptine, moclobemide, and imipramine, belong to different groups that have been proven to have anti-apoptotic effects in neuronal culture or neural stem cells (Drzyzga et al., 2009; Jantas et al., 2014). Nevertheless, the effect of antidepressants on apoptosis is multifarious and cell-type dependent (Jantas et al., 2014; Xia et al., 1999).

Amitriptyline, one of the earliest TCAs that has been in use, continues to be used in therapy for a number of mental disorders, as well as for neuropathic pain (Leucht et al., 2012; Moore et al., 2015); it appears to be highly effective compared with newer SSRIs (Anderson, 2000). In addition, in patients with depression, serum BDNF concentration was increased by 13% after treatment with amitriptyline, but decreased by 12% after treatment with paroxetine, an SSRI (Hellweg et al., 2008). One study reported that amitriptyline induced global CCpGG hypomethylation in rat primary astrocytes, although no changes in specific gene expressions were observed (Perisic et al., 2010); the study suggested further investigation into epigenetic alterations by amitriptyline. Amitriptyline also appears to have an anti-apoptotic effect by preventing PC12 cells from cell death induced by hydrogen peroxide (Kolla et al., 2005) and by attenuating DNA damage induced by neurotoxins, *N*-(2-chloroethyl)-*N*-ehtyl-2-bromobenzylamine (DSP4) and camptothecin (CPT), in SH-SY5Y cells (Wang et al., 2015). Jang et al. (2009) demonstrated that amitriptyline, but not other TCAs (imipramine and clomipramine), protected primary cultured hippocampal neurons and in vivo hippocampal neurons from oxygen-glucose deprivation- and kainic acid-induced apoptosis, respectively; and that amitriptyline could independently activate tropomyosin receptor kinase A (TrKA) and TrKB and provoke their dimerization, which could not be seen with other TCAs (desipramine, imipramine, and trimipramine) or fluoxetine. Moreover, TCAs, particularly amitriptyline, have been shown to play a role in the treatment of neurodegenerative disorders. In a mouse model of AD, amitriptyline treatment significantly improved long- and short-term memory and increased neurogenesis and neurosynaptic marker proteins (Chadwick et al., 2011). In PD patients, nortriptyline was shown to be superior in effectiveness when compared to paroxetine or placebo in a randomized controlled trial (Menza et al., 2009). Furthermore, a retrospective cohort study pointed out that amitriptyline delayed the initiation of dopaminergic therapy in early PD, although this may possibly result from the increase in quality of life of patients with depression, or from the neuroprotective effect of the drug itself (Paumier et al., 2012). Later, the same group showed that in 6-hydroxydopamine model rats, pre-treatment with amitriptyline prevented tyrosine hydroxylase positive and NeuN positive cells from cell loss (Paumier et al., 2015). Amitriptyline and tranylcypromine (a monoamine oxidase inhibitor) protected PC12 cells from cell death induced by 1-methyl-4-phenylpyridinum (MPP^+); in contrast, fluoxetine increased the toxicity of MPP^+ (Han and Lee, 2009). Recently, a study in rats treated with rotenone as a model of PD showed that pre-treatment with amitriptyline and imipramine improved motor performance and coordination and attenuated the noxious effects of rotenone (Kandil et al., 2016). Taken together, these data support that amitriptyline may have neuroprotective effects besides its well-known anti-depressive effects. However, as mentioned above, the neuroprotective effects of amitriptyline in neuronal cells need to be verified. Moreover, it is unclear whether amitriptyline has epigenetic effects and whether its neuroprotective effects occur via epigenetic mechanisms.

In the present study, we sought to identify amitriptyline's epigenetic mechanism in gene regulation and to support its neuroprotective effects. First, we examined changes in gene expression after TCA exposure (imipramine and amitriptyline). Interestingly, we found that genes associated with anti-apoptotic action are commonly up-regulated by TCAs; therefore, we selected and screened them for epigenetic changes. Lastly, we used MPP^+ and amyloid β peptide 1–42 ($A\beta_{1–42}$) as a model of neurotoxicity in PD and AD and confirmed the anti-apoptotic effect of amitriptyline. These analyses indicated that amitriptyline might have an epigenetic-related anti-apoptotic action in neuronal cells.

2. Materials and methods

2.1. Cell culture and treatment

Primary cultures of neocortical neuronal cells were prepared from ICR (CD-1) mouse fetuses on embryonic day 15. Briefly, neocortices were carefully dissected, minced, and digested with 0.25% trypsin. Isolated cells were suspended in Neurobasal medium (Life Technologies Ltd., Carlsbad, CA, USA) supplemented with 1% L-glutamine, penicillin/streptomycin, and B27 serum-free supplement (Life Technologies), and plated at a density of 2.5×10^5 cells/cm^2 on poly-L-ornithine-coated plastic dishes. This method provided > 95% of cells positive for microtubule-associated protein 2 (MAP2), a general marker for neurons, as confirmed by immunofluorescence (Supplementary Fig. 1). These cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Our experimental protocols were approved by the Institute Animal Care and Use Committee of the University of Yamanashi.

2.2. Drugs and treatments

The antidepressant drugs used in this study, imipramine and amitriptyline, were obtained from Sigma Aldrich, St. Louis, MO, USA and prepared at a concentration of 100 mM in distilled water for stocking. On the day of treatment, drugs were diluted to the desired concentration in culture medium. Drug concentrations were selected following a preliminary 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay to identify the maximum dose that did not significantly reduce cell survival rates after 48 h (Supplementary Fig. 2). First, we evaluated the effects of 48 h exposure to antidepressant drugs in cultured cells. Briefly, cultured neuronal cells at in vitro day 3 (DIV3) were treated with 5 μM imipramine or 5 μM amitriptyline. The cultures were harvested after 48 h (DIV5) for further investigation. We used $A\beta_{1–42}$ (Abcam, Tokyo, Japan) and MPP^+ (Sigma Aldrich) to induce apoptosis in neuronal cell cultures. Stocking solution was prepared in line with product protocols. Treatment solution was prepared before each experiment by diluting in Neurobasal medium supplemented with 1% L-glutamine, penicillin/streptomycin, and B27 serum-free supplement minus AO (Life Technologies). In co-treatment experiments, 5 μM amitriptyline was added to $A\beta_{1–42}$ treatment solution and treated to cultured neuronal cells at DIV5 for 24 h.

HDAC inhibitor SAHA was obtained from Histone Deacetylase

(HDAC) Inhibitor Set II (Sigma-Aldrich). 10 mM stocking solution was prepared in DMSO and stored at -20 °C. The drug was diluted to the desired concentration just before treatment. Cells grown in 96-well plates were treated with 0.5 or 1 μM SAHA, or a combination of 5 μM amitriptyline and 0.25, 0.5, or 1 μM SAHA for 48 h. After removal of the drug, cells were treated with 5 or 10 μM $A\beta_{1-42}$ for 24 h. MTT assay was performed as described.

2.3. Microarray analysis

RNA was isolated from amitriptyline- and imipramine-treated cells and from untreated cells using High Pure RNA Isolation Kit (Roche Applied Science, Manheim, Germany) following the manufacturer's protocol. Total RNA (100 ng) was amplified, converted to double-stranded cDNA, and labeled with biotin according to the manufacturer's protocol (Affymetrix, Santa Clara, CA, USA). Biotinylated cDNA was hybridized to Mouse Gene 2.0 ST chips (Affymetrix). Gene chips were scanned using an Affymetrix Scanner 3000. All probe sets were normalized by using the RMA algorithm on the Affymetrix Expression Console. The data analysis was performed using Subio Platform version 1.19 (Subio Inc., Kagoshima, Japan). The statistical significance of the differences between the subjects and the controls was evaluated using the cross-gene-error model in combination with one-way ANOVA ($P < 0.05$) and Bonferroni's multiple testing correction. The data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE63005.

