available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Astrocyte-conditioned medium protecting hippocampal neurons in primary cultures against corticosterone-induced damages via PI3-K/Akt signal pathway

Ze-Hua Zhu[a], *Ru Yang*[b], *Xin Fu*[a], *Yan-Qing Wang*[a], *Gen-Cheng Wu*[a,*]

[a]*Department of Integrative Medicine and Neurobiology, Institute of Acupuncture Research, Shanghai Medical College, Fudan University, P.O. Box 291 138, Yi Xue Yuan Road, Shanghai 200032, PR China*

[b]*State Key Laboratory of Medical Neurobiology, Shanghai Medical College, Fudan University, 138 Yi-Xue-Yuan Road, Shanghai 200032, PR China*

ARTICLE INFO

Article history:
Accepted 17 July 2006
Available online 23 August 2006

Keywords:
Rat
Astrocyte-conditioned medium
Corticosterone
Hippocampal neuron
TUNEL
Signal pathway

ABSTRACT

Prolonged or excessive exposure to corticosterone leads to neuronal damages in the brain regions, including hippocampus. We reported that astrocyte-conditioned medium (ACM) protected the neurons of the primary hippocampal cultures against the corticosterone-induced damages. Corticosterone added to the cultures resulted in a significant number of TUNEL-positive cells. However, corticosterone-induced TUNEL labeling was suppressed as for ACM-cultured neurons. To delineate the molecular basis underlying the neuroprotection of ACM, we assessed the activation of ERK1/2 and (PI3-K)/Akt signal pathways in response to corticosterone-induced neuronal damages. Western blot test revealed that corticosterone increased the phosphorylation of ERK1/2 and PI3-K/Akt in hippocampal neurons grown in Neurobasal medium supplemented with B27 and 500 μm L-glutamine (NBM^+). Interestingly, the increase of phospho-ERK1/2 and Akt levels was much pronounced and the time course of phosphorylation was altered in ACM, suggesting that both signaling pathways might participate in ACM protection. Furthermore, the selective inhibitor of Akt, rather than ERK1/2, blocked the neuroprotective activity against corticosterone in ACM-cultured neurons. In summary, our data showed that ACM had a potent neuroprotective effect in cultured neurons. PI3-K/Akt signal pathway, but not ERK1/2, was involved in the protective activity against the corticosterone-induced damages.

* *Corresponding author*. Fax: +86 21 54237023.
E-mail address: gcwu@shmu.edu.cn (G.-C. Wu).

0006-8993/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2006.07.070

Abbreviations:
ACM, astrocyte-conditioned medium
BSA, bovine serum albumin
CNS, central nervous system
CORT, corticosterone
DMEM, Dulbecco's modified Eagle's medium
DAPI, 4′,6′-diamidino-2-phenylindole dihydrochloride
ERK1/2, extracellular signal-regulated kinases 1 and 2
FBS, fetal bovine serum
LY294002, 2-(4-morpholino)-8-phenyl-4*H*-1-benzopyran-4-one
NTFs, neurotrophic factors
PI3-K, phosphatidylinositide 3-kinase
U0126, 1,4-diamino-2,3-dicyano-1,4-bis (2-aminophenylthio)
TUNEL, Terminal transferase-mediated dUTP nick-labeling

1. Introduction

Astrocyte-conditioned medium (ACM) is a nutritious liquid for neuronal growth containing soluble factors released from astrocytes. Like a layer of astrocytes wherein neurons are cultured, ACM constructs an environment of certain glial–neuron interaction. Experimentally, ACM has been used to study the role of astrocytes in neuroprotection and astrocyte–neuronal interactions (Mahesh et al., 2006). Glial cells outnumber neurons by 9:1 and thus are of the most abundant cell type in the brain, and they interact with neurons in a more complex way than simply by providing structural, metabolic and trophic support. Astrocytes, which are probably the most interesting neuronal partners, support proliferation, survival and maturation of the developing neurons and neuroblasts that are already committed to the neuronal lineage (de Sampaio e Spohr et al., 2002). And also astrocytes participate in the regulation of the ionic environment and take up and release several neurotransmitters, thus modulating neuronal activities by monitoring the concentration of such substances at the synaptic cleft (Laming et al., 2000). Astrocytes are bi-directional communication partners in the central nervous system (CNS), receiving signals from neighboring neurons and responding by releasing neuroactive substances. Neurons cultured on a layer of astrocytes obtained from different brain regions develop different phenotypes. Similar data could be obtained by culturing neurons in the presence of medium previously conditioned by different astroglial populations (Hatten et al., 1986). Although the mechanism and its implications of interactions between glial components and neurons are not fully understood, the soluble factors from ACM present signaling molecules in triggering, influencing and guiding the ultimate destiny of neuronal development and function.

Glucocorticoids can be apoptotic, which is one form of neuronal damages. As adrenal hormones released under stress, glucocorticoids function to maintain or restore homeostasis through energy mobilization and regulate immune responses to infection. Glucocorticoids target the tissues in the sense organs, internal organs, and in the peripheral and CNS with a high concentration of hippocampal glucocorticoid receptors (Pryce et al., 2005). Prolonged elevations of glucocorticoids occur in numerous disorders, such as anorexia nervosa (Seed et al., 2000), Cushing's disease (Starkman et al., 1992), suggesting that the overloading of glucocorticoids is one cause of neuronal damages, particularly in the hippocampus. For example, both stress and physiologic elevation of glucocorticoids levels impair the ability of hippocampal neurons to survive after epileptic seizure in vivo (Stein-Behrens et al., 1994). Similar effects are also observed in primary hippocampal cultured neurons in vitro (Goodman et al., 1996).

Considering the potent neuroprotective action of astrocytes and the deleterious effects of glucocorticoids particularly on hippocampal neurons, the present study was undertaken to compare corticosterone (CORT)-induced damages in ACM- and NBM^+-cultured hippocampal neurons with a view to evaluating neuroprotective effects of ACM. To further investigate molecular mechanisms involved in acquired resistance against CORT-induced damages in ACM-cultured neurons, we examined the activation of two signal pathways, i.e. extracellular signal-regulated kinases 1 and 2 (ERK1/2) and phosphoinositide 3-kinase (PI3-K)/Akt. Both are known to be important in mediating neuronal survival (Yuan et al., 2003).

