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Nebracetam Inhibited Hippocampus Neurons Injury Induced by Aβ 25-35 in MRTF-A-CArG Manner via ERK1/2 Pathway

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Abstract
Nebracetam has been recently proposed to have a neuroprotective action and cognitive enhancing effect being characteristic of a nootropic drug, while the mechanisms remained ambiguity. In this study, we investigated the protective effects of nebracetam on hippocampus neurons injury-induced by β-amyloid protein(Aβ25-35) and its mechanisms. Hippocampus neurons were treated with nebracetam (0.05 mM, 0.2 mM or 0.8 mM) or Aβ25-35 (20 uM/L). We found that Nebracetam significantly reduced apoptotic induced by Aβ25-35 and increase in the total number of dendritic spines and dendritic spine density in a dose-dependent manner. RT-PCR assay and Western blotting analysis revealed that nebracetam increased the expression of myeloid cell leukemia-1 (Mcl-1), B-cell lymphoma-2 (Bcl-2) and activity regulated cytoskeleton associated protein (Arc) in Aβ25-35-treated hippocampus neurons. Co-treatment nebracetam with MRTF-A (Myocardin-related transcription factor-A) siRNA reversed Mcl-1, Bcl-2 and Arc mRNA and protein levels. What's more, the luciferase assays indicated that the transcriptional activities of Mcl-1, Bcl-2 and Arc genes were significantly abolished by MRTF-A siRNA while it showed no changes on activities of mut Mcl-1-promoter-luc, mut Bcl-2-promoter-luc and mut Arc-promoter-luc. Additionally, the up-regulation of Mcl-1, Bcl-2 and Arc protein expression in nebracetam-treated group was inhibited by extracellular signal regulated protein kinase 1/2 (ERK1/2) inhibitor PH80509. These results demonstrated that nebracetam inhibited Aβ25-35-induced hippocampus neurons injury by enhancing the transactivity of Mcl-1, Bcl-2 and Arc, which may actively based in MRTF-A-CArG-dependent manner by thwarting the ERK1/2 pathway.

Keywords: Nebracetam; Apoptosis; Synaptic plasticity; MRTF-A; ERK1/2 signaling pathway

Introduction
Nebracetam, 4-aminomethyl-1-benzylpyrroloidin-2-one, has been recently proposed to have a neuroprotective action and considered to improve the cognitive effect of nootropics [1]. Nebracetam enhances cholinergic neuro-transmission, and acts as a partial agonist presynaptically at muscarinic receptors. It also reduces dopaminergic and serotonin uptake and inhibits intracellular calcium flux in response to glutamnergic stimulation [2]. Although the neuroprotection activity of nebracetam has been demonstrated, the mechanisms remained ambiguity.

Alzheimer disease (AD) is a neurodegenerative disease characterized by neuronal cell death, accumulation of extracellular amyloidplaques, neurofibrillary tangles (NTF) [3]. Various deranged mechanisms such as chronic oxidative stress [4], mitochondrial dysfunction [5,6], Aβ production [7], neurofibrillary tangles accumulation, hormone imbalance [8], inflammation, mitotic dysfunction [9], calcium mishandling [10], and genetic components [11] play a role in the disease process. Although the mechanisms are diverse, neuronal death, the inevitable event occurs resulting in AD. Therefore, inhibiting neuronal apoptosis may be beneficial to prevent AD. Bcl-2 family proteins are central regulators of apoptosis and consist of both anti-apoptotic and pro-apoptotic members based on their structure and function [12]. Mcl-1 (Myeloidcellleukemia-1) and Bcl-2 (B cell lymphoma/ leukemia-2) are the anti-apoptotic members, and increasing evidence has demonstrated that modifying neuronal synapses may play a major role in the pathophysiology of AD. The strategies to affect synaptic plasticity could have an enormous impact in improving outcomes of AD. The activity-regulated cytoskeletal (Arc) gene encodes a protein that is critical for memory consolidation [2]. Arc regulates synaptic strength through multiple mechanisms and is involved in essentially synaptic plasticity [13]. Studies of the transcriptional control of Bcl-2 and Mcl-1 expression have highlighted the regulation of MRTF-A on the CArG box in the promoters of Bcl-2 and Mcl-1 [14,15]. And we also found the CArG box in Arc promoter. Based on the importance of MRTF-A-mediated transcriptional regulation signal on apoptotic associated proteins, we speculated that MRTF-A and its target molecules might contribute to the protective effect of nebracetam on hippocampus neuron injury induced by Aβ25-35. Furthermore, Luciferase report assay suggested that the CArG boxes in Mcl-1, Bcl-2 and Arc promoters were required for the transcriptional modulation of these target genes by nebracetam via MRTF-A-dependent mechanism. Therefore, it is conceivable that MRTF-A may mediate nebracetam-induced the modulation of neuronal Mcl-1, Bcl-2 and Arc expressions. Moreover, the activation of the ERK1/2 pathway might involve in the upregulation of Mcl-1, Bcl-2 and Arc expression and transcription activity in hippocampus neurons.