2.4. Quantitative reverse transcription PCR

Quantitative reverse transcription PCR (qRT-PCR) was performed using a StepOnePlus real time PCR system (Applied Biosystems, CA, USA). cDNA was prepared using a Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was then performed with Fast SYBR Green Master Mix (Applied Biosystems). Primer sequences are described in Supplementary Table 1. Gene expression levels were calculated using the comparative CT method after normalization against β-actin.

2.5. Western blotting

Primary cultures of neocortical neuronal cells were lysed in buffer containing 50 mM Tris, 25 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose, 5% Triton X-100, and a protease inhibitor cocktail or phosphatase inhibitor cocktail (both from Nacalai Tesque Inc., Kyoto, Japan). Ten-microgram protein extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked using Blocking One P solution (Nacalai Tesque Inc., Kyoto, Japan) for 40 min in order to be used with phospho-specific antibodies. Membranes were incubated overnight at 4 °C with one of the following primary antibodies: rabbit anti-Hmox1 (diluted 1:200, sc-10789, Santa Cruz Biotechnology, Dallas, TX, USA); rabbit anti-Atf3 (diluted 1:500, ab180842, Abcam); goat anti-Gdf15 (diluted 1:1000, ab39999, Abcam); rabbit anti-cleaved-caspase 3 (diluted 1:1000. #9664S); rabbit anti-ERK1/2 (diluted 1:1000; #4695S); rabbit anti-p-ERK1/2 (diluted 1:2000, #4370S); rabbit anti-CREB (diluted 1:1000, #4820S); rabbit anti-p-CREB (diluted 1:1000, #9198S); or mouse anti-β-actin (diluted 1:1000, #3700S) (all from Cell Signaling Technology Inc., Danvers, MA, USA). We then incubated the membranes with the appropriate secondary horseradish peroxidase-linked antibodies. Immunoreactivity was visualized by a chemiluminescence detection system (Nacalai Tesque) and the images were analyzed using LAS-4000 software (Fujifilm Inc., Tokyo, Japan). Staining intensities were normalized as a ratio relative to β-actin. For relative ratio of p-ERK/ERK, the quantification of ERK1/2 and pERK1/2 was first normalized against beta-actin, after which the normalized values were used for quantification of p-ERK/ERK. Representative bands for each protein were selected from one single membrane per experiment; however, for simplicity, we cropped the unused lanes, as indicated by black vertical lines in the figures.

2.6. Quantitative chromatin immunoprecipitation assay

Quantitative chromatin immunoprecipitation (qChIP) assays were performed using ChIP-IT Express Enzymatic Kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer's instructions. In brief, primary cultured neocortical neuronal cells were fixed with 1% formaldehyde for 10 min at room temperature to form DNA-protein cross-links. Fixation was stopped by the addition of glycine. Cells were collected and re-suspended in lysis buffer, then homogenized on ice using a dounce homogenizer. After centrifugation for 10 min at 5000 rpm, the pellets were re-suspended in digestion buffer for 5 min at 37 °C, and the chromatin was sheared to an average length of 200–500 base pairs. Sheared chromatin was incubated with rotation at 4 °C overnight in the presence of 5 μg mouse anti-acetyl-H3K9 (Cat#. 61,251, Active Motif), 10 μg rabbit anti-acetyl-H3K27 (#4353S, Cell Signaling Technology), or 5 μg rabbit anti-tri-methyl-H3K4 (#9727S, Cell Signaling Technology), or with IgG (Jackson ImmunoResearch, West Grove, PA, USA) and protein G beads. The beads were then washed and eluted. Cross-links were reversed by heating at 95 °C for 15 min. After treatment with proteinase K for 1 h at 37 °C, the reaction was stopped using a Proteinase K Stop Solution. Precipitated DNA was analyzed by real-time PCR with primer pairs corresponding to the upstream and downstream regions of mouse *Atf3* and *Hmox1*. Primer sequences are described in Supplementary Table 1.

2.7. MTT and lactate dehydrogenase (LDH)-release assays

Apoptosis was induced in neuronal cell cultures using either MPP^+ or $A\beta_{1-42}$. Cultured neuronal cells at DIV3 were treated with 5 μM amitriptyline for 48 h. After removal of the drug, the cultures were maintained in medium without antioxidants with either MPP^+ (50 or 200 μM) for 48 h or with $A\beta_{1-42}$ (5 or 10 μM) for 24 h. Control group was maintained in medium without antioxidants. Cell viability in all treatment groups was examined by MTT reduction assays as described previously (Miyake and Nagai, 2009). Cell death rates were determined by LDH-release assays using Cytotoxicity Detection Kit^{PLUS} (Roche Ltd., Basel, Switzerland) in accordance with the manufacturer's instructions. For the MTT assay, the untreated control was set as 100% survival. For the LDH-release assay, the untreated control was set as 0% dead and the positive control was set as 100% dead.

2.8. Immunocytochemistry

Cells used for immunostaining were washed 2 times with PBS, and then fixed in 4% paraformaldehyde. After fixing, cells were washed 2 times with PBS and permeabilized using 0.1% Triton X-100 in PBS. 1% skim milk in PBS was used to block unspecific binding. The primary antibodies were diluted in PBS, added to cells after removing the blocking solution, and incubated overnight at 4 °C. The following primary antibodies were used: rabbit anti-MAP2 (diluted 1:200, #8707S, CST) and mouse anti-glial fibrillary acidic protein (anti-GFAP) (diluted 1:200, MAB3402, Millipore). The next day, cells were rinsed 5 times with PBS, and proper fluorophore-conjugated secondary antibodies were diluted 1:1000 in blocking solution and added to cells. Cells were incubated with secondary antibodies for 1 h at room temperature, and then washed again 5 times with PBS. After the last washing, Hoechst 33342 (Dojindo) was diluted in distilled water, added to cells, and incubated at room temperature for 15 min. After incubating, Hoechst 33342 was replaced by PBS. Images were acquired using EVOS® FL Cell Imaging System (Life Technologies).

2.9. Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM) for *n* independent observations. Comparisons of multiple groups were performed with one-way analysis of variance (ANOVA), followed by Tukey's post hoc test or Dunnett's T3 test. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Identification of genes whose expression is commonly altered by antidepressants

Our first goal was to identify genes whose expression was altered by the tested antidepressants, namely, imipramine and amitriptyline (TCAs), because such genes potentially could be associated with the therapeutic mechanisms of the antidepressants. For this purpose, we considered that 1 μM and 10 μM concentrations of amitriptyline are at plasma therapeutic level and cumulative level in the brain, respectively (Perisic et al., 2010) and are sufficient to induce changes in the mRNA level of neuroprotective enzyme superoxide dismutase (Li et al., 2000). We treated mouse primary neocortices neuron cultures with 5 μM amitriptyline and confirmed with MTT assay that this concentration did not induce cell death (Supplementary Fig. 2). Next, we performed a DNA microarray assay and a based-cluster analysis. The expression profiles of the two TCAs showed some similarity (Fig. 1A).

From the comparative analysis, we selected genes showing at least 1.5-fold higher or lower fold changes in expression between the treated and non-treated cells. In total, 60 and 43 genes were up-regulated by amitriptyline and imipramine, respectively (Fig. 1B). Of these, 17 genes were up-regulated by both antidepressants (Supplementary Table 2). We identified 74 and 45 genes that were down-regulated by amitriptyline and imipramine, respectively (Fig. 1C). Of these, 27 genes were down-regulated by both antidepressants (Supplementary Table 3).

