

Brain-derived neurotrophic factor protects against acrylamide-induced neuronal and synaptic injury via the TrkB-MAPK-Erk1/2 pathway

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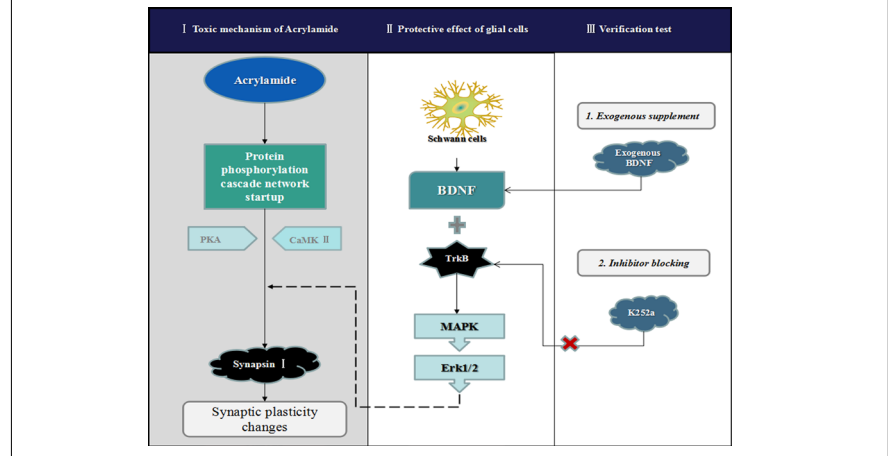
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Graphical Abstract Effect of brain-derived neurotrophic factor (BDNF) on neurosynaptic injury induced by acrylamide



Abstract

Acrylamide has been shown to be neurotoxic. Brain-derived neurotrophic factor (BDNF) can alleviate acrylamide-induced synaptic injury; however, the underlying mechanism remains unclear. In this study, dibutylryl-cyclic adenosine monophosphate-induced mature human neuroblastoma (NB-1) cells were exposed with 0–100 µg/mL acrylamide for 24–72 hours. Acrylamide decreased cell viability and destroyed synapses. Exposure of co-cultured NB-1 cells and Schwann cells to 0–100 µg/mL acrylamide for 48 hours resulted in upregulated expression of synapsin I and BDNF, suggesting that Schwann cells can activate self-protection of neurons. Under co-culture conditions, activation of the downstream TrkB-MAPK-Erk1/2 pathway strengthened the protective effect. Exogenous BDNF can increase expression of TrkB, Erk1/2, and synapsin I, while exogenous BDNF or the TrkB inhibitor K252a could inhibit these changes. Taken together, Schwann cells may act through the BDNF-TrkB-MAPK-Erk1/2 signaling pathway, indicating that BDNF plays an important role in this process. Therefore, exogenous BDNF may be an effective treatment strategy for acrylamide-induced nerve injury. This study was approved by the Laboratory Animal Welfare and Ethics Committee of the National Institute of Occupational Health and Poison Control, a division of the Chinese Center for Disease Control and Prevention (approval No. EAWE-2017-008) on May 29, 2017.

Key Words: factor; injury; pathway; peripheral nerve; protection; protein; regeneration; repair

Chinese Library Classification No. R466; R741; R388.1+3

Introduction

Acrylamide (ACR) is a water-soluble vinyl monomer used in various industries (e.g. water management, cosmetic and dye manufacturing, ore processing, paper production, and permanent press fabrics), mainly for the synthesis of polyamide (Friedman, 2003; Blancher and Cormick, 2012). Its frequent discovery in heated foods has been regarded an issue of concern in the public and medical communities (Tareke et al., 2002). In 2001, the Scientific Committee on

Toxicity, Ecotoxicity, and the Environment reported the neurotoxic, genotoxic, and carcinogenic effects of ACR, as well as its reproductive toxicity (Myers and Macun, 1991; Kjuus et al., 2004; Carere, 2006; Huang et al., 2011; Keramat, 2011; Pennisi et al., 2013). Because damage to the central and peripheral nervous systems caused by ACR poisoning varies and can exhibit a consistent phenotype with some neurodegenerative diseases, ACR has become a typical model for exploring mechanisms of neurodegenerative diseases