2. Results

2.1. *TUNEL study*

We performed TUNEL staining so as to establish an assay to identify the neuroprotective effects of ACM against neuronal damages induced by CORT. We compared the TUNEL-positive cells grown in astrocyte-conditioned medium (ACM) with

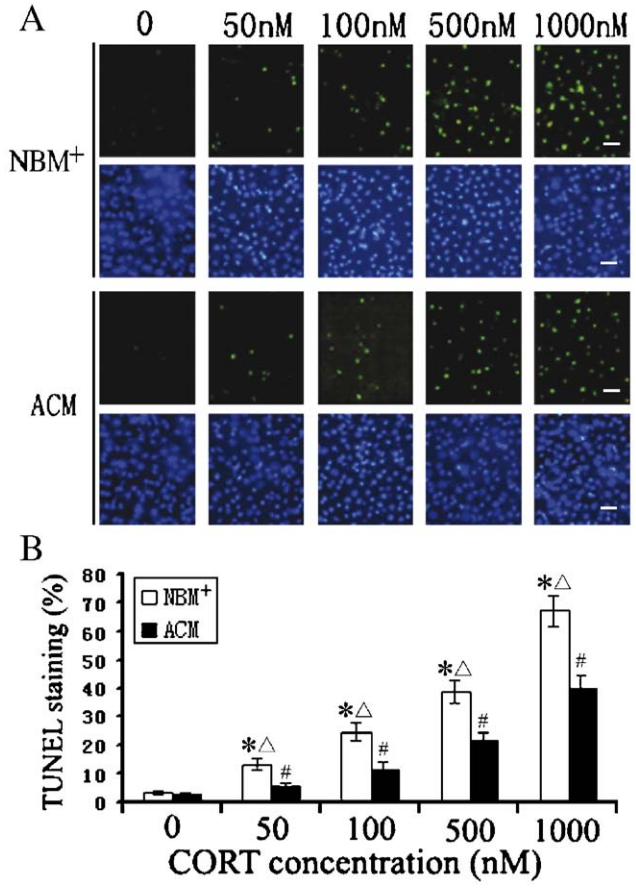

Fig. 1 – CORT-induced concentration-dependent TUNEL-positive cells (green) in ACM- and NBM$^+$-cultured hippocampal neurons. The cells were treated with CORT for 24 h. The nuclei of the cells were also stained with DAPI (blue) (A). The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei. Each bar represented the mean ± SEM of three independent experiments (B). Without CORT treatments were used as respective controls. *$P<0.05$ indicated statistical significance compared with control (non-CORT-treated cultures) in NBM$^+$-cultured hippocampal neurons. $^{\#}P<0.05$ vs that with ACM. $^{\triangle}P<0.05$ indicated statistical significance compared with ACM-cultured hippocampal neurons treated with CORT at same concentrations. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

those in a Neurobasal medium (NBM) supplemented with B27 and 500 μm L-glutamine (NBM$^+$) during CORT treatment. CORT induced the production of TUNEL-positive cells in a concentration-dependent manner in ACM- or NBM$^+$-cultured hippocampal neurons (Figs. 1A and B) and significant TUNEL labeling at a concentration as low as 50 nM. Its higher concentrations (up to 1 μM) brought about even greater TUNEL labeling with 500 nM, which produced a robust effect. We thus chose 500 nM for subsequent studies. Interestingly, the number of TUNEL-positive cells caused by corticosterone 24 h following the treatment (Fig. 1B) more significantly decreased in the ACM- than in the NBM$^+$-cultured hippocampal neurons. The analysis demonstrated that approximately 42% of hippocampal neurons cultured in NBM$^+$ were TUNEL-positive with 500 nM CORT. However, in response to the same concentration of CORT, the number of TUNEL-positive cells was significantly reduced to 21% in the ACM-cultured hippocampal neurons, suggesting a significantly enhanced neuroprotective effect against CORT.

2.2. *Effect of CORT on ERK1/2 phosphorylation in cultured hippocampal neurons*

In order to determine whether ERK1/2 signal pathway was activated following CORT-induced insults in hippocampal neurons, phosphorylation of ERK1/2 (i.e. the phospho-ERK1/2 levels) was assessed. CORT induced a 1.5-fold increase in phospho-ERK1/2 levels at the point of 6 h and a significant 3-fold in phospho-ERK1/2 levels at the point of 12 h following the treatment in NBM$^+$-cultured hippocampal neurons in comparison to the control (Fig. 2). The increase sustained for at least 24 h. Hippocampal neurons cultured in ACM also exhibited an increase in phospho-ERK1/2. However, the magnitude of the increase was not significantly different as in the hippocampal neurons grown in NBM$^+$ in response to

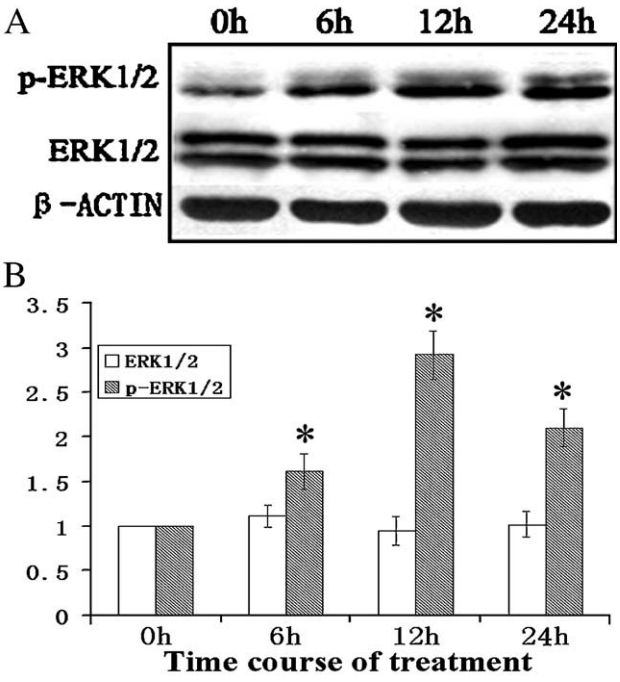

Fig. 2 – Effects of CORT on the levels of phosphorylated ERK1/2 and total ERK in NBM$^+$-cultured hippocampal neurons. Hippocampal neurons were treated with 500 nM of CORT for the indicated periods of time, and the whole cell lysates were collected. Western blots analysis of the phosphorylation of ERK1/2 and total ERK in hippocampal neurons (A). Panel B indicated the relative values of phosphorylated ERK1/2 and total ERK compared with respective controls. Data represented mean ± SEM of at least three independent experiments. Neurons not subjected to CORT were used as controls. *$P<0.05$ indicated statistical significance compared with control.