3.2. Up-regulation of neuroprotection-associated genes

Of the 17 genes that were up-regulated by both imipramine and amitriptyline, we selected three genes, activating transcription factor 3 (*Atf3*), heme oxygenase 1 (*Hmox1*), and growth differentiation factor 15 (*Gdf15*), which are known to have neuroprotective actions (Hettiarachchi et al., 2014; Liang et al., 2009; Strelau et al., 2000). We performed qRT-PCR and western blot to confirm the up-regulation of these three genes by both antidepressants at both mRNA and protein levels. Result analyses demonstrated that, at both mRNA and protein levels, *Atf3* was significantly up-regulated solely by amitriptyline (Fig. 2A and C), while *Hmox1* was significantly up-regulated by both imipramine and amitriptyline (Fig. 2B and D).

However, for *Gdf15*, although both antidepressants up-regulated at mRNA level, none was significantly up-regulated at protein level (Supplementary Fig. 3). Therefore, we excluded *Gdf15* from further investigation.

3.3. Amitriptyline-induced active histone modifications in the Atf3 and Hmox1 promoter regions

As imipramine is known to induce epigenetic changes by increasing histone acetylation, we asked whether the up-regulation of *Atf3* and *Hmox1* by amitriptyline was related to changes in histone modification. To screen for changes to active histone modifications in the promoter regions of the up-regulated genes, we performed ChIP against histone H3 lysine 4 trimethylation (H3K4me3), H3K9 acetylation (H3K9ac), or H3K27 acetylation (H3K27ac), followed by qPCR. These analyses showed that amitriptyline significantly increased the level of histone H3K4me3 and H3K9ac within the *Atf3* promoter region encompassing the MEF2 and CRE binding sites (Zhang et al., 2011) (Fig. 3A and B). ChIP qPCR for *Hmox1* promoter showed that amitriptyline significantly increased H3K4me3, but not H3K9ac, in the region encompassing the AP-1 and HSF binding sites (Alam and Cook, 2007) (Fig. 3C and D). H3K27ac was not enriched in the promoter region of either gene (Fig. 3B and D).

Given that H3K4me3 and H3K9ac are well-defined as histone codes for active promoters and H3K27ac is enriched at active promoters and enhancers, collectively, our results suggest that amitriptyline up-regulated *Atf3* and *Hmox1* genes by inducing active histone modifications in the promoter regions of *Atf3* and *Hmox1*.

3.4. Neuroprotective action of amitriptyline in primary cultures of mouse neuronal cells

Since amitriptyline induced up-regulation of the neuroprotection-associated *Atf3* and *Hmox1* genes at both the mRNA and protein levels in mouse primary neocortical neuron culture, we theorized that amitriptyline might have a neuroprotective effect on neuronal cells. We induced cell damage by treating cells with either MPP^+ (50 or 200 μM) or $A\beta_{1-42}$ (5 or 10 μM) for 48 h or 24 h, respectively. Previous studies have shown that at these concentrations, MPP^+ had a neurotoxic effect and $A\beta_{1-42}$ significantly induced apoptosis and increased caspase-3 level in rat neurons (Marini and Nowak, 2000; Wei et al., 2008). Amitriptyline was found to increase cell survival rates slightly but significantly in an MTT assay of cultures treated with 200 μM MPP^+ (Fig. 4A) and with 5 or 10 μM $A\beta_{1-42}$ (Fig. 4C).

Amitriptyline significantly decreased the proportion of dead cells in an LDH-release assay in cultures treated with 50 or 200 μM MPP^+ (Fig. 4B), and in cultures treated with 5 or 10 μM $A\beta_{1-42}$ (Fig. 4E). Furthermore, amitriptyline significantly reduced cleaved caspase 3, a critical marker for neuronal cell death (Ferrer and Planas, 2003), in cultures treated with 200 μM MPP^+ (Fig. 4C) or 5 μM $A\beta_{1-42}$ (Fig.4F). When results are considered in toto, amitriptyline appeared to exercise epigenetic regulation of neuroprotection-associated genes and exhib-

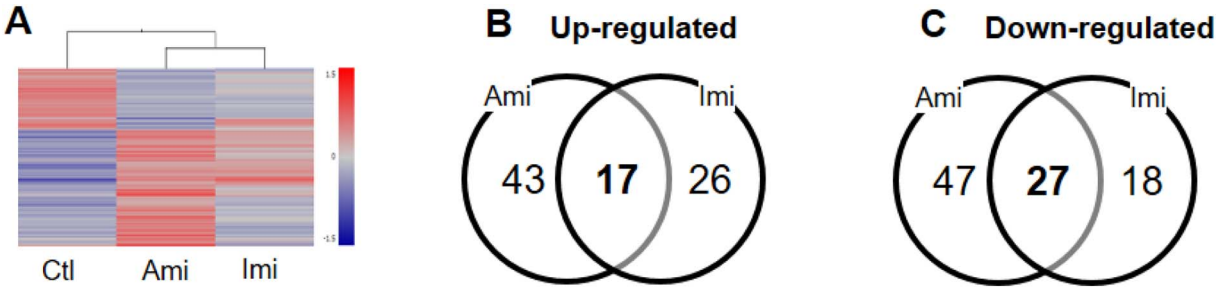

Fig. 1. Global gene expression patterns in primary cultures of mouse neuronal cells treated with amitriptyline (Ami) or imipramine (Imi). (A) Hierarchical clustergram of transcription profiles. Red: higher gene expression. Blue: lower gene expression. Venn diagram of the genes that were (B) up-regulated or (C) down-regulated by antidepressant treatment. Cultured neuronal cells at DIV3 were treated with 5 μM imipramine or 5 μM amitriptyline for 48 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

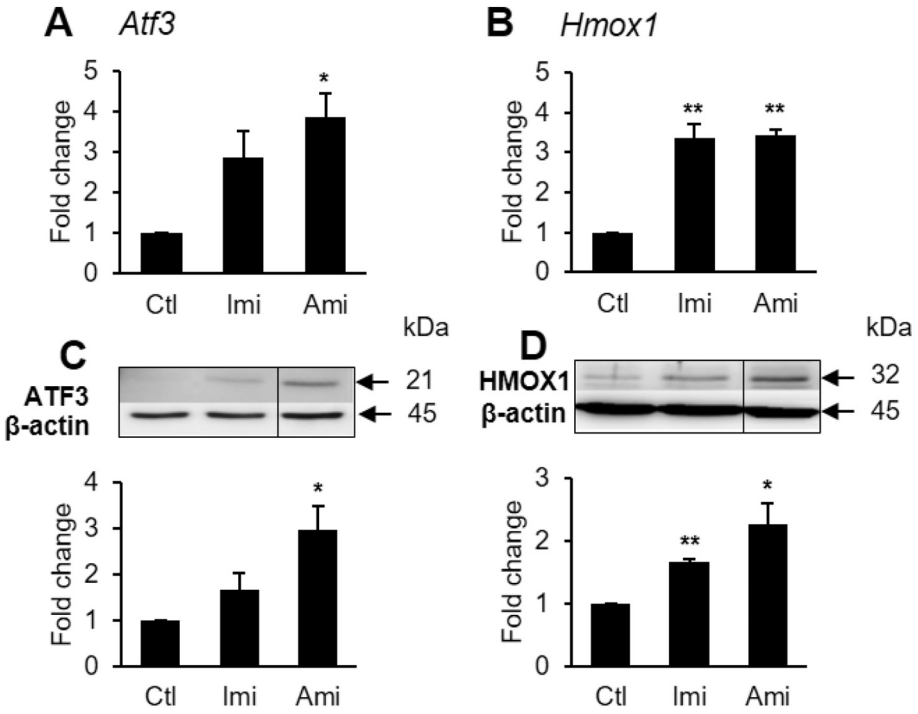

Fig. 2. qRT-PCR assay and western blots of *Atf3* and *Hmox1* in mouse neuronal cells treated with 5 μM imipramine or 5 μM amitriptyline for 48 h. (A and B) qRT-PCR analysis of *Atf3* and *Hmox1*. (C and D) Western blot of *Atf3* and *Hmox1*. Bars represent the relative ratios of treated to untreated controls. Data are based on three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ versus control.

ited neuroprotective effects at a concentration as low as 5 μM.