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(Erkekoglu and Baydar, 2014). In 2002, the World Health Organization and U.S. Food and Drug Administration reported that “the impact of ACR on the population’s nervous system in the current occupation and environment has become a practical issue of concern in the occupational health field and the government” (U.S. Food and Drug Administration, 2002; World Health Organization, 2002). In recent decades, many studies have investigated the neurotoxic and toxic mechanisms of ACR, which are now well understood (He et al., 1989; Deng et al., 1993; LoPachin and Lehning, 1994; LoPachin et al., 2002, 2003; Lopachin, 2004). However, current treatments for ACR are still symptomatic, and no available therapies are specifically targeted.

Synapses, the sites of neurotransmitter release, are the basis for normal regulation of neurological activity. Morphological and functional changes (synaptic plasticity) of synapses vary in response to the influence of external harmful factors (Jovanovic et al., 1996; Cesca et al., 2010; Bykhovskaia, 2011). Previous studies have demonstrated that synapsin I is strongly associated with the neurological damaging effects of ACR (Xiao et al., 2009, 2011b). ACR shortens the length of synaptic activity bands in rats, and significantly reduces mRNA and protein expression of synapsin I (Xiao et al., 2009, 2011b). Moreover, the supplementation of exogenous brain-derived neurotrophic factor (BDNF; 50–75 ng/mL) can alleviate the effects of ACR toxicity on synaptic morphology, and is accompanied by upregulation of synapsin I protein (Wang, 2015). There is a close link between neurons and glial cells upon interaction with exogenous chemicals in *in vivo* microenvironments. Although the focus is usually on synaptic plasticity, feedback from glial cells and their secreted functional proteins (such as BDNF) and receptors on neuronal damage signals cannot be ignored. Together, these studies suggest that synaptic structural and functional plasticity may be important mechanisms of nerve damage induced by ACR, and BDNF can attenuate ACR-induced synaptic damage within a certain concentration range.

BDNF has a high affinity for tropomyosin receptor kinase B (TrkB), which is involved in both development and maturation of the central and peripheral nervous systems (Yoshii and Constantine-Paton, 2010). After ligand binding, TrkB recruits and activates downstream signaling molecules to regulate gene expression, and may provide protection to neurons (Gómez-Palacio-Schjetnan and Escobar, 2013). BDNF-TrkB signaling has three main downstream pathways (Zheng and Wang, 2009): mitogen-activated protein kinase (MAPK) signaling, phosphatidylinositol-3-kinase (PI3K)/AKT signaling, and Ca²⁺/calmodulin-dependent kinase signaling. Almeida et al. (2005) first proposed the involvement of PI3K and MAPK signaling pathways in BDNF-related neuronal protection. Both pathways promote neuronal survival by altering gene expression and participating in synaptic remodeling. Further study indicated that MAPK inhibitors partially block the neuroprotective effect of BDNF, whereas PI3K inhibitors do not affect this protection. Almeida et al. (2005) concluded that the MAPK signaling pathway might be the dominant pathway involved in BDNF-related neuroprotection.

In this study, we aimed to determine the neurotoxic effects of ACR, along with the underlying mechanisms of subsequent neuronal repair, in a mature neuron model based on human neuroblastoma cells (NB-1 cells) with dibutyryl-cyclic adenosine monophosphate (db-cAMP) treatment. We also examined the effect of BDNF on ACR-induced neurotoxicity, including underlying signaling pathways.

Materials and Methods

Cell culture

NB-1 cells were a gift from Professor Masaki Kameyama (Kagoshima University, Japan). Cells were cultured in

Dulbecco’s Modified Eagle’s Medium (DMEM; Gibco BRL, Paisley, Scotland, UK) supplemented with 1% (v/v) penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) heat inactivated fetal bovine serum (FBS; Gibco BRL), and 10% (v/v) horse serum (Solarbio, Beijing, China). NB-1 cells were passaged when they reached 80% confluency, approximately every 3 days. Before the experiment, db-cAMP (Sigma-Aldrich) was used to induce differentiation of NB-1 cells into mature neuronal cells.