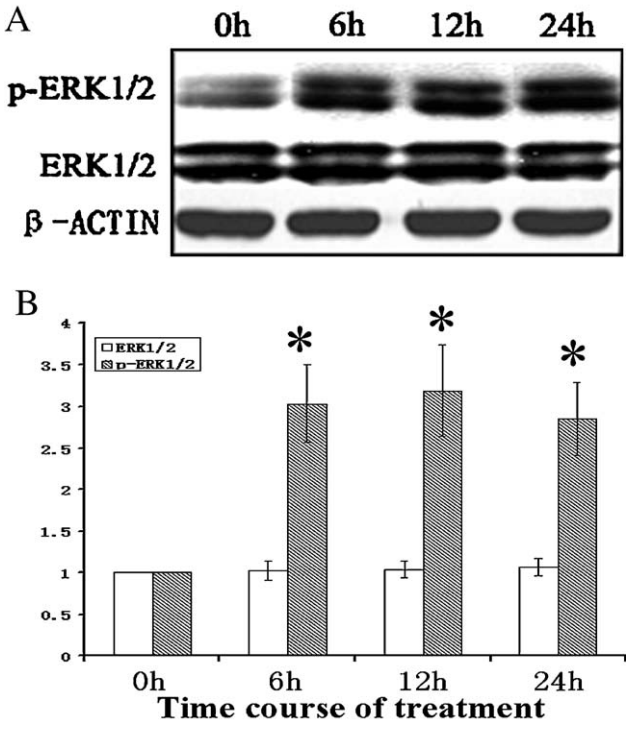

Fig. 3 – Effects of CORT on levels of phosphorylated ERK1/2 and total ERK in ACM-cultured hippocampal neurons. Hippocampal neurons were treated with 500 nM of CORT for the indicated periods of time, and the whole cell lysates were collected. Western blots analysis of the phosphorylation of ERK1/2 and total ERK in hippocampal neurons (A). Panel B indicated the relative values of phosphorylated ERK1/2 and total ERK compared with respective controls. Data represented mean ± SEM of at least three independent experiments. Neurons not subjected to CORT treatment were used as controls. *$P<0.05$ indicated statistical significance compared with control.

CORT treatment (Fig. 3), with the total ERK1/2 not significantly different as in both ACM- and NBM$^+$-cultured hippocampal neurons. The increase in phospho-ERK1/2 levels was due to the increase in activation of ERK1/2 since the total amount of constitutive non-phosphorylated ERK1/2 did not change during the 24 h incubation either in the presence or absence of CORT, or in the ACM and NBM$^+$, which suggested that ERK1/2 signal pathway was activated during the process of the damages to the hippocampal neurons caused by CORT.

2.3. *Effect of CORT on Akt phosphorylation in cultured hippocampal neurons*

The activation of (PI3-K)/Akt signal pathway by CORT in cultured hippocampal neurons was measured by phospho-Akt levels. Western blot test revealed a 3-fold increase of phospho-Akt at 6 h following the treatment with CORT in NBM$^+$-cultured hippocampal neurons, but the levels of phospho-Akt were then reduced to the control levels at 12 h (Fig. 4). Under the ACM-cultured condition, CORT induced a significant 12-fold increase in phospho-Akt levels in cultured hippocampal neurons at 6 h following the treatment in comparison to the control (Fig. 5). The increase in phospho-Akt levels sustained for at least 24 h, while the total non-phosphorylated Akt levels under the experimental conditions remained the same in the control and in the ACM and NBM$^+$ neurons either treated with or without CORT. The results demonstrated that (PI3-K)/Akt signal pathway might play an important role in the anti-apoptotic effect of ACM-cultured hippocampal neurons against CORT effects.

2.4. *Effect of CORT on Bcl-2 expression in cultured hippocampal neurons*

We also examined the expression of Bcl-2, one of the important anti-apoptotic members. Western blot test results revealed that Bcl-2 levels increased significantly in the cultured hippocampal neurons at 6 h following the CORT treatment under the ACM-cultured condition (Figs. 6C, D), which, however, could not be detected in the NBM$^+$-cultured hippocampal neurons (Figs. 6A, B), suggesting that the anti-apoptotic effect against corticosterone was mediated by Bcl-2 in ACM-cultured hippocampal neurons. In this experiment, we used total Akt as an internal control to monitor equal protein loading since total Akt did not change in this model.

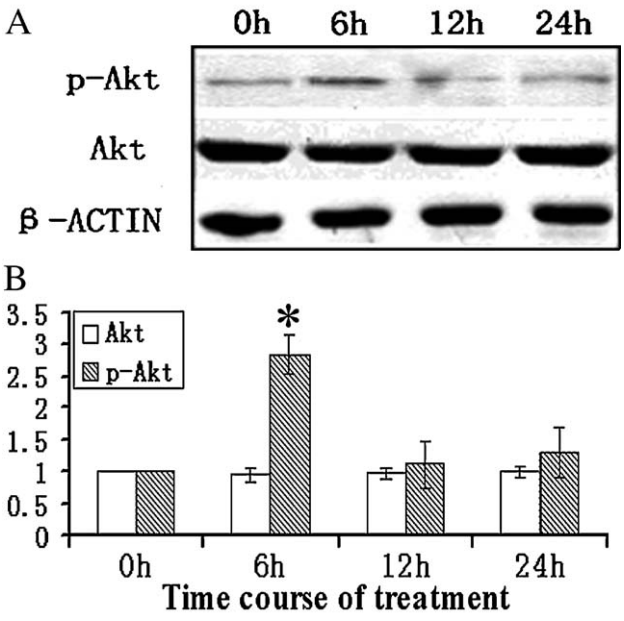

Fig. 4 – Effects of CORT on levels of phosphorylated Akt and total Akt in NBM$^+$-cultured hippocampal neurons. Hippocampal neurons were treated with 500 nM of CORT as indicated periods of time, and the whole cell lysates were collected. Western blots analysis of the phosphorylation of Akt and total Akt in hippocampal neurons (A). Panel B indicated the relative values of phosphorylated Akt and total Akt compared with respective controls. Data represented mean ± SEM of at least three independent experiments. Neurons not subjected to CORT treatment were used as controls. *$P<0.05$ indicated statistical significance compared with control.

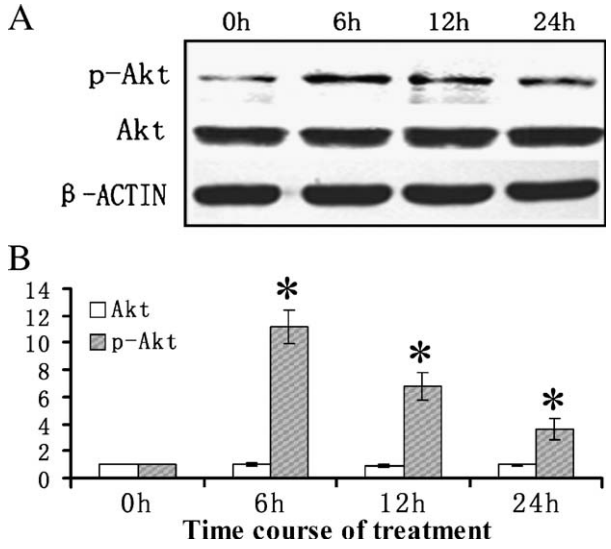

Fig. 5 – Effects of CORT on the levels of phosphorylated Akt and total Akt in ACM-cultured hippocampal neurons. Hippocampal neurons were treated with 500 nM of CORT for the indicated periods of time, and the whole cell lysates were collected. Western blots analysis of the phosphorylation of Akt and total Akt in hippocampal neurons (A). Panel B indicated the relative values of phosphorylated Akt and total Akt compared with respective controls. Data represented mean ± SEM of at least three independent experiments. Neurons not subjected to CORT treatment were used as controls. *$P<0.05$ indicated statistical significance compared with control.