3.5. Amitriptyline inhibited the activation of mitogen-activated protein kinase (MAPK) family members extracellular signal-regulated kinase 1 and 2 (ERK1/2)

MAPK families contain c-Jun N-terminal kinase (JNK), p38-MAP kinase, and ERK1 and 2, which control many intracellular processes, including gene expression, cell growth, cell differentiation, cell survival, and cell death (Chen et al., 2001). MAPK sub-families ERK1/2 have both anti-apoptotic and pro-apoptotic functions. Activation of ERK1/2 may result either in the activation of anti-apoptotic proteins such as cAMP response element-binding protein (CREB) and CREB-binding protein (CBP) and the inhibition of pro-apoptotic proteins such as caspase 8 and 9 or lead to the enhancement of pro-apoptotic molecules such as caspase 3 and p53 under some specific statuses (Lu and Xu, 2006). Primary cortical neuron cultures and SH-SY5Y cells exposed to oxidative stress induced by H_2O_2, ethanol, or $A\beta_{1-42}$ were reported to have increased phosphorylated ERK1/2 (p-ERK1/2) in cells (Bartov et al., 2006; Liu et al., 2010; Yu et al., 2016). Therefore, we presumed that ERK1/2 and p-ERK1/2 were altered in our experiments. To investigate this presumption, we performed western blot on control and amitriptyline-treated primary neocortices neuron cultures with and without MPP^+ or $A\beta_{1-42}$.

As expected, p-ERK1/2 increased in cells exposed to 200 μM MPP^+ (Fig. 5B) or 5 μM $A\beta_{1-42}$ (Fig. 5E), while total ERK1/2 remained unchanged (Fig. 5A and D). Amitriptyline neither altered total ERK1/2 in control and cell damage-induced groups (Fig. 5A and D), nor changed p-ERK1/2 in control groups (Fig. 5B and E). However, amitriptyline significantly reduced the elevated p-ERK1/2 caused by MPP^+ or $A\beta_{1-42}$ (Fig. 5B and D). Analyses of the ratio of p-ERK1/2 to total ERK1/2 also revealed that amitriptyline successfully reduced the p-ERK1/2 to ERK1/2 ratio compared with the control group treated with MPP^+ (Fig. 5C) or showing a trend toward significance compared with the control group treated with $A\beta_{1-42}$ (Fig. 5F). To exclude the possibility that activation of ERK1/2 gave rise to the MAPK anti-apoptotic pathway, we examined changes in total CREB and p-CREB. As predicted, we found no alteration in total CREB or p-CREB between control and cell damage-induced groups (Supplementary Fig. 4).

Taken together, our results demonstrated that MPP^+ and $A\beta_{1-42}$ activated ERK1/2 followed the pro-apoptotic pathway, and consequently increased activation of caspase 3. Further, amitriptyline exercised a neuroprotective effect partly involved in inhibiting ERK1/2 activation and reducing cleaved-caspase 3.

3.6. Co-treatment also up-regulated Atf3 and Hmox1 but not retained neuroprotection

We further asked whether amitriptyline also possessed neuroprotective effect when treated at the same time with $A\beta_{1-42}$. In MTT assay of co-treatment cultures, amitriptyline failed to increase cell survival rates compared to control (Fig. 6A), suggesting that the effect on cell survival of amitriptyline is related to the changes induced by pre-treatment. For this reason, we wondered how *Atf3* and *Hmox1* changed followed treatment with $A\beta_{1-42}$ and co-treatment. To this end, we performed RT-PCR and western blot to further examine the alterations of *Atf3* and *Hmox1* after $A\beta_{1-42}$ treatment and after co-treatment with $A\beta_{1-42}$ and amitriptyline. Amitriptyline slightly but significantly up-regulated *Atf3* and *Hmox1* at both mRNA and protein levels after 24 h of treatment, which were further increased after 48 h (Fig. 6B, C, D, and E). Interestingly, treatment with $A\beta_{1-42}$ only for 24 h up-regulated *Atf3* and *Hmox1* similar to the effect of treatment with amitriptyline for 48 h (Fig. 6B, C, D, and E).

When comparing between treatment with $A\beta_{1-42}$ only, pre-treatment, and co-treatment with 5 μM amitriptyline, we found that, at mRNA level, *Atf3* and *Hmox1* were significantly up-regulated after pre-treatment with amitriptyline compared to treatment with $A\beta_{1-42}$ alone. Unexpectedly, the highest increased mRNA level of *Atf3* and *Hmox1* was observed after co-treatment, which significantly higher than pre-treatment (Fig. 6B and C). However, protein level of ATF3 only showed a trend of increasing similarly with mRNA level without significance (Fig. 6D). Western blot assay for HMOX1 revealed that pre-treatment