The sciatic nerve was isolated from 3-day-old Sprague-Dawley rats (Vital River Experimental Animal Technology Co., Ltd., Beijing, China) and cultured into primary Schwann cells (SCs). SCs were isolated from bilateral sciatic nerves by a previously described double enzyme digestion-differential attachment method (Jiang, 2014). Animal use and experimental protocols were approved by the Laboratory Animal Welfare and Ethics Committee of the National Institute of Occupational Health and Poison Control, a division of the Chinese Center for Disease Control and Prevention (approval No. EAWE-2017-008) on May 29, 2017. Dissociated SCs were inoculated into poly-L-lysine-treated culture flasks and cultured in DMEM containing FBS, penicillin/streptomycin, and SC growth supplement (Sigma-Aldrich). NB-1 cells and SCs were maintained in a 5% CO₂ and 37°C humidified incubator.

Co-culture of NB-1 cells and SCs

A modified Transwell® culture protocol was used, as reported in a previous study (Chen et al., 2018). Briefly, SCs ($1 \times 10^5/\text{cm}^2$) were seeded into the upper chamber of Millicell® six-well plates (Sigma-Aldrich) and co-cultured with 5×10^3 NB-1 cells, which were seeded into the lower chamber. When SCs are cultured, precoating of culture plates poly-L-lysine is needed. For co-culture experiments, NB-1 cells were co-cultured with SCs in DMEM containing 10% FBS. The culture medium was replaced every 3 days. NB-1 cells cultured alone were used as negative controls.

Cell viability assay

Differentiated mature NB-1 cells were seeded into 96-well plates for continued culturing (1×10^5 cells per well). Cells were divided into a control group and different concentrations ACR groups (25, 50, 75, 100, 125, 150, and 200 µg/mL; Sigma-Aldrich), which were incubated for 24, 48, and 72 hours.

Similarly, to determine an adequate concentration and inhibition time of K252a (Sigma-Aldrich) for subsequent experiments, and establish an inhibition model of TrkB, cells were exposed to various concentrations of K252a. We also investigated the effect of BDNF (Sigma-Aldrich) intervention on the survival of cells exposed to ACR. NB-1 cells were cultured in a 5% CO₂ incubator at 37°C. After 24 hours, cells were randomly divided into blank control, 60 µg/mL ACR, and 60 µg/mL ACR + BDNF groups. Intervention dosages of BDNF were 50, 75, and 100 ng/mL.

The viability of mature NB-1 cells after treatment with ACR and K252a was monitored using a Cell Proliferation and Cytotoxicity Assay Kit [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Sigma-Aldrich]. Cell viability was assessed according to the manufacturer’s protocol. Briefly, the culture medium was replaced with fresh medium containing 0.5 mg/mL MTT and cells were incubated for 90 minutes at 37°C and 5% CO₂. Next, the medium was removed, and cells were incubated with formazan, which was dissolved in dimethyl sulfoxide. Plates were shaken for 10 minutes, and the absorbance of each well was recorded at 490 nm with a plate reader (M200 Pro, TECAM Infinite, Männedorf, Switzerland). The results are expressed as a percentage of cell survival inhibition.