2.5. *Effects of selective inhibitors of ERK1/2 and Akt signal pathways on the changes of CORT-induced TUNEL staining*

To determine whether the activation of either the ERK1/2 pathway or the (PI3-K)/Akt pathway was involved in the neuroprotective activity of ACM against CORT-induced neuronal damages which were assessed using TUNEL staining, an ERK1/2 inhibitor (U0126) and a (PI3-K)/Akt inhibitor (LY294002) were used to prevent the activation of their respective cascade. Western blots test showed that 40 μM U0126 and 10 μM LY294002 blocked effectively the phosphorylation of ERK1/2 and (PI3-K)/Akt respectively in the cultured hippocampal neurons (data not shown). In the NBM^+-cultured hippocampal neurons, both ERK1/2 inhibitor U0126 and (PI3-K)/Akt inhibitor LY294002 significantly enhanced the number of TUNEL-positive cells caused by CORT at 24 h. U0126 (40 μM) and LY294002 (10 μM) themselves had no effects on TUNEL labeling of the neurons (data not shown). The effect of Akt inhibitor was more pronounced than that of ERK1/2 inhibitor. The presence of U0126 failed to enhance CORT-induced TUNEL labeling of the ACM-cultured neurons. However, it was significantly enhanced by Akt inhibitor LY294002 (Fig. 7). Altogether, the results indicated that a (PI3-K)/Akt-dependent mechanism was involved in the neuroprotective activity of ACM.

3. Discussion

In the present study, it was observed that ACM-cultured hippocampal neurons became dramatically resistant to CORT-induced damages. This was consistent with the evidence

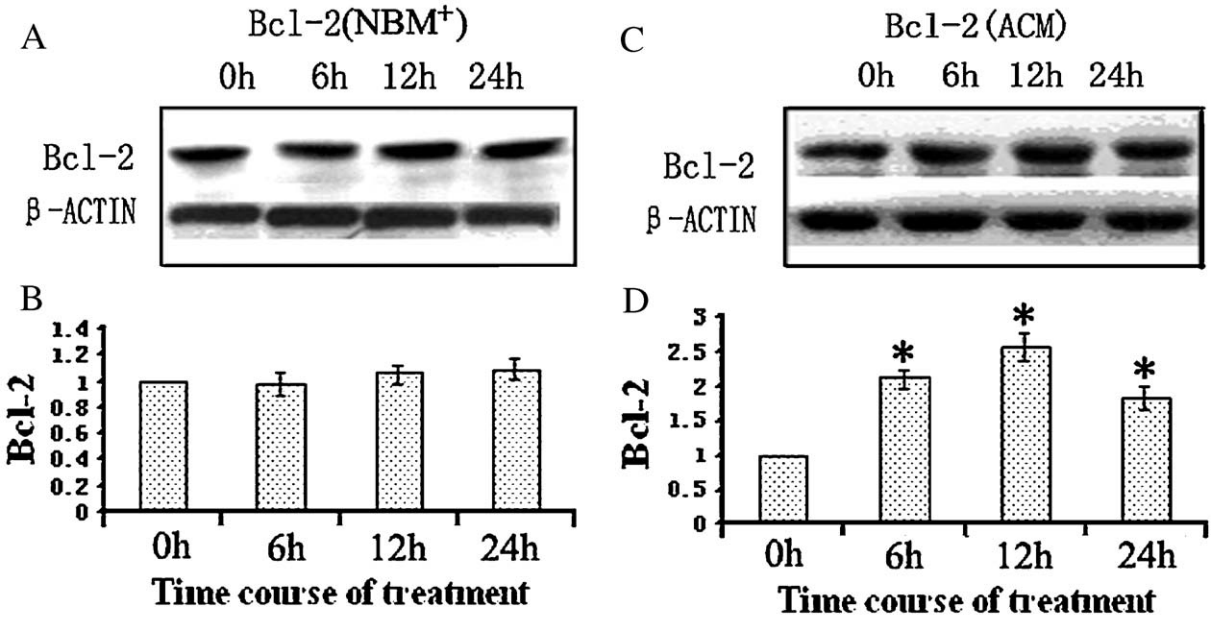

Fig. 6 – Effects of CORT on Bcl-2 expression in ACM- and NBM^+-cultured hippocampal neurons. Hippocampal neurons were treated with 500 nM of CORT for the indicated periods of time, and the whole cell lysates were collected. Panels A and C showed Western blot analysis of Bcl-2 expression in hippocampal neurons. Panels B and D indicated the relative values of Bcl-2 expression compared with control. Data represented mean ± SEM of at least three independent experiments. Neurons untreated with CORT were used as controls. *$P<0.05$ indicated statistical significance compared with control.