In this study, we found nebracetam inhibited Aβ25-35-induced neuronal apoptosis and promoted neuronal synaptic plasticity, which is related to the up regulation of the mRNA and protein levels of Mcl-1, Bcl-2 and Arc. Furthermore, we speculated that MRTF-A is a key molecule in the up regulation of these target genes by nebracetam, while the ERK1/2 pathway was also involved.

Materials and Method
Reagents
Nebracetam was prepared in our laboratory (purity ≥ 98.77%). Neurobasal - Medium and antibiotics were purchased from Gibco...
In vitro

Invitrogen (Carlsbad, CA, USA), and fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China). Annexin V-FITC/PI cell apoptosis detection kit was purchased from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibodies against M2F6, Mcl-1, Bcl-2, Arc and β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol Reagent was purchased from Invitrogen. RevertAid First strand cDNA synthesis kit was from Thermo scientific.

Cell culture

Rat hippocampus neurons were dissociated from the rats born in 24 h and cultured at a density of 1.5 × 10^5 cells/cm² onto poly-D-lysine-coated 96- or 6-well plates, or cover slips. The cells were cultured in Neurobasal – Medium with 15% horse serum, 2.5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and were maintained in a humidified incubator in air with 5% CO₂. For this study, the cells were pretreated with vehicle (PBS), nebracetam (freshly made) at different dosage (0.05 mM, 0.2 mM or 0.8 mM) for 24 h and then exposed to Aβ25-35 for another 24 h.

Apoptosis analysis by flow cytometry

Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC/PI apoptosis detection kit. After preincubated with nebracetam (0.05 mM, 0.2 mM or 0.8 mM) for 24 h, and exposed with Aβ25-35 (20 μM/L) for 24 h, cells cultured on 6-well plates were washed twice with ice-cold phosphate buffered saline (PBS), suspended in PBS, followed by centrifugating at 1200 rpm for 5 min. After discarding the supernatant, the cells were resuspended in 200 μl binding buffer and incubated with 10 μl Annexin V-FITC and 10 μl PI at room temperature for 15min in the dark. Then the cells were added 300μl binding buffer before the fluorescence analysis was performed using a flow cytometer [16] (BD-LSRII, U.S.A.).

Synaptic morphological analysis

Hippocampus neurons were fixed with 4% Paraformaldehyde and rinsed extensively in PBS, and then exposed for 2 h at room temperature to the drebrin (M2F6) mouse monoclonal antibody at a concentration of 1:200. Following the PBS rinses, covers lips were incubated for 1 h at room temperature with the secondary antibody. Wash Five times for 5 min with 2 ml of PBS. Mount slide with anti-fading agent. Images were acquired using a confocal laser scanning unit [17].

Reverse-transcription PCR (RT-PCR) assay

Total RNA was isolated from hippocampus neurons using Trizol Reagent (Invitrogen, U.S.A.) according to the manufacturer’s protocol. Aliquots (2 μg) of total RNA were reverse-transcribed into cDNA by using oligo-dT as a primer in 20 μl reverse-transcription solution and amplified by a thermal cycler (Bio-Rad, CA). Complementary DNA was then used as a template for PCR carried out in 18 μl reaction mixture containing 0.6 μl of each primer, 1.8 μl 10X Taq Buffer, and 0.11 μl of Taq polymerase (Takara, Japan). Primers sequences were designed using primer 5.0 as follows: β-actin forward: 5’-GAGGGAAATCGTGGTGAC-3’; β-actin reverse: 5’-CTGGGAAGGTGGACAGTGAG-3’.

Western blotting analysis

The cells were washed twice with cold PBS before lysed in ProtoJet™ Mammalian Cell Lysis Reagent (MBI Fermentas). The lysate was centrifugated at 4°C, 16000 rpm for 45 min. Equal amounts of proteins were subjected to electrophoresis on 4.12% Bis-Tris NuPAGE Mini-gel (Invitrogen), followed by transfer to PVDF membrane (Millipore).The membrane was blocked with 5% non-fat milk for 1 h and incubated with primary antibody overnight at 4°C. After incubation with secondary conjugated antibody for 1 h, the membrane was subjected to enhanced chemiluminescence detection using SuperSignal West Pico Chemiluminescent Substrate (Pierce). The protein expression levels were quantified by the Adobe Photoshop software.