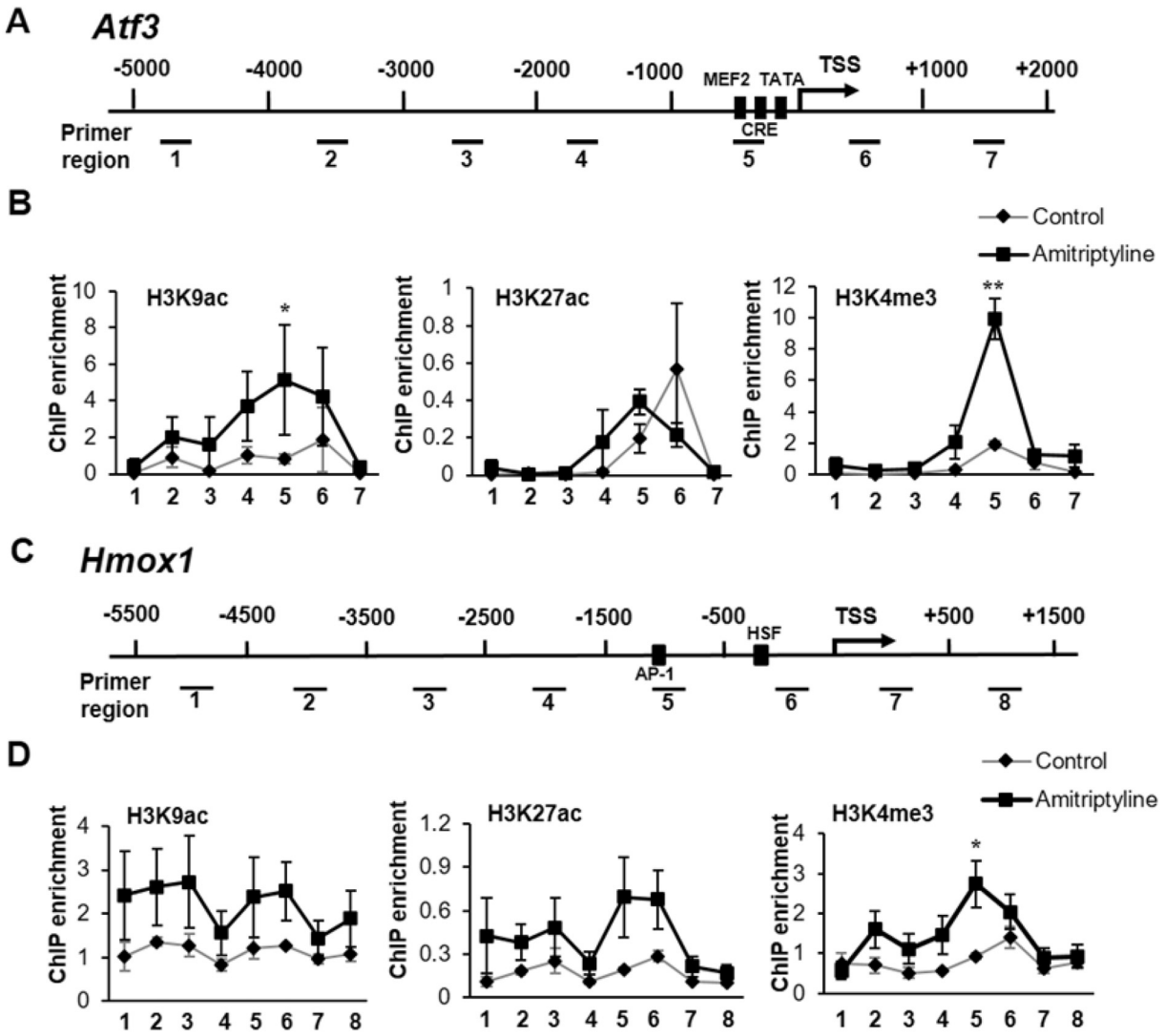

Fig. 3. Changes in histone modifications in the promoter regions of *Atf3* and *Hmox1*. (A) Schematic representation of the upstream region of *Atf3* encompassing the MEF2 and CRE binding sites and the TATA box. Numbers (1 to 7) represent the regions assayed by ChIP qPCR. (B) Changes in histone H3K9ac, H3K27ac, and H3K4me3 modifications after treatment with 5 μM amitriptyline for 48 h. (C) Schematic representation of the upstream region of *Hmox1* encompassing the AP-1 and HSF binding sites. Numbers (1 to 8) represent the regions assayed by ChIP qPCR. (D) Changes in histone H3K9ac, H3K27ac, and H3K4me3 modifications after treatment with 5 μM amitriptyline for 48 h. Data were normalized over input and based on three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus control.

with amitriptyline significantly up-regulated HMOX1 compared to treatment with $A\beta_{1–42}$ alone. In co-treatment group, HMOX1 was also up-regulated but without significance compared to treatment with $A\beta_{1–42}$ only (Fig. 6E).

4. Discussion

In this study, we analyzed changes in the expression of a number of genes, investigated the underlying epigenetic mechanisms for altered gene expression levels following treatment with tricyclic antidepressants, and verified the neuroprotective action of amitriptyline. First, by screening for genes whose expression was altered by imipramine and amitriptyline, we identified 17 up-regulated genes and 27 down-regulated genes that responded to both tested drugs. Next, we confirmed the up-regulation at both mRNA and protein levels of two neuroprotection-related genes, *Atf3* and *Hmox1*, after treatment with amitriptyline, and of *Hmox1* after treatment with imipramine. In addition, we found that the changes in *Atf3* and *Hmox1* expression induced by amitriptyline were associated with epigenetic alterations to their promoter regions. Finally, we demonstrated neuroprotective action of amitriptyline in mouse primary cultured neocortical neuronal cells potentially by lessening ERK1/2 activation.

Hmox1 is known to have neuroprotective effect against various toxic stimuli (Reviewed by Schipper et al., 2009), including $A\beta_{1–42}$ and MPP^+ (Hettiarachchi et al., 2014; Huang and Chuang, 2010; Hung et al., 2008). Overexpression of HMOX1 in rat protected Dopaminergic neurons from MPP^+-induced cell death and attenuated asymmetric behavior (Hung et al., 2008). Moreover, HMOX1 overexpressed 24 h before and at the same time with adding MPP^+ had higher effectiveness than overexpressed after adding MPP^+ (Hung et al., 2008). In primary cortical neurons, up-regulated Hmox1 was also proven to have protective effect possibly by reducing H_2O_2 level (Huang and Chuang, 2010). Previous study showed that antidepressants, desipramine (a TCA) and fluoxetine, up-regulated *Hmox1* by activating nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and MAPK pathways, although the activation of MAPK pathway was only observed up to 2 h after treatment (Lin et al., 2012), which is discussed later in this paper; the authors also pointed out that up-regulation of Hmox1 by desipramine helped to protect from neurotoxicity induced by 6-OHDA and rotenone. Another gene we selected for further investigation was *Atf3*, of which neuroprotection is still controversial. Indeed, overexpression of ATF3 in CA3 hippocampal neurons of rat prior to kainic acid administration significantly reduced

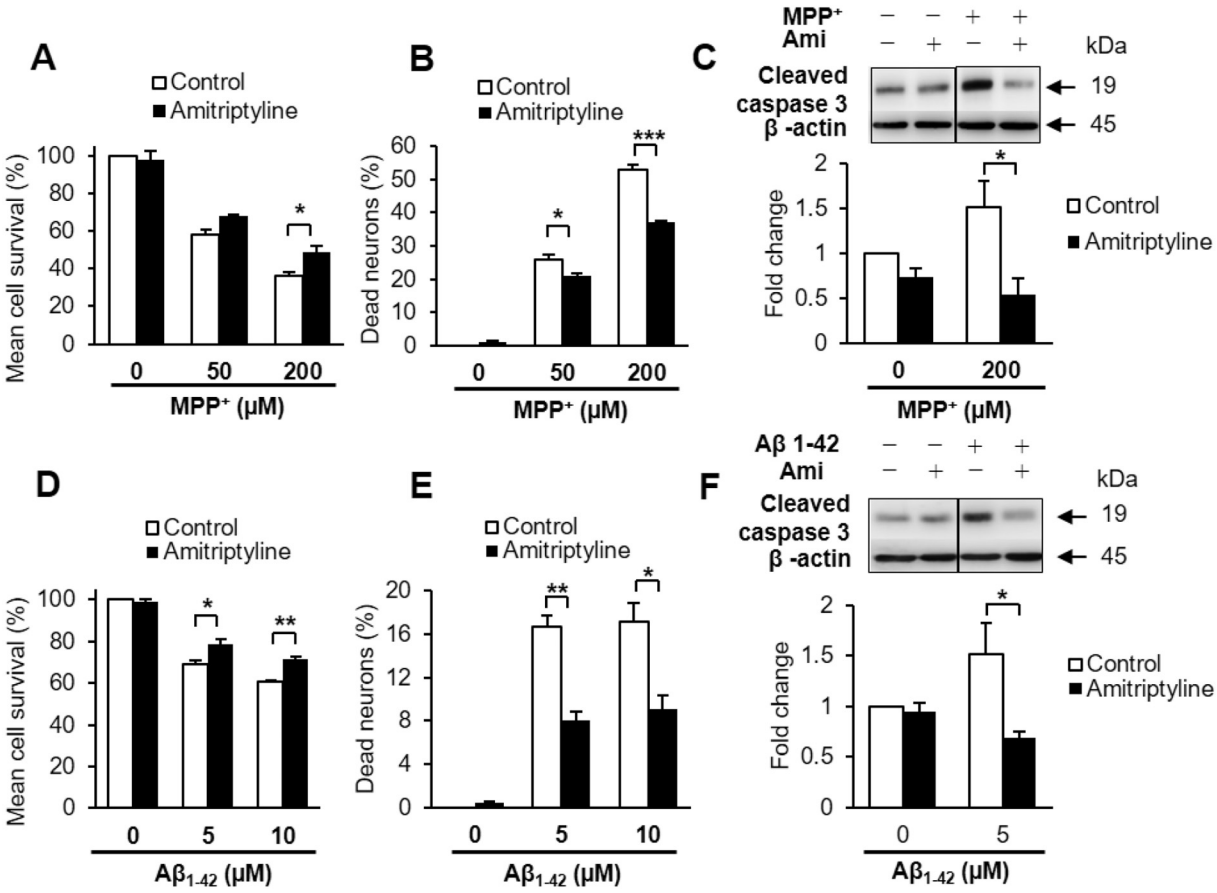

Fig. 4. Assessment of the neuroprotective effects of amitriptyline in mouse neuronal cells. Cells at DIV3 were treated with 5 μM amitriptyline for 48 h, after removal of the drug, cells were treated with either MPP^+ (50 or 200 μM) for 48 h or $A\beta_{1-42}$ (5 or 10 μM) for 24 h. (A) MTT assay for MPP^+-induced cell damage. (B) LDH-release assay for MPP^+-induced cell damage. (C) Western blot for MPP^+-induced changes in cleaved caspase 3 expression. (D) MTT assay for $A\beta_{1-42}$ -induced cell damage. (E) LDH-release assay for $A\beta_{1-42}$-induced cell damage. (F) Western blot for $A\beta_{1-42}$ -induced changes in cleaved caspase 3 expression. For cell survival and cell death assay, each value represents the mean ± SEM of 12 wells in three independent experiments. For western blot of cleaved caspase 3, each value represents the mean ± SEM in three independent experiments. Bars represent the relative ratios of treated to untreated controls. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