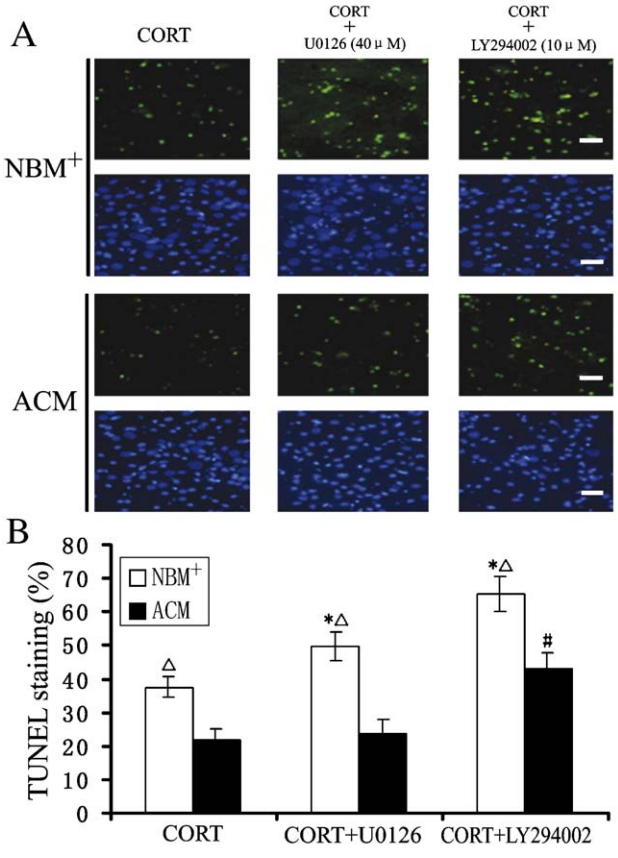

Fig. 7 – Effects of selective inhibitors of ERK1/2 (U0126) and (PI3-K)/Akt (LY294002) signal pathways on CORT-induced TUNEL-positive cells (green) in ACM- and NBM$^+$-cultured hippocampal neurons. Neurons were pretreated with 40 μM U0126 or 10 μM LY294002 for 45 min and subjected to 24 h of 500 nM CORT treatment. Apoptosis of hippocampal neurons was assessed by TUNEL staining (green), the nuclei of the cells were also stained with DAPI (blue) (A). The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei. Each bar represented the mean ± SEM of three independent experiments (B). CORT treatments alone were used as respective controls. $^*P<0.05$ indicated statistical significance compared with CORT control grown in NBM$^+$-cultured hippocampal neurons. $^\#P<0.05$ indicated statistical significance compared with CORT control grown in ACM-cultured hippocampal neurons. $^\triangle P<0.05$ indicated statistical significance compared with ACM-cultured hippocampal neurons for CORT treatment alone, CORT + U0126 and CORT + LY294002 treatment neurons. Scale bar, 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that ACM protected against neuronal injury induced by amyloid-β protein in cultured cortical neurons (Yamamuro et al., 2003). ACM also up-regulated rapidly apolipoprotein (apo) E expression in cultured mouse primary cortical and hippocampal neurons (Harris et al., 2004). Especially, when rat retinal ganglion cells (RGCs) were cultured in ACM, there was an increase in the number of structural synapses (Christopherson et al., 2005). Neuronal damages depend on multiple factors and occur through diverse pathways. Although neurons may die as a normal physiological process during their development or as a pathological process, any approach that could prevent neuronal death would benefit their survival, development and function. ACM was found to be of neuroprotective function in the current work. As reported, astrocytes, rather than neurons, are implicated in the neuronal survival. Previous studies have shown that astrocytes perform a number of well-documented neuroprotective roles such as in buffering toxic raises in glutamate and promoting ions metabolic support for neurons and releasing neurotrophic peptides. The soluble factors released from astrocytes can be neuroprotective, which is in correspondence with the effect of astrocytes themselves. In addition, ACM could be one meaningful medium in studying the actions of astrocytes on neurons. In some future studies, we would focus on soluble factors independent of whole cells, which are easier to handle, detect and calculate, without temporal consideration of the synaptic action between glia and neurons.

The neuroprotection of ACM against CORT casts a light on the explorations of potential cure of CORT-induced neurological disorders. CORT is well known to induce various neuronal deaths under stressful conditions in vivo. For example, CORT can induce neuronal damages and reduce the expression of brain-derived neurotrophic factor (BDNF) mRNA and protein contents in the hippocampus (Nitta et al., 1999b). Moreover, repeated exposure to mild stressors in rats leads to the increase of the circulating levels of CORT, accelerating cellular death in the hippocampal formation (Sapolsky et al., 1984). It has been suggested that increased neuronal death was due to chronic stimulation of CORT receptors, which were particularly abundant in the hippocampus (Wong and Herbert, 2004).

It is likely that multiple mechanisms are involved in glucocorticoid-induced neurotoxicity including impairment of energy metabolism, inhibition of glucose transport, and energy-dependent disruption of neuronal Ca^{2+} regulation. These effects may lead to decreased energy supplies, adenosine 5′-triphosphate (ATP) depletion, failure of Ca^{2+} buffering systems (Karst et al., 2002) and excessive glutamate release leading to glutamate receptor hyperactivity (Huxtable, 1989). Elevated Ca^{2+} levels could then activate Ca^{2+}-dependent proteases, hence promoting the degradation of cytoskeletal proteins and lipases, leading to increased levels of free radicals and resulting in membrane damage (Welch, 1992). Another aspect of the negative action of glucocorticoids on neuronal homeostasis is the disruption of neuronal protective mechanisms such as feedback regulation of Ca^{2+} channel functions (de Leon et al., 1995), induction of stress proteins and release of inhibitory neurotransmitters to block subsequent glutamate release (Dragunow et al., 1985; Huxtable, 1989). It thus seems that glucocorticoids not only can induce direct deleterious effects, but also can impair neuroprotective components of neuronal survival. In this study, CORT induced an apoptotic process in cultured hippocampal neurons by means of a concentration-dependent manner. However, ACM-cultured hippocampal neurons became dramatically resistant to CORT-induced damages.

In the present study, to further understand the mechanisms and pathways involved in acquired resistance against neurotoxitin of CORT in ACM-cultured hippocampal neurons, we investigated the role of MAPK (ERK1/2) and (PI3-K)/Akt signal pathways, both of which are important to neuronal survival (Yuan et al., 2003), in CORT-treated hippocampal neurons under different culture conditions.

Evolutionarily, the components of MAPK are highly conserved in terms of their functions and regulations. They are responsible for transferring of signals from cell-surface receptors (i.e. tyrosine kinase receptors and G-protein-coupled receptors) to the intracellular response machinery. We found out that ERK1/2 signal pathways were activated in the hippocampal neurons cultured in ACM and NBM^+ following the treatment with CORT. This was, as expected, a response to external damages. But the magnitude of the increase of ERK1/2 phosphorylation was not significantly different between NBM^+- and ACM-cultured hippocampal neurons in response to CORT treatment. Interestingly, under the ACM-cultured condition, a significant enhancement of activation of (PI3-K)/Akt signal pathway was detected in the neurons in response to CORT damages, the time course of activation of (PI3-K)/Akt signal pathway was altered, and CORT induced significantly a 12-fold increase in phospho-Akt levels in the neurons at 6 h following the treatment in comparison to the control. The increase sustained for at least 24 h, but the levels just increased at 6 h and then declined to the control levels at 12 h following the treatment with CORT in the neurons under the condition of NBM^+-cultured.