Luciferase reporter assay

The promoter-linked luciferase reporters including Mcl-1, Bcl-2, Arc CARG or their mutants (0.5 μg) were transiently transfected with 1ng CMV-Renilla Luciferase reporter (Promega) into hippocampus neurons using the Lipofectamine and Plus transfection reagents (Invitrogen) for 24 h. The promoter activities were determined by the firefly luciferase activity relative to the internal control renilla luciferase activity using the dual luciferase assay system described by the manufacturer (Promega, Madison, WI).

Statistical analysis

Experiments were repeated three times and the results were statistically evaluated with SPSS11.0 software. Hypothesis testing methods included one way analysis of variance (ANOVA) followed by least significant difference (LSD) test; p value of less than 0.05 were considered to indicate statistical significance. All the results were expressed as the mean ± S.D.

Results

Nebracetam inhibited apoptosis in neuronal hippocampus induced by Aβ25-35 and reduced Aβ25-35 induced hippocampus neuronal synaptic injury in vitro

The primary hippocampus neurons were pretreated with different dosages of nebracetam (0.05 mM, 0.2 mM and 0.8 mM) for 24 h, and then, the cells were exposed to Aβ25-35 for another 24 h. The apoptotic rate was determined by flow cytometry analysis. The results (Figure 1A) showed that pre-treated with nebracetam significantly decreased the apoptosis rate at the concentration of 0.2 mM (apoptotic rate: 16 ± 1.5%, **p<0.01) and 0.8 mM (apoptotic rate: 8.4 ± 1.4%, **p<0.01) compared to Aβ25-35-treated group (apoptotic rate: 39 ± 2.9%) in primary hippocampus neurons culture in a dose-dependent manner (Figure 1A).

Drebrin is an actin-binding protein mainly expressed in neurons [18],

and play a role in the structure-based plasticity [19]. The antibody M2F6 reacts with both the embryonic (drebrinE) and adult (drebrinA) isoforms of drebrin [20]. Confocal images showed that a significant degeneration of dendritic spines was observed in Aβ25-35 treated group (Figure 1B). However, nebracetam significantly increased the number of dendritic spine and the density of dendritic spine in hippocampus neurons at the concentration of 0.2 mM or 0.8 mM for 24 h in a dose-dependent manner.

Nebracetam upregulated the mRNA and protein levels of Mcl-1, Bcl-2 and Arc

The data (Figure 2) showed that pre-treated with Aβ25-35 significantly decreased the mRNA and protein levels of Mcl-1, Bcl-2 and Arc in hippocampus neurons. However, preincubation with nebracetam obviously upregulated the mRNA and protein levels of Mcl-1, Bcl-2 and Arc at the concentration of 0.2 mM or 0.8 mM (p<0.05 or **p<0.01) compared to Aβ25-35-treated group, which suggested that the neuroprotective effect of nebracetam against neuronal apoptosis might be associated with its upregulation the mRNA and protein expressions of Mcl-1, Bcl-2 and Arc.

MRTF-A involved in Nebracetam inhibiting Aβ25-35 induced hippocampus neuronal apoptosis and reducing Aβ25-35 induced hippocampus neuronal synaptic injury in vitro

Our previous study [17] has shown that MRTF-A, as a co-transcription factory of SRF, plays an important role in promoting neuronal survival against apoptosis, which is dependent on the binding of SRF and CArG boxes located on anti-apoptosis related gene (such as Mcl-1 and Bcl-2). To determine the exact role of MRTF-A in the effect of nebracetam on Aβ25-35-induced hippocampus neuron apoptosis, we knocked down hippocampus neuronal MRTF-A using MRTF-A siRNAs. Interestingly, we found that the inhibition of nebracetam on neuronal apoptosis was also reduced markedly by MRTF-A siRNA (Figure 3A). We next explored whether MRTF-A also participated in the effect of nebracetam on hippocampus neuronal synapse. Confocal laser scanning was used to observe the changes in dendritic spines of hippocampus neuron. As expect, preincubation with nebracetam significantly increased in the number and the density of dendritic spine in hippocampus neurons. However, the protect effects of nebracetam
on synaptic injury was markedly weakened by MRTF-A siRNA in a dose-dependent manner (Figure 3B).