Western blot analysis

NB-1 cells in different ACR groups were processed according

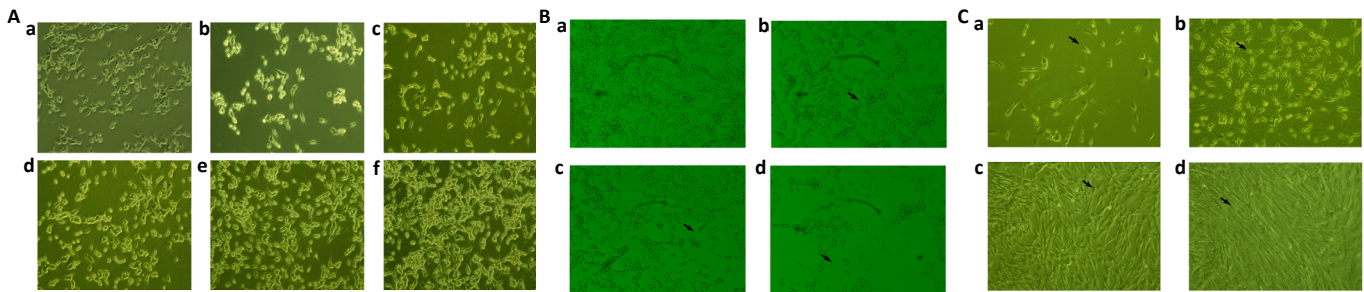


Figure 1 | Effects of different treatments on the morphology of NB-1 cells and Schwann cells.

(A) Morphology of NB-1 cells. (a) Morphology of normal NB-1 cells; (b–f) morphologies of NB-1 cells induced by db-cAMP for 1, 3, 5, 7, and 10 days. After 5 days of induction, the morphology began to change significantly. Most NB-1 cells matured after more than 7 days of db-cAMP induction. (B) Morphological changes of NB-1 cells after ACR exposure. (a) Normal control group; (b–d) 25, 50, and 100 µg/mL ACR groups. After ACR exposure, the

surface of NB-1 cells appeared blocky and the protrusions shortened. In addition, reduced cell-to-cell connections resulted in fewer adherent cells and an increase in dead cells (black arrows). (C) Morphology of Schwann cells (black arrows) at 24 hours (a), 48 hours (b), 5 days (c), and 10 days (d). Cultured Schwann cells had a typical spindle shape and were arranged in a bundle, generally bipolar, and occasionally multipolar shape. ACR: Acrylamide; db-cAMP: dibutyl-cyclic adenosine monophosphate (original magnification, 100×).

to the following three stages. Stage I: Observe the damaging effects of ACR on synapses treated with 25, 50, and 100 µg/mL ACR. Stage II: Observe the protective effect of BDNF intervention on synaptic damage, in which cells were divided into control, ACR (50 µg/mL), BDNF (75 ng/mL), and ACR + BDNF groups. Stage III: Observe the role of TrkB in regulating the MAPK pathway, in which cells were divided into control, ACR (50 µg/mL), K252a inhibition (1 nmol/mL), and ACR + K252a inhibition groups. Next, NB-1 cells in each group were collected and washed three times with 0.01 M phosphate-buffered saline. After protein extraction using a protein extraction buffer and phenylmethylsulphonyl fluoride, followed by centrifugation at 16,000 × g at 4°C for 15 minutes, we detected total protein concentrations with a Bicinchoninic Acid Protein Assay Kit (Beyotime Biotechnology Co., Ltd., Beijing, China).

In stages I and II, we used primary antibodies raised in rabbit from Cell Signaling Technology (Danvers, MA, USA) to detect protein expression of total and phosphorylated synapsin I (1:1000), extracellular signal-regulated kinase (Erk)1/2 (1:10,000), phosphorylated Erk1/2 (1:10,000), BDNF (1:2000), and TrkB (1:1000) by western blot assay. In stage III, we only analyzed changes in MAPK expression. After transfer, non-specific binding was blocked by immersing the nitrocellulose membrane in 5% skimmed milk solution and incubating overnight with the primary antibody at 4°C. Following incubation with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000; Beyotime Biotechnology) at 37°C for 1 hour, an enhanced chemiluminescence substrate (Beyotime Biotechnology) was used for detection. Band densities were analyzed by Quantity One software (Bio-Rad, Hercules, CA, USA). Protein expression was normalized to the optical density of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (rabbit; 1:1000; Beyotime Biotechnology).

