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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