**Nebracetam upregulating the transactivity and expression of Mcl-1, Bcl-2 and Arc via a MRTF-A-dependent mechanism**

Based on the above results, we next explored whether MRTF-A also participated in the regulation of nebracetam on Bcl-2, Mcl-1 and Arc levels. Extensive studies have been carried out to characterize the molecular mechanism of MRTF-A regulates Mcl-1, Bcl-2 and Arc expression [17]. As expect, the increasing of Bcl-2, Mcl-1 and Arc mRNA and protein levels induced by nebracetam was weakened when cotransfected with MRTF-A siRNA (Figure 4).

Next, we hypothesized that the effect of nebracetam enhancing the expressions of Bcl-2, Mcl-1 and Arc via MRTF-A mediated transcription mechanism. To test this hypothesis, we investigated the transactivity of Bcl-2, Mcl-1 and Arc promoters in primary hippocampus neurons after treated with nebracetam alone or cotransfected with MRTF-A siRNA. The transient luciferase assays showed that nebracetam activated the luciferase gene expressions driven by WT Bcl-2, Mcl-1 and Arc promoters dose-dependently, whereas this effects was significantly repressed by cotransfection with MRTF-A siRNA in a dose dependent manner (Figures 5C and 5D).

To determine whether the transactivation of Bcl-2, Mcl-1 and Arc promoters induced by nebracetam is dependent on the CarG box, we constructed the dominant negative luciferase reporter vectors of Bcl-2, Mcl-1 and Arc gene promoter (mut-Bcl-2 luc, mut-Mcl-1 luc and mut-Arc luc). Next, we transfected primary hippocampus neurons with WT and mut-luciferase reporter vectors, respectively, and measured the luciferase activity. The luciferase activity of WT Bcl-2, Mcl-1 and Arc promoters was significantly higher than that of mut-luciferase reporter vectors (Figures 6A, 6B and 6C). These results indicated that MRTF-A activates the luciferase gene expressions driven by WT Bcl-2, Mcl-1 and Arc promoters dose-dependently, whereas this effects was significantly repressed by cotransfection with MRTF-A siRNA in a dose dependent manner (Figures 5C and 5D).
Arc luc, respectively). Our dates showed that the nebracetam induced transactivation of Bcl-2, Mcl-1 and Arc promoters was reduced or abolished after the mutation of the CArG boxes in Bcl-2, Mcl-1 and Arc promoters (Figures 5E and 5F), suggesting that nebracetam induced transactivation of Bcl-2, Mcl-1 and Arc promoters through activating the key CArG box, which was the transcriptional regulatory element transactivated by MRTF-A.

**Figure 3:** Nebracetam inhibited hippocampus neuronal apoptosis and synaptic injury involved MRTF-A. (A) The apoptotic of co-treatment nebracetam with MRTF-A siRNA on primary hippocampus neurons was determined by flow cytometry. (B) Morphology of hippocampus neurons treated with nebracetam and MRTF-A siRNA by immunofluorescence assay (40X). Results are presented as means ± S.D (n=3). *p<0.05 or **p<0.01 vs. Control, *p<0.05 or **p<0.01 vs. model. #p<0.05 or ##p<0.01 vs. the same dosage of nebracetam without MRTF-A siRNA groups, respectively.

ERK-dependent pathways involved in the effect of Nebracetam on MRTF-A induction.

To determine whether the anti-apoptotic effect of nebracetam was associated with ERK pathway, Cells were treated with PD98059 (the inhibitor for ERK1/2 signal pathway) to inhibit ERK pathway in Aβ25-35-treated hippocampus neurons after pretreated with nebracetam.
Mcl-1, Bcl-2 and Arc expressions were determined by western blot. As shown in (Figure 6), Mcl-1, Bcl-2 and Arc expressions were decreased obviously by Aβ25-35. However, preincubate with nebracetam upregulated the expressions of Mcl-1, Bcl-2 and Arc in a concentration manner (*p<0.05 or **p<0.01), while at the presence of PD98059, the expression of Mcl-1, Bcl-2 and Arc was slightly lower than those in control.
control group. As for the cells were co-treated nebracetam (0.05 mM, 0.2 mM, 0.8 mM) with PD98059, the increasing effect of nebracetam on Mcl-1, Bcl-2 and Arc expression was inhibited (#p<0.05 or ##p<0.01).