RT-PCR analysis

Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate gene expression of synapsin I and MAPK1 in NB-1 cells exposed to 0, 25, 50, and 100 µg/mL ACR, as well as the impact of BDNF on synapsin I mRNA expression after ACR exposure in NB-1 cells. First, purified total RNA was extracted. Next, the oligonucleotide primer and Superscript™ II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) were used for synthesizing first-strand complementary DNA. Finally, real-time RT-PCR analysis was carried out with a Line Gene 9600 Plus sequence detection instrument (Bioer Technology, Hangzhou, China) using a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). All RT-PCR quantification procedures were performed in duplicate. Primers used were as follows: GAPDH, 5'-CTG GGC TAC ACT GAG CAC C-3' and 5'-AAG TGG TCG TTG AGG GCA ATG-3';

MAPK, 5'-TGC TGC TCA ACA CCA CCT-3' and 5'-AAT GCA GCC TAC AGA CCA A-3'; synapsin I, 5'-GGA AGT GCC CAA ATA CCA G-3' and 5'-GTG TTG GGT GGC ATT TGA AG-3'. GAPDH was used as the housekeeping gene for analysis.

Statistical analysis

All values are presented as mean ± standard error of mean (SEM). One-way analysis of variance was applied to compare differences between multiple groups, followed by least significant difference test for comparison of two groups using SPSS version 13.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered to be statistically significant. Correlation analysis results are expressed as the Pearson correlation coefficient. All statistical graphs were made with GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Damaging effect of ACR exposure on neurons

db-cAMP can induce neurite formation in NB-1 cells (Ishikawa, 1977; Kunimoto, 1995). Therefore, we first observed neurite formation in NB-1 cells cultured with or without db-cAMP. The typical morphology of NB-1 cells is tear-drop-shaped or round with relatively short extensions. Following db-cAMP treatment, the cells became spindle-shaped with both ends protruding and intersecting. At 7–10 days after treatment, > 90% of NB-1 cells had differentiated and matured. After the mature NB-1 cell model was prepared, we investigated the effect of ACR. After ACR treatment, cell agglomeration increased, cell extensions were significantly shortened, numbers of round cells significantly increased, cell-to-cell connections were reduced, cell adhesion was weakened, and viable cells were significantly reduced (**Figure 1**).

The relative survival rate of NB-1 cells changed after ACR exposure, as detected by MTT assay. The optimal linear relationship between cell viability and ACR dosage was observed at 48 hours ($R^2 = 0.9901$) according to Pearson correlation analysis. When the concentration of ACR was > 100 µg/mL, the decrease in viability of NB-1 cells was approximately 50%. Therefore, to better observe changes in cells, concentrations of ACR in subsequent experiments were set to 0, 25, 50, and 100 µg/mL, and the exposure time was set to 48 hours (**Figure 2**).

Damaging effect of ACR on synapse-related protein expression in NB-1 cells

ACR damages the synapses of neuronal cells, as reflected by changes in the key protein synapsin I. As shown in **Figure 3A**, a statistically significant difference in expression of synapsin I was observed in the ACR groups compared with the control group ($P < 0.05$). There was a negative correlation between

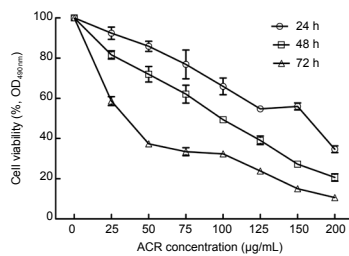


Figure 2 | Effect of ACR on viability of NB-1 cells detected by MTT assay. Viability of NB-1 cells, as a percentage of cell survival inhibition, exhibited a dose- and time-dependent trend after ACR exposure. Data are expressed as the mean \pm SEM ($n = 6$). ACR: Acrylamide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OD: optical density.

the concentration of ACR and expression of total synapsin I ($R^2 = 0.8751$, $P < 0.0001$).

To determine whether BDNF or its receptor play a role in neuronal and synapse damage repair, we assessed changes in BDNF, TrkB, and the downstream MAPK-Erk1/2 pathway. As illustrated in **Figure 3B–D**, compared with the control group, expression of TrkB and Erk1/2 decreased with higher concentrations of ACR ($P < 0.05$), revealing a negative dose-response relationship. These results indicate that BDNF, its receptors, and downstream signaling pathways are largely involved in neuronal cells exposed to ACR.

Protective effect of SCs on neurons and synapses after ACR exposure

Glial cells regulate the excitability and synaptic plasticity of neurons by regulating signal transmission between synaptically-controlled neurons and the exchange of substances (e.g. neurotrophic factors) (Perea and Araque, 2005). To understand the initiation and progression of neuroprotection in the presence of glial cells, we co-cultured NB-1 cells with SCs to simulate the actual damage repair process in the body.