The ERK1/2 is serine/threonine kinases, which are closely related in terms of their functions. They are activated via phosphorylation of a threonine and a tyrosine residue by MEK1/2. Activated ERK1/2 would phosphorylate their specific substrates and activate downstream signal pathways related to neural survival, i.e. the 90-kDa ribosomal protein S6 kinase (p90 RSK) dependent phosphorylation of the pro-apoptotic Bcl-2 family protein Bad and Bim and phosphorylation of cAMP response element binding protein (CREB). Phosphorylation of CREB induces the expression of anti-apoptotic members of the Bcl-2 family, i.e. Bcl-2 and bag-1 (Riccio et al., 1999a). ERK1/2 may also inhibit neural death via the inhibition of a pro-apoptotic kinase GSK3β (Hetman et al., 2002). ERK1/2 plays a protective role in CORT-induced injuries to the NBM^+-cultured hippocampal neurons. However, the neurotoxin of CORT on the ACM-cultured hippocampal neurons was not affected by ERK1/2 inhibitor U0126, demonstrating that ERK1/2 pathway was not primarily responsible for their protection against CORT under the condition of ACM-cultured.

In opposition to ERK1/2, the involvement of (PI3-K)/Akt signal pathway in neuronal survival occurs via different mechanisms. Akt targets several key proteins so that they keep neurons alive, such as apoptosis regulators and transcription factors. For example, Bad is a pro-apoptotic member of the Bcl-2 family, which in its unphosphorylated form can bind to Bcl-x_L, thus blocking survival. But the activation of Akt induces the phosphorylation of Bad and promotes its interaction with the chaperone protein 14-3-3, which sequesters Bad in the cytoplasm and inhibits Bad's pro-apoptotic activity (Datta et al., 1997). Akt has been shown to affect, directly or indirectly, three transcription factor families: Forkhead, CREB and NF-κB, all of which are involved in regulating cell survival, and whereas the phosphorylation of Forkhead family members by Akt negatively regulates death-promoting signals (Brunet et al., 1999), the phosphorylation of CREB and IkB kinase (IKK) stimulates survival pathways (Kane et al., 1999; Riccio et al., 1999b). Because CREB is known to activate transcription of Bcl-2, it can stimulate cell survival directly (Middleton et al., 2000). Thus, although there is a divergence in the survival pathways downstream after activation, both the (PI3-K)/Akt and ERK1/2 pathways converge on the same set of proteins, Bad and CREB, to induce survival genes expression. Bcl-2 has a crucial role in the maintenance of neuronal survival. In the present study, Bcl-2 levels remained the same in NBM^+- and ACM-cultured neurons not exposed to CORT. Interestingly, Bcl-2 increased significantly following CORT treatment in the ACM-cultured ones, but not in the NBM^+-cultured ones. Although it is speculated that CORT-induced apoptosis may be due to the inhibition of Bcl-2 expression in hippocampal neuronal cultures, the effect of CORT on the modulation of Bcl-2 proteins must be discussed in the context of cell types and cultures used (Wassim et al., 2004). It is reasonable to conclude that the resistance increase against CORT in ACM-cultured neurons may be due to the increase in Bcl-2. But further explorations are still needed. In the future studies, we intend to assess the expression of anti-apoptotic and pro-apoptotic Bcl-2 family proteins in NBM^+ and ACM cultures exposed to CORT and the relation between the expression of Bcl-2 family proteins and MAPK (ERK1/2) activation or (PI3-K)/Akt signal pathways.

When hippocampal neurons were cultured in the ACM, the (PI3-K)/Akt signal pathway was activated and their death was suppressed against the neurotoxin of CORT. However, the mechanism was still unclear. A possible mechanism might involve its receptors activated by unidentified astrocyte-derived neurotrophic factors (NTFs), which protected the neurons or induced their enhanced resistance against CORT damages. Astrocytes can provide neurotrophic factors and enhance neuronal survival. NTFs, such as insulin-like growth factor 1 (IGF-1), never growth factor (NGF), fibroblast growth factor 2 (FGF2) and neurotrophin 3 (NT3), perform a critical role in neuronal survival, outgrowth of processes and differentiation during development. For example, IGF-1 has been shown to be capable of enhancing the resistance of hippocampal neurons against CORT (Nitta et al., 2004). NGF binding to its receptor tyrosine kinase TrkA activates a host of prosurvival proteins such as PI3 kinase and Akt, which regulate apoptosis by inhibiting pro-apoptotic proteins such as Bad and Forkhead and by activating prosurvival proteins such as CREB and NF-κB (Yuan and Yankner, 2000), directly or indirectly regulate the core apoptotic machinery. It is reasonable to hypothesize that neurotrophic factors of astrocytes secreted directly or indirectly activate ERK1/2 and (PI3-K)/Akt signal pathways. Furthermore, under the condition of ACM-cultured, the way the signal pathways respond to CORT in the neurons changed, suggesting that the factors from the astrocytes might bind to the selective receptors on the neurons, causing a differential effect on the activation pattern of two signal pathways. However, further explorations are still needed; we will address which component within the soluble factors from astrocytes is the key to mediating the anti-apoptotic actions against CORT.

Altogether, the present study demonstrated that both ERK1/2 and (PI3-K)/Akt signal pathways were activated in hippocampal neurons against CORT under the ACM-cultured condition. The inhibition of (PI3-K)/Akt by LY294002 increased the CORT-induced TUNEL labeling. However, the ERK1/2 inhibitor (U0126) failed to influence TUNEL labeling of the ACM-cultured neurons, namely, (PI3-K)/Akt pathway, but ERK1/2 was not involved in enhanced resistance against CORT neurotoxin in ACM-cultured neurons. The neuroprotective effect of (PI3-K)/Akt pathway was probably mediated by promoting Bcl-2 expression, and the neuronal cells might act via selective anti-apoptotic mechanisms against damages under the ACM conditions.

4. Experimental procedures

4.1. *Preparation of hippocampal neurons cultures*

Animal care was conducted according to protocols and guidelines approved by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publications No. 8023, revised 1978). Cultured hippocampal neurons were from fetuses (embryonic Days 17–18) obtained from pregnant Sprague–Dawley rats (Experimental Animal Center, Shanghai Medical College of Fudan University, China) as described previously (Nitta et al., 1999a). In brief, fetuses were removed by caesarean section under pentobarbital anesthesia (50 mg/kg). Dissected hippocampi were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin (Gibco BRL, Grand Island, NY) for 20 min at 37 °C and triturated with a fire-polished Pasteur pipette to dissociate the tissue into single cells. After centrifugation (900×*g*, 3 min), the cell pellet was resuspended in Dulbecco's modified Eagle's minimum essential medium (DMEM, Gibco, Grand Island, NY). Neurons were plated at a density of 2×10^6 to 2.5×10^6 cells/ml in 10 cm culture dishes [coated with poly-L-lysine (molecular weight 30,000–70,000, 1 mg/ml; Sigma, St. Louis, MO, USA)] in DMEM containing 10% fetal calf serum. After a 24 h culture period, the medium was changed to Neurobasal medium (NBM) (Gibco BRL, Grand Island, NY) supplemented with B27 (Invitrogen Corp.) and 500 μm L-glutamine (NBM^+) (Gibco BRL, Grand Island, NY).