**Discussion**

ß-amyloid protein (Aß) accumulation has been implicated as one of the most important pathogenic traits of Alzheimer’s disease [21]. Evidence in vivo and vitro experiments showed that Aß induced inflammatory response, oxidative stress, neuronal apoptosis, and modifying neuronal synapses resulting in neurodegeneration and cognitive dysfunctions [22]. In this study, hippocampus neurons injury induced by Aß25-35 has been used as AD model to investigate the protective effects of nebracetam and its possible mechanism [23]. It was found that the average apoptotic rate was obviously increased after cells were treated with Aß25-35. Nebracetam inhibited apoptosis in hippocampus neurons exposed to Aß25-35 by flow cytometry. Confocal images indicated an increase in synaptic loss treated with...
Aβ25-35, while nebracetam significantly suppressed synaptic loss induced by Aβ25-35 in a dose-dependent manner. Our experiment also demonstrated that the neuroprotection of nebracetam is related to enhancing the transactivity and protein expression of Mcl-1, Bcl-2 and Arc genes.

MRTF-A, an important expressed transcription factor that plays an important role in cellular growth, and cardiac development, and mostly found in neurons such as cerebral cortex, hippocampus, etc. [24,25], is a co-factor of SRF necessary for gene expression, plays a vital role in neuronal survival [26]. Previous studies have indicated that MRTF-A elevates SRF-driven transcription and enhances its stimulation by brain-derived neurotrophic factor (BDNF) in primary cortical neurons, which can regulate SRF and hence contributes to specific gene expression. In order to investigate nebracetam promoting expressions of Bcl-2, Mcl-1 and Arc involved MRTF-A manner, the cells were transfected with an expression plasmid for MRTF-A siRNA (0.5 μg), the expressions of Mcl-1, Bcl-2 and Arc were determined by RT-PCR and Western blot. The results showed that the upregulation of Mcl-1, Bcl-2 and Arc expression by nebracetam were inhibited by MRTF-A siRNA, providing the evidence that MRTF-A was involved in the upregulation of Mcl-1, Bcl-2 and Arc expression by nebracetam.

How do nebracetam upregulate expressions of Mcl-1, Bcl-2 and Arc by MRTF-A manner? Several studies have showed that MRTF-A can regulate SRF through binding to it [27]. This complex activates target gene expression, requiring MRTF-A contacting a definite DNA sequence flanking the CArG box [28]. The promoter regions of Mcl-1, Bcl-2 and Arc contained confirmed transcription factor binding sites including the CArG box [CC (A/T) 6GG] DNA sequences. In order to investigate whether nebracetam promoting expressions of Mcl-1, Bcl-2 and Arc is correlated to MRTF-A enhancing CArG box of their promoters, we cloned fragments of murine Mcl-1, Bcl-2 and Arc gene whose promoter contains the CArG box and their mutants in the site of CArG box. The results showed that MRTF-A siRNA inhibited the up-regulation of nebracetam on the transcription activities of Mcl-1, Bcl-2 and Arc genes. On the other hand, it showed no significantly difference on these luciferase reporter gene activities at the presence of these gene mutants. Our data strongly confirmed the above hypothesis that the up-regulation of Mcl-1, Bcl-2 and Arc expression by nebracetam is correlated to MRTF-A enhancing CArG box in their promoters.

ERK1/2 as an important approach to AD, the pathway is being widely studied [29-32]. Previous studies have demonstrated that this pathway can be involved in the regulation of synaptic structure and function [31,33]. Activation of ERK1/2 pathway plays a key role in the regulation of functional and structural synaptic plasticity [34]. It is hypothesized that the up-regulation of nebracetam on Mcl-1, Bcl-
2 and Arc modulation may mediated through ERK1/2 pathway. To determine whether nebracetam mediates its protective effect against 
AJβ25-35-induced neurotoxicity in hippocampus neurons through the 
activation of the ERK1/2 pathway, we tested the influence of the ERK1/2 
pathway inhibitor, PD98059. The result showed that upregulation of 
Mcl-1, Bcl-2 and Arc expressions in nebracetam-treated group was 
inhibited by PD98059, which suggested that the effect of nebracetam 
on hippocampus neurons injury induced by AJβ25-35 through ERK1/2 
signal pathway.

In short, our study described that nebracetam prevent hippocampus 
nurons injury induced by AJβ25-35 via the mechanisms of inhibiting 
apoptosis and promoting synaptic plasticity via MRTF-A-mediated 
transactivation in ERK1/2-dependent pathway. The results showed 
that nebracetam could directly prevent the apoptosis of hippocampus 
nurons and the loss of synaptic plasticity by up regulating of Mcl-1, 
Bcl-2 and Arc expressions. Furthermore, the expressions of Mcl-1, Bcl- 
2, and Arc genes regulated by nebracetam were correlated to MRTF-A 
manner which may be related to its triggering on the CaRGe box of their 
promoters. Meanwhile, these neuroprotective effects were associated 
with ERK1/2 signaling pathway.

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