MTT assay results indicated that SCs protected NB-1 cells from ACR exposure (Chen et al., 2018). In this study, we also observed that SCs alleviated the inhibitory effect of ACR on synapsin I. As illustrated in **Figure 4A**, compared with the control group, the NB-1 + SC group had no significant dose-response relationship to the decline of synapsin I expression ($R^2 = 0.8057$, $P < 0.0001$).

To further explore the protective mechanisms of SCs, we examined changes in BDNF, its receptor, and downstream pathways in the co-culture system following exposure to different ACR concentrations. We observed significantly increased BDNF expression ($R^2 = 0.9562$, $P < 0.0001$; **Figure 4B**), and levels of TrkB and Erk1/2 in co-cultured cells (NB-1 + SC) were positively correlated with the concentration of ACR under this condition ($R^2 = 0.9257$, $P < 0.0001$; $R^2 = 0.9662$, $P < 0.0001$; **Figure 4C and D**). These results indicate that glial cells may protect against reductions in synapsin I caused by ACR by secreting a large amount of BDNF, thus triggering BDNF-related signaling pathways.

BDNF promotes TrkB expression and activation of its downstream pathway in neurons after synapse damage

We further validated the role of BDNF by adding exogenous BDNF. According to the inhibitory effect of ACR on synapsin I (**Figure 3A**), and the intervention effect of BDNF on NB-1 cells exposed to a cytotoxic dose of ACR (**Figure 5A**), we selected appropriate ACR exposure (50 µg/mL) and BDNF intervention (75 ng/mL) doses. Compared with the control group, expression of TrkB, Erk1/2, and phosphorylated Erk1/2 were markedly increased in cells treated with BDNF, particularly in the ACR + BDNF group (**Figure 5B and C**). Moreover, we also

observed a significant increase in synapsin I expression, as well as a decrease in phosphorylated synapsin I expression, in BDNF and ACR + BDNF groups compared with control and ACR groups ($P < 0.05$; **Figure 5D**).

To verify whether activation of the MAPK-Erk1/2 signaling pathway by BDNF was involved in neuronal damage repair, we inhibited BDNF activity with the TrkB inhibitor K252a. Preliminary experiments were performed to evaluate the toxicity of K252a using an MTT assay. The results indicated that the inhibitory effect of K252a on NB-1 cells was dose-dependent, but not time-dependent. Moreover, when the K252a concentration was 1 nmol/mL, the inhibitory rate of NB-1 cells can be maintained within 10%. Considering the inhibitory effect of K252a and cell survival rates, the concentration of K252a was set to 1 nmol/mL for subsequent experiments, and the inhibition time was set to 1 hour. As shown in **Figure 6**, K252a markedly inhibited TrkB expression; once again, we demonstrated that ACR could inhibit the activation of neuronal protection pathways by inhibiting TrkB. In addition, Erk1/2 expression in K252a and K252a + ACR groups was markedly lower than observed in control and ACR groups ($P < 0.05$), indicating a vital role for BDNF.

BDNF increases mRNA expression of MAPK and synapsin I in NB-1 cells after ACR exposure

To further confirm that BDNF promoted expression of MAPK and synapsin I, mRNA levels were examined using RT-PCR. Consistent with our previous results, there was an apparent difference in MAPK mRNA expression between NB-1 cells and co-cultured NB-1 cells. These results indicate that BDNF secretion by SCs altered MAPK expression (**Figure 7A**). In addition, compared with the control group, mRNA expression of synapsin I was significantly decreased in the ACR group ($P < 0.05$), and significantly increased in the 75 ng/mL BDNF group ($P < 0.05$), which also suggests that BDNF has a protective effect on synapsin I (**Figure 7B**).