apoptotic cell death (Francis et al., 2004). In a stable PC12 cell model of Huntington disease induced by mutant huntingtin (mHTT), overexpression of ATF3 exerted a neuroprotective effect against mHTT while *Atf3* knockout enhanced the effects of mHTT (Liang et al., 2009). The evidences above support our hypothesis that amitriptyline up-regulated ATF3 before induced cell death with $A\beta_{1-42}$ or MPP^+ may aid in amitriptyline-induced neuroprotection. On the contrary, a recent study showed that *Atf3* knockdown prevented SH-SY5Y cells from MPP^+-induced apoptosis (Zhao et al., 2016), which was also contrast to an earlier study showing that primary neuron cultures from *Atf3* knockout mice did not exhibit increased survival compared to cultures from wild-type mice (Bernstein and O'Malley, 2013). We also looked at *Gdf15*, which was reported to rescue phenotype and have neuroprotection effect on dopaminergic neurons from 6OHDA-induced neurotoxicity (Strelau et al., 2000). However, we observed a discrepancy between *Gdf15* mRNA and protein level. While treatment with imipramine and amitriptyline increased *Gdf15* mRNA significantly compared to control, our results showed only a trend toward increasing GDF15 protein after treatment with these two antidepressants. The translation from mRNAs to proteins is under the control of complicated post-transcriptional and -translational mechanisms. In fact, a previous study showed that only 44% of changes in protein level can be expected from mRNA level (Schwanhausser et al., 2011). Moreover, it is suggested that, the correlation between mRNA and protein levels remarkably change over time when cells are under stimulation (Cheng et al., 2016). Thus, in our study, one possible explanation is that we only examined genes' expression for only one timepoint, 48 h after treatment with antidepressants, which may not be enough for the increase of GDF15 protein level.

We found that *Atf3 and Hmox1* were up-regulated by treatment with 5 μM $A\beta_{1-42}$ alone in a same manner with pre-treatment with amitriptyline for 48 h, further increased by pre-treatment with amitriptyline, but unexpectedly, even further increased, at least at mRNA level, in co-treated cells. A possible explanation is that co-treatment suddenly up-regulated *Atf3* and *Hmox1* up to a very high level, which made cells unstable and led to the loss of neuroprotective effect. In addition, earlier studies have highlighted the neuroprotection of preconditioning, mainly in the context of ischemia and injury (recently reviewed by Li et al., 2017). Noticeably, *Atf3* and *Hmox1* were implicated to participate in the effect of preconditioning. Although studies on ischemic preconditioning of the heart have pointed out changes in *Atf3* expression, location, and activation in cardioprotective effect (Brooks et al., 2014; Liu et al., 2009), as far as we can tell, there are not many studies focus on *Atf3* in brain preconditioning. *Atf*3 was found to be one of the early response genes that up-regulated after one hour of hypoxia preconditioning (Tang et al., 2006). Thus, together with the controversial findings in neuroprotective effect of *Atf*3 mentioned above, further studies on gene knockout as well as overexpression are needed to elucidate the mechanisms and confirm the roles of *Atf3* in preconditioning and neuroprotection. In contrast, many studies have subjected the up-regulation of *Hmox1* as an important and common event in preconditioning by different factors, for instance, hypoxia (Shu et al., 2016), sublethal ischemia (Lee et al., 2015a), *Ginkgo biloba* (Nada and Shah, 2012), or helium (Li et al., 2016). Furthermore, our

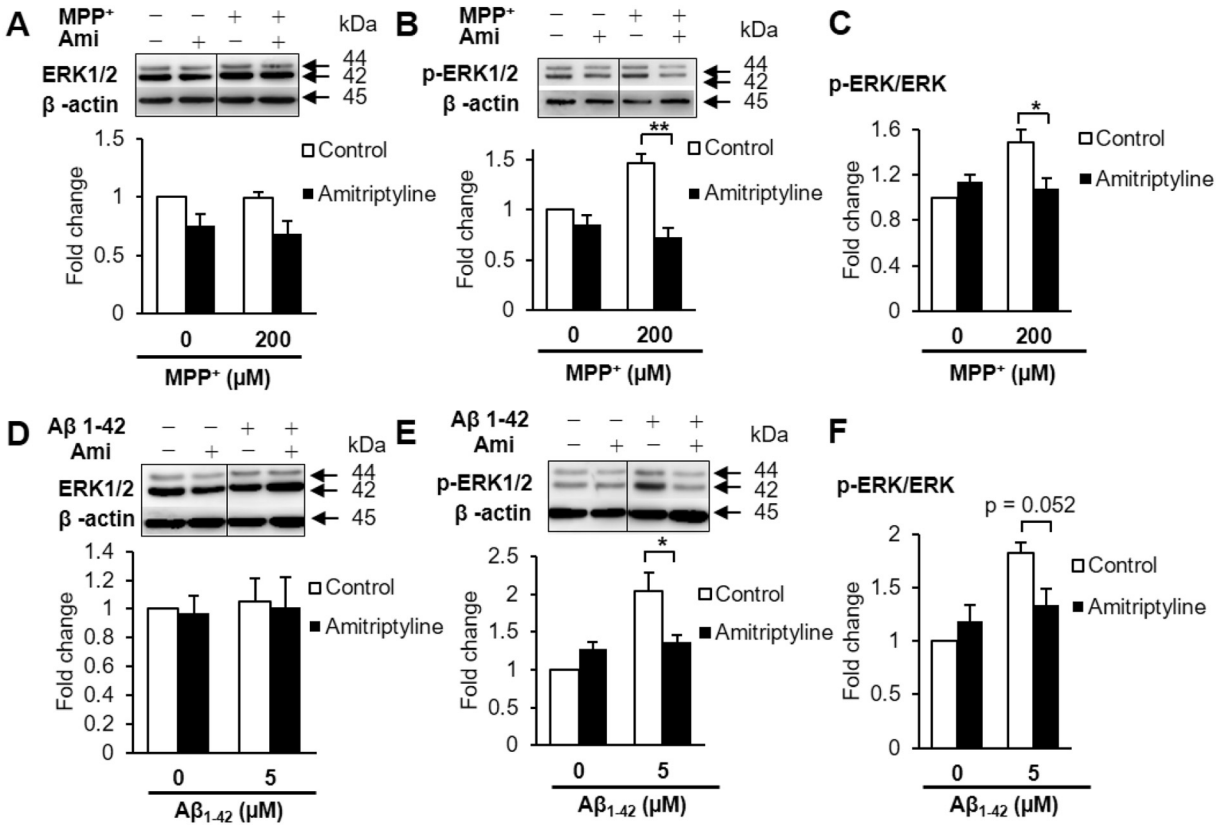

Fig. 5. Assessment of the changes in ERK1/2 and p-ERK1/2 in mouse neuronal cells. Cells at DIV3 were treated with 5 μM amitriptyline for 48 h, after removal of the drug, cells were treated with either 200 μM MPP^+ for 48 h or 10 μM $A\beta_{1-42}$ for 24 h. (A and B) Western blot for MPP^+-induced changes in ERK1/2 and p-ERK1/2 expressions. (C) Ratio of p-ERK1/2 to ERK1/2 in MPP^+-induced groups. (D and E) Western blot for $A\beta_{1-42}$-induced changes in ERK1/2 and p-ERK1/2 expressions. (F) Ratio of p-ERK1/2 to ERK1/2 in $A\beta_{1-42}$-induced groups. Each value represents the mean ± SEM in three independent experiments. Bars represent the relative ratios of treated to untreated controls; $^*P < 0.05$; $^{**}P < 0.01$.

observation was similar to studies that investigated *Hmox1* after cell damage initiation followed preconditioning, where the results showed a higher increase compared to preconditioning or cell damage initiation alone (Lee et al., 2015a; Nada and Shah, 2012; Shu et al., 2016). Taken with the loss of neuroprotective effect in co-treatment, our results suggested that pre-treatment with amitriptyline may bring about preconditioning effect that increased tolerance of primary neuron cultures to $A\beta_{1-42}$-induced neurotoxicity.