4.2. *Preparation of astrocytes and ACM*

Hippocampal astrocyte-enriched cultures were prepared from 1-day-old SD rats as described (Chou, 1998). Hippocampi were dissected and digested with trypsin, and the resultant dissociated cells were suspended in DMEM containing 10% fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY) followed by inoculation into 10 cm culture dishes. After 3 days in culture, cells were re-fed with fresh 10% FBS/DMEM and maintained at 37 °C for an additional 3 days. At this time, cells were dissociated with trypsin, resuspended in 10% FBS/DMEM and lastly plated in culture vessels. These cultures were grown for 7–8 days prior to use. Cultures grown by this method contain approximately 90–95% astrocytes as determined by immunochemical staining with an antibody against glial fibrillary acidic protein (GFAP; Santa Cruz, California, USA), a specific marker for astrocytes. For preparation of ACM, confluent cultures of astrocytes in 10 cm dishes were washed three times in PBS and fed with 10 ml hippocampal neurons medium (without CNTF, BDNF or forskolin). ACM was harvested after 4–6 days of conditioning and filtered through a 0.2 μm syringe filter. Hippocampal neurons were cultured for 4 days to allow robust process outgrowth and then cultured with ACM for an additional 6 days.

4.3. *Treatment of cells*

In the experiments, hippocampal neurons grown in astrocyte-conditioned medium (ACM) or Neurobasal medium (NBM) supplemented with B27 and 500 μm L-glutamine (NBM^+) were allowed to grow for 10 days before treatment. In the ACM-cultured experiments, hippocampal neurons were cultured for 4 days to allow robust process outgrowth and then cultured with ACM for an additional 6 days in culture dishes before treatment. CORT (Sigma, St. Louis, MO, USA) was dissolved initially in ethanol as a stock solution and then in culture media (final concentration of ethanol was 0.1%). U0126 [1,4-diamino-2, 3-dicyano-1,4-bis (2-aminophenylthio); (Beverly, MA, USA)] (40 μM) and LY294002 [2-(4-morpholino)-8-phenyl-4*H*-1-benzopyran-4-one; (Beverly, MA, USA)] (10 μM) were used as specific ERK1/2 and (PI3-K)/Akt inhibitors, which block ERK1/2 and (PI3-K)/Akt signal pathways, respectively. To reveal effects of these inhibitors against CORT-induced cell death, all cultures were pretreated with U0126 [40 mM in dimethyl sulfoxide (DMSO) as a stock] or LY294002 (10 mM in DMSO as a stock) for 45 min before addition of CORT. These inhibitors were maintained in the media throughout the experiment. Only the respective vehicles were used in the controls.

4.4. *Immunoblotting of ERK1/2, Akt and Bcl-2*

After treatments, cells were washed three times with ice-cold PBS and lysed with Lysis buffer [20 mM Tris, 1 mM EDTA, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml pepstatin and 1 μg/ml leupeptin] 15 min. Lysates were centrifuged in 4 °C at 14,000×*g* for 15 min. Protein concentrations were determined with BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots of the protein (40 μg) were loaded per well and separated on a 12% sodium dodecyl sulfate polyacrylamide gel. The separated proteins were electrophoretically transferred to a PVDF membrane (Bio-Rad Laboratories, Hercules, CA, USA). Subsequently, membranes were incubated with 5% nonfat dried milk in TBST [20 mM Tris-buffered saline (pH 7.5) containing 0.1% Tween-20] at room temperature for 1 h to block nonspecific binding. For detection of designated proteins, the membranes were incubated with primary antibodies in TBST containing 5% BSA overnight at 4 °C. The antibodies were diluted at 1:1000 for ERK1/2 (San Diego, CA, USA), phospho-ERK1/2 (San Diego, CA, USA), Akt (Santa Cruz, California, USA) and phospho-Akt (San Diego, CA, USA) and at 1:8000 for Bcl-2 (Santa Cruz, California, USA). Membranes were washed with TBST and incubated for 1 h with horseradish-peroxidase (HRP)-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) (1:5000 dilution in TBST). Enhanced chemiluminescence method (Amersham

Pharmacia Biotech, UK) was used to visualize immune complexes on the membrane. The intensity of the selected bands was captured and analyzed using GeneSnap Image Analysis Software (Syngene, UK).

4.5. *TUNEL assay*

Terminal transferase-mediated dUTP nick-labeling (TUNEL) was performed to detect cells undergoing apoptosis. An In Situ Apoptosis Detection Kit (Roche, Indianapolis, USA) was employed. For the TUNEL analysis, the cells were fixed with 4% paraformaldehyde in PBS for 20 min at 4 °C and subjected to permeabilization for 20 min at room temperature with 0.1% sodium citrate containing 0.1% Triton X-100. The fixed and permeabilized hippocampal neurons were labeled with the TUNEL reaction mixture for 60 min at 37 °C. The nuclei of these hippocampal neurons were counter-stained with 4′,6′-diamino-2-phenylindole (DAPI). Fluorescent-labeled DNA, indicating DNA fragmentation, was analyzed by using a laser scanning confocal microscope (Leica TCS-NT). The percentage of TUNEL labeling was expressed as the number of TUNEL-positive nuclei divided by the total number of nuclei stained with DAPI.

4.6. *Statistical analysis*

Band intensities of Western blots were quantified by a densitometer and expressed as relative values to the controls. All values were expressed as means ± SEM. The SPSS statistical package (SPSS Inc., Chicago, IL, USA) was used for analysis, with ANOVA and Tukey's post hoc test at a significance level of 0.05.

Acknowledgments

We thank members of Wu laboratory for their technical assistance and provision of primary cell culture and their valuable suggestions on the manuscript. This study, a part of 973 Project, was supported by a grant from National Basic Research Program of China (no. 2005CB523306).

REFERENCES

Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96 (6), 857–868.

Chou, Y.C., 1998. Corticosterone exacerbates cyanide-induced cell death in hippocampal cultures: role of astrocytes. Neurochem. Int. 32 (3), 219–226.

Christopherson, K.S., Ullian, E.M., Stokes, C.C., Mullowney, C.E., Hell, J.W., Agah, A., Lawler, J., Mosher, D.F., Bornstein, P., Barres, B.A., 2005. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell 120 (3), 421–433.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M.E., 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell 91 (2), 231–241.

de Leon, M., Wang, Y., Jones, L., Perez-Reyes, E., Wei, X., Soong, T.W., Snutch, T.P., Yue, D.T., 1995. Essential Ca(2+)-binding motif for Ca(2+)-sensitive inactivation of L-type Ca2+ channels. Science 270 (5241), 1502–1506.

de Sampaio e Spohr, T.C., Martinez, R., da Silva, E.F., Neto, V.M., Gomes, F.C., 2002. Neuro–glia interaction effects on GFAP gene: a novel role for transforming growth factor-beta1. Eur. J. Neurosci. 16 (11), 2059–2069.