Discussion

As a cell model that has been applied in many basic medical research fields, NB-1 cells can rapidly divide and proliferate, exhibit an appropriate diversity of electrophysiological properties, and have clear calcium channel dependence (Song et al., 2000). In response to db-cAMP, NB-1 cells can differentiate into mature neurons (Miyake et al., 1975; Ishikawa, 1977). With regard to the functional basis of NB-1 cells, our team has completed a lot of previous work (Wang et al., 2015; Chen et al., 2018). Indeed, we established a mature neuron model for *in vitro* synaptic research by applying appropriate inducers to NB-1 cells and performing a series of experiments that indicated the utility of this integrated *in vitro* system in terms of the physical signs of mature neurons, material basis of synapses, calcium channel ions, and key enzymes.

Previous studies have described relationships between various enzymes in the phosphorylation cascade network, initiation of synapsin I-mediated conformational adjustments, and conditions of SNARE complex polymerization and dissociation (Xiao et al., 2011a, b, 2014; Wang et al., 2015; Chen et al., 2018). The results suggest that BDNF influences the synaptic plasticity of neurons after ACR exposure and provides a protective effect. Based on these previous results, this study further explored the specific role of BDNF in this neuroprotective process.

ACR causes damage to neuronal cells and synapses

In terms of health hazards, ACR-induced nervous system

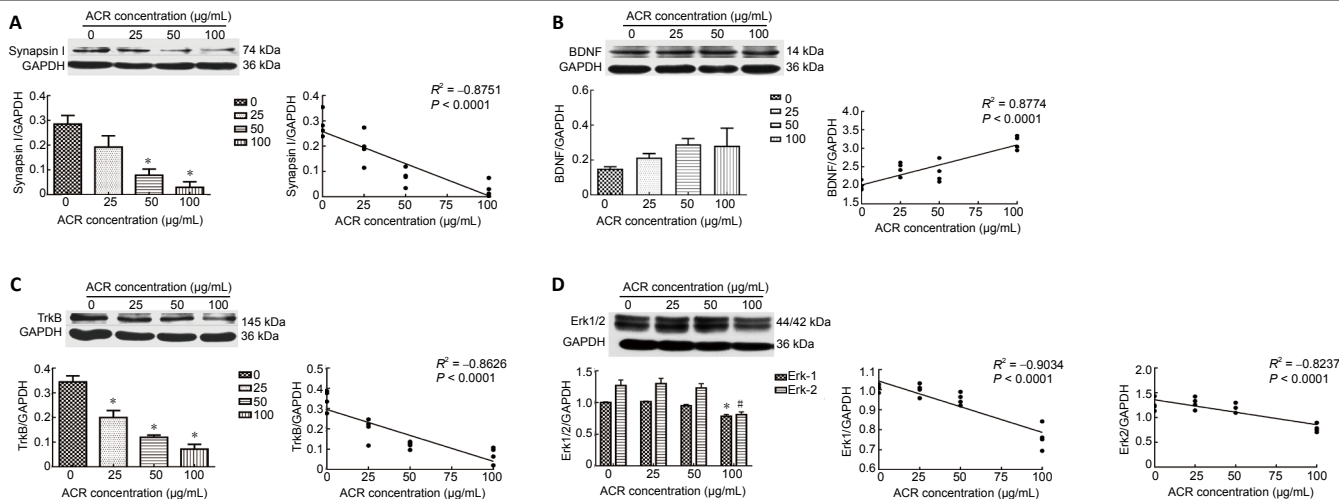


Figure 3 | Potential protection mechanism of ACR on NB-1 cells. (A–D) NB-1 cells were treated with ACR for 48 hours at different concentrations, and then synapsin I, BDNF, TrkB, and Erk1/2 protein expression was detected by western blot analysis. Relationships between these protein levels and ACR dose were assessed by Pearson correlation analysis. Relative expression of each target protein was expressed as OD ratio to GAPDH. Relative expression of all target proteins was negatively correlated with ACR concentration (synapsin I: $R^2 = -0.8751$, $P < 0.0001$;

BDNF: $R^2 = -0.8774$, $P < 0.0001$; TrkB: $R^2 = -0.8626$, $P < 0.0001$; Erk1: $R^2 = -0.9034$, $P < 0.0001$; Erk2: $R^2 = -0.8237$, $P < 0.0001$). Data are expressed as the mean \pm SEM ($n = 3$). * $P < 0.05$, vs. control group (0 $\mu\text{g/mL}$ ACR group); # $P < 0.05$, vs. 50 $\mu\text{g/mL}$ ACR group (one-way analysis of variance followed by the least significant difference test). ACR: Acrylamide; BDNF: brain-derived neurotrophic factor; Erk: extracellular signal-regulated kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; OD: optical density; TrkB: tropomyosin receptor kinase B.