According to our results, amitriptyline induced active epigenetic changes, including H3K4me3 and H3K9ac but not H3K27ac, in promoters of *Atf3* and *Hmox1* of neurons. Previous study has provided evidence that amitriptyline induced minor cytosine demethylation by reducing DNA methyl transferase (DMNT) activities but did not cause any changes in histone acetylation and astrocyte-specific genes in rat primary astrocytes (Perisic et al., 2010). To our knowledge, this is the first report that amitriptyline alters histone modifications and consequently changes gene expression in neurons. However, the mechanism is still unclear; future studies may usefully focus on histone methyltransferases and histone demethylases after antidepressant exposure. Moreover, an earlier study of myeloma cells has shown that amitriptyline inhibited several HDACs and increased histone H3 acetylation in tumor suppressor genes *p21* and *p27* (Mao et al., 2011); thus, amitriptyline acted as an HDAC inhibitor in myeloma cells. In line with this earlier study, our results suggest that amitriptyline may have HDAC inhibitor properties. However, this property of amitriptyline may be cell-type dependent and warrants further investigation. Amitriptyline is used as an antidepressant as well as being the first line of treatment for neuropathic pain, a common problem in clinical practice that results from many peripheral and central nervous system diseases including diabetic neuropathy and cancer (Moore et al., 2015). Therefore, further studies are needed to compare the alterations in HDAC activities and HDAC expressions in several cell types to understand more about this ability of amitriptyline.

Amitriptyline is known to inhibit neurotransmitter reuptake, and thereby reverse serotonin and norepinephrine accumulation in synapses in patients with depression (Guaiana et al., 2007). It is also reported that amitriptyline has ability as a sodium channel blockade, which may involve in the effect of a painkiller and migraine prophylaxis of amitriptyline (Yan et al., 2010). Additionally, amitriptyline has a neuroprotective effect by countering mental stress-induced down-regulation of anti-apoptotic genes through the up-regulation of nerve growth factor 1A, mineralocorticoid receptors, and glucocorticoid receptors (Johansson et al., 1998; Okugawa et al., 1999). Together with these findings, our study suggests that epigenetic up-regulation of anti-apoptotic genes, such as *Atf3* and *Hmox1*, may contribute to the neuroprotective effect of amitriptyline. It is worth mentioning that HDAC inhibitors have been proven to improve cell viability and inhibit the activation of caspase 3 in SK-N-SH and MES 23.5 cells treated with MPP^+ (Kidd and Schneider, 2010). We also showed that suberoylanilide hydroxamic acid (SAHA), also known as vorinostat, improved cell viability in primary neurons treated with $A\beta_{1-42}$ (Supplementary Fig. 5A). In our result, SAHA showed a higher neuroprotective effect compared to amitriptyline. Moreover, in control cells, treated with 1 μM SAHA showed a significant increase in MTT assay compare to untreated cells (Supplementary Fig. 5A). These observations could be explained by the fact that, in addition to its innate neuroprotection against oxidative stress (Ryu et al., 2003), SAHA was also reported to protect neurons against the gradual shrinkage in primary cultures (Chen et al., 2012). The neuroprotective effect of SAHA may rely on glial cells (Chen et al., 2012), which were lacked in our experiments, thus, the neuroprotection of SAHA may not be at full potential, resulted in similar effect between SAHA 0.5 and 1 μM. However, combination of

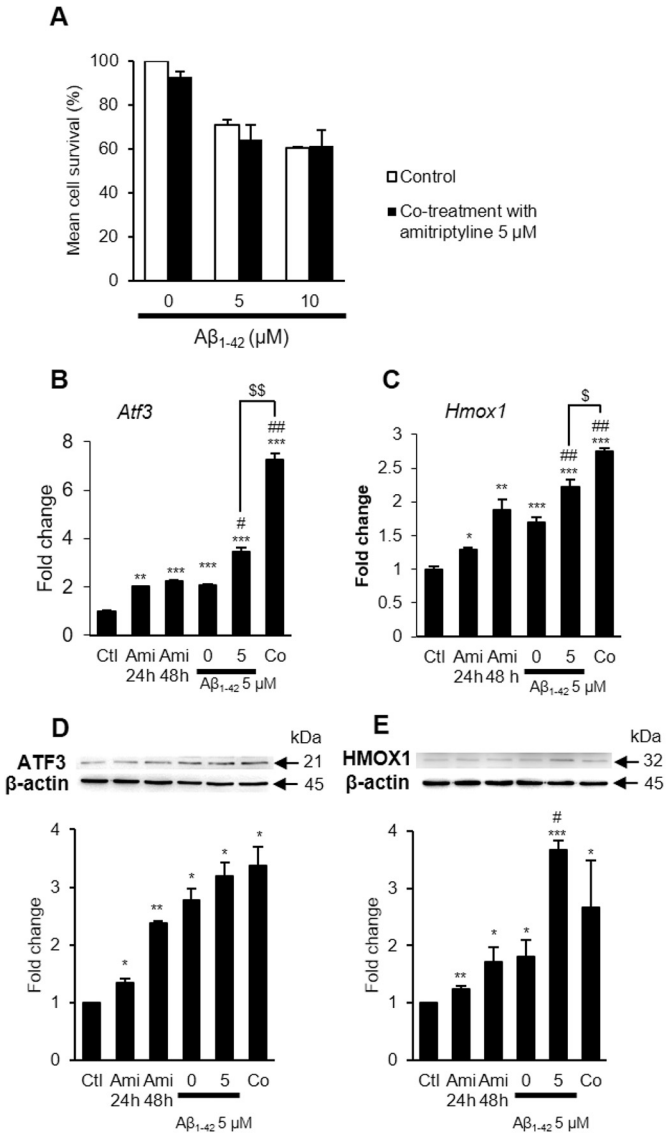

Fig. 6. (A) The neuroprotective effect of amitriptyline was lost when treated at the same time with Aβ1–42 in mouse neuronal cells as assessment by MTT assay. Cells at DIV5 were treated with 5 μM amitriptyline and $A\beta_{1-42}$ (5 or 10 μM) at the same time (Co-treatment) for 24 h. Each value represents the mean ± SEM of 12 wells in three independent experiments. (B and C) qRT-PCR analysis of *Atf3* and *Hmox1* before and after $A\beta_{1-42}$ -induced cell damage. (D and E) Western blot of *Atf3* and *Hmox1* before and after $A\beta_{1-42}$ -induced cell damage. Cells were treated with only 5 μM amitriptyline for 24 h or 48 h (Ami 24 h and Ami 48 h, respectively); or with 5 μM $A\beta_{1-42}$ with or without pre-treatment with 5 μM amitriptyline; or co-treated with 5 μM amitriptyline and 5 μM $A\beta_{1-42}$ for 24 h. Each value represents the mean ± SEM in three independent experiments. Bars represent the relative ratios of treated to untreated controls. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus treated with 5 μM $A\beta_{1-42}$ only; $^{\$}P < 0.05$, $^{\$\$}PP < 0.01$ between pre-treatment and co-treatment with 5 μM amitriptyline.