Dragunow, M., Goddard, G.V., Laverty, R., 1985. Is adenosine an endogenous anticonvulsant? Epilepsia 26 (5), 480–487.

Goodman, Y., Bruce, A.J., Cheng, B., Mattson, M.P., 1996. Estrogens attenuate and corticosterone exacerbates excitotoxicity, oxidative injury, and amyloid beta-peptide toxicity in hippocampal neurons. J. Neurochem. 66 (5), 1836–1844.

Harris, F.M., Tesseur, I., Brecht, W.J., Xu, Q., Mullendorff, K., Chang, S., Wyss-Coray, T., Mahley, R.W., Huang, Y., 2004. Astroglial regulation of apolipoprotein E expression in neuronal cells. Implications for Alzheimer's disease. J. Biol. Chem. 279 (5), 3862–3868.

Hatten, M.E., Liem, R.K., Mason, C.A., 1986. Weaver mouse cerebellar granule neurons fail to migrate on wild-type astroglial processes in vitro. J. Neurosci. 6 (9), 2676–2683.

Hetman, M., Hsuan, S.L., Habas, A., Higgins, M.J., Xia, Z., 2002. ERK1/2 antagonizes glycogen synthase kinase-3beta-induced apoptosis in cortical neurons. J. Biol. Chem. 277 (51), 49577–49584.

Huxtable, R.J., 1989. Taurine in the central nervous system and the mammalian actions of taurine. Prog. Neurobiol. 32 (6), 471–533.

Kane, L.P., Shapiro, V.S., Stokoe, D., Weiss, A., 1999. Induction of NF-kappaB by the Akt/PKB kinase. Curr. Biol. 9 (11), 601–604.

Karst, H., Nair, S., Velzing, E., Rumpff-van Essen, L., Slagter, E., Shinnick-Gallagher, P., Joels, M., 2002. Glucocorticoids alter calcium conductances and calcium channel subunit expression in basolateral amygdala neurons. Eur. J. Neurosci. 16 (6), 1083–1089.

Laming, P.R., Kimelberg, H., Robinson, S., Salm, A., Hawrylak, N., Muller, C., Roots, B., Ng, K., 2000. Neuronal–glial interactions and behaviour. Neurosci. Biobehav. Rev. 24 (3), 295–340.

Mahesh, V.B., Dhandapani, K.M., Brann, D.W., 2006. Role of astrocytes in reproduction and neuroprotection. Mol. Cell. Endocrinol. 246 (1–2), 1–9.

Middleton, G., Cox, S.W., Korsmeyer, S., Davies, A.M., 2000. Differences in bcl-2- and bax-independent function in regulating apoptosis in sensory neuron populations. Eur. J. Neurosci. 12 (3), 819–827.

Nitta, A., Ito, M., Fukumitsu, H., Ohmiya, M., Ito, H., Sometani, A., Nomoto, H., Furukawa, Y., Furukawa, S., 1999a. 4-Methylcatechol increases brain-derived neurotrophic factor content and mRNA expression in cultured brain cells and in rat brain in vivo. J. Pharmacol. Exp. Ther. 291 (3), 1276–1283.

Nitta, A., Ohmiya, M., Sometani, A., Itoh, M., Nomoto, H., Furukawa, Y., Furukawa, S., 1999b. Brain-derived neurotrophic factor prevents neuronal cell death induced by corticosterone. J. Neurosci. Res. 57 (2), 227–235.

Nitta, A., Zheng, W.H., Quirion, R., 2004. Insulin-like growth factor 1 prevents neuronal cell death induced by corticosterone through activation of the PI3k/Akt pathway. J. Neurosci. Res. 76 (1), 98–103.

Pryce, C.R., Feldon, J., Fuchs, E., Knuesel, I., Oertle, T., Sengstag, C., Spengler, M., Weber, E., Weston, A., Jongen-Relo, A., 2005. Postnatal ontogeny of hippocampal expression of the mineralocorticoid and glucocorticoid receptors in the common marmoset monkey. Eur. J. Neurosci. 21 (6), 1521–1535.

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A., Ginty, D.D., 1999a. Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286 (5448), 2358–2361.

Riccio, A., Ahn, S., Davenport, C.M., Blendy, J.A., Ginty, D.D., 1999b. Mediation by a CREB family transcription factor of

NGF-dependent survival of sympathetic neurons. Science 286 (5448), 2358–2361.
Sapolsky, R.M., Krey, L.C., McEwen, B.S., 1984. Glucocorticoid-sensitive hippocampal neurons are involved in terminating the adrenocortical stress response. Proc. Natl. Acad. Sci. U. S. A. 81 (19), 6174–6177.
Seed, J.A., Dixon, R.A., McCluskey, S.E., Young, A.H., 2000. Basal activity of the hypothalamic–pituitary–adrenal axis and cognitive function in anorexia nervosa. Eur. Arch. Psychiatry Clin. Neurosci. 250 (1), 11–15.
Starkman, M.N., Gebarski, S.S., Berent, S., Schteingart, D.E., 1992. Hippocampal formation volume, memory dysfunction, and cortisol levels in patients with Cushing's syndrome. Biol. Psychiatry 32 (9), 756–765.
Stein-Behrens, B., Mattson, M.P., Chang, I., Yeh, M., Sapolsky, R., 1994. Stress exacerbates neuron loss and cytoskeletal pathology in the hippocampus. J. Neurosci. 14 (9), 5373–5380.
Wassim, Y.A., Ohannes, K., Maroun, M.A., 2004. On the link between Bcl-2 family proteins and glucocorticoid-induced apoptosis. J. Leukocyte Biol. 76 (1), 7–14.
Welch, W.J., 1992. Mammalian stress response: cell physiology, structure/function of stress proteins, and implications for medicine and disease. Physiol. Rev. 72 (4), 1063–1081.
Wong, E.Y., Herbert, J., 2004. The corticoid environment: a determining factor for neural progenitors' survival in the adult hippocampus. Eur. J. Neurosci. 20 (10), 2491–2498.
Yamamuro, A., Ago, Y., Takuma, K., Maeda, S., Sakai, Y., Baba, A., Matsuda, T., 2003. Possible involvement of astrocytes in neuroprotection by the cognitive enhancer T-588. Neurochem. Res. 28 (12), 1779–1783.
Yuan, J., Yankner, B.A., 2000. Apoptosis in the nervous system. Nature 407 (6805), 802–809.
Yuan, J., Lipinski, M., Degterev, A., 2003. Diversity in the mechanisms of neuronal cell death Neuron 40 (2), 401–413.