amitriptyline and SAHA exhibited neurotoxicity in a dose dependent manner of SAHA (Supplementary Fig. 5B). SAHA is known as a pan-HDAC inhibitor that targets a range of HDACs class I and II. At high concentration, HDAC inhibitors, particularly SAHA at 2.5 μM, may resulted in aberrant gene expression and induced cell death (Peart et al., 2005; Rouaux et al., 2004). Furthermore, neuronal cells were suggested to be more susceptible to changes in acetylation level and both hyper- and hypo-methylation may induced cell death (Rouaux et al., 2004). Therefore, we supposed that SAHA and amitriptyline, when combined together, may exceedingly perturb gene expression and induced cell damage. In the present study, amitriptyline showed neuroprotective effects at the concentration examined for epigenetic changes. This concentration is lower than that reported in a previous study of PC12 cells, which used 50 μM and 100 μM (Kolla et al., 2005). As mentioned earlier in this discussion, the neuroprotective effect of antidepressants, particularly amitriptyline, is presumed to depend on cell types and doses. Recently, amitriptyline has been shown to diminish lidocaine-induced rat dorsal root ganglion neurons in a dose-dependent manner up to 10 μM, but exerted toxicity at higher concentrations (Zheng et al., 2016). Previous studies showed that amitriptyline at 50 μM reduced cell viability in colon carcinoma cells (Arimochi and Morita, 2006), and more recently, concentrations at 60 μM and 120 μM caused mitochondria-mediated neurotoxicity and oxidative stress in SH-SY5Y cells (Lee et al., 2015b). Collectively, we hypothesized that amitriptyline when treated in low dose, induced preconditioning of neuronal cells without causing any harm to cells viability and, subsequently, had to neuroprotective effect. On the other hand, when treated with a high dose, amitriptyline resulted in neurotoxicity.

Our detailed results showed that amitriptyline exhibited an anti-apoptotic effect by suppressing the activation of ERK1/2 and its downstream caspase 3. The interplay between amitriptyline and the MAPK pathways seems to be more complex and relate to cell types and cell conditions. In brief, our data identified that amitriptyline inhibits ERK1/2 activation in neurons induced by MPP^+ or $A\beta_{1-42}$. On the other hand, others have shown that amitriptyline up-regulates early growth response-1 (*Egr-1*) and *Bdnf* mRNA by promoting the activation of ERK1/2 in rat astrocytes and microglia (Chung et al., 2007; Hisaoka-Nakashima et al., 2016). The length of time of exposure to oxidative stress is another concern, as some studies have identified different consequences from transient and sustained ERK1/2 activation (Marshall, 1995; Rybakova et al., 2012). When transiently activated, ERK1/2 assists anti-apoptotic factors and cell survival; however, sustained activation boosts pro-apoptotic factors and leads to cell death. The same complexity in the MAPK pathway involving ERK1/2 was also observed in MPP^+- or $A\beta_{1-42}$-induced neurotoxicology models. Previous studies on PC12 cells showed that treatment with MPP^+ for up to 120 min inhibited the activation of ERK1/2 and compounds with the effects of activating ERK1/2 successfully prevented MPP^+-induced cell death, which was lost by ERK1/2 inhibition (Di Segni et al., 2006; Hashimoto et al., 2012). In contrast, p-ERK started increasing after 8 h treatment with MPP^+ and this effect increased overtime up to at least 32 h (Zhu et al., 2007). Moreover, adding MAPK/ERK Inhibitor UO126 before or up to 12 h after MPP^+ initiation significantly prevented cell death induced by MPP^+, however, if administered after 20 h, the protective effect of UO126 was lost (Zhu et al., 2007). In primary cortical neurons, treatment with MPP^+ for 42 h was shown to increase caspase 3 activation, which could not be observed after 6 h treatment (Sun and Chang, 2003), and treatment with MPP^+ for 24 h increased p-ERK in a dose dependent manner (Fragkouli and Doxakis, 2014). In our study, the activation of ERK and caspase 3 after treatment with $A\beta_{1-42}$ was in line with other studies in SH-SY5Y cells, mouse primary cortical neurons and primary neural progenitor cells (Ha et al., 2012; Li and Qian, 2016; Mendell et al., 2016). Moreover, chemicals that can inhibit the activation of ERK1/2 were found to reduce the neurotoxicity of $A\beta_{1-42}$ (Li and Qian, 2016; Mendell et al., 2016), supporting our finding in amitriptyline. $A\beta_{1-42}$ was shown to induce phosphorylation of ERK in a dose dependent manner, however, this p-ERK level returned to normal after 60 min of treatment (Young et al., 2009). In contrast to our result, Thangnipon et al. (2013) showed that on rat primary cortical neuron cultures at DIV5, treatment with 10 μM $A\beta_{1-42}$ did not affect p-ERK but increased activated caspase-3. Collectively, the activation of ERK1/2 and its downstream caspase 3 played important role in the neurotoxicity induced by MPP^+ or $A\beta_{1-42}$. Together with other studies, we exposed primary neuron cultures to MPP^+ or $A\beta_{1-42}$ for 48 h and 24 h, respectively, which would activate ERK1/2 and lead to apoptosis, and showed that pre-treatment with amitriptyline successfully prevented

this effect.

Although we have adequately investigated the epigenetic and neuroprotective effects of amitriptyline, there are limitations remaining to be explored. Firstly, in our study, we treated primary neuron cultures at DIV3 with amitriptyline, and after 48 h (DIV5) with $A\beta_{1-42}$ or MPP^+. Therefore, the observation was limited to immature neurons. There are evidences that the neurotoxicity effects of staurosporine, doxorubicin, cyclosporine, *N*-methyl-D-aspartate, and glutamate are different in primary neuronal cultures at different DIVs. In fact, neurons at earlier stages were more prone to apoptosis induced by staurosporine, doxorubicin, and cyclosporine, while neurons at later stages showed higher susceptibility to glutamate and *N*-methyl-D-aspartate (Jantas-Skotniczna et al., 2006; Jantas et al., 2016; Jantas and Lason, 2009; McDonald et al., 1997). Related to models used in our study, hippocampal neurons at DIV30 treated with Aβ caused neurite degeneration without affecting cell survival (Rapoport and Ferreira, 2000). Thus, the changes in gene expression and the neuroprotective effect of amitriptyline maybe different in mature primary cultures of neurons. Secondly, in our study, we examined the neuroprotection of amitriptyline with only one concentration, which corresponded to the concentration investigated for epigenetic effect. Moreover, as discussed above, the MPP^+- or $A\beta_{1-42}$-induced changes in ERK1/2 were different over time. To comprehensively understand the neuroprotection effects of amitriptyline, further study is needed to investigate in dose- and time-dependent changes in gene expression, neuroprotection, and ERK1/2 pathway activation.

5. Conclusion

In conclusion, our study has provided insights into the epigenetic regulation of the preconditioning and neuroprotective effect of the tricyclic antidepressant amitriptyline in mouse primary cultured neurons. However, the pathways that taken place in amitriptyline's preconditioning and neuroprotection require further investigations. Moreover, it remains to be determined whether these neuroprotective effects occur in vivo (e.g., in the brains of mice under stress) or in the patients' neurons. To address this question, we plan to investigate the neuroprotective mechanism of antidepressants using recently established neuronal cells derived from human induced pluripotent stem cells derived from patients with a mental disorder (Andoh-Noda et al., 2015).

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ntt.2017.05.002.

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