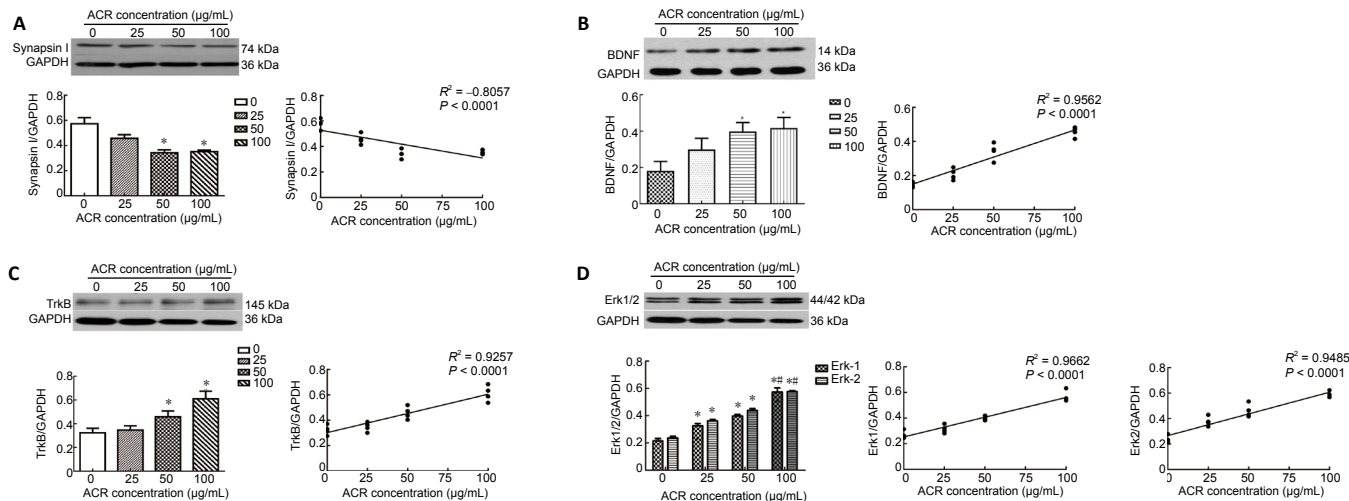


Figure 4 | Protective effects of Schwann cells on synapses and changes in NB-1 cells.

(A–D) NB-1 cells co-cultured with Schwann cells were treated with ACR for 48 hours, and then protein expression of synapsin I, BDNF, TrkB, and Erk1/2 was detected by western blot analysis. Relationships between levels of these proteins and ACR dose were assessed by Pearson correlation analysis. Relative expression of each target protein is expressed as OD ratio to GAPDH. Relative expression of all target proteins was negatively correlated with ACR

concentration (synapsin I: $R^2 = -0.8057$, $P < 0.0001$; BDNF: $R^2 = -0.9562$, $P < 0.0001$; TrkB: $R^2 = -0.9257$, $P < 0.0001$; Erk1: $R^2 = -0.9662$, $P < 0.0001$; Erk2: $R^2 = -0.9485$, $P < 0.0001$). Data are expressed as the mean \pm SEM ($n = 3$). * $P < 0.05$, vs. control group (0 $\mu\text{g/mL}$ ACR group); # $P < 0.05$, vs. 50 $\mu\text{g/mL}$ ACR group (one-way analysis of variance followed by least significant difference test). ACR: Acrylamide; BDNF: brain-derived neurotrophic factor; Erk: extracellular signal-regulated kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; OD: optical density; TrkB: tropomyosin receptor kinase B.

damage is of particular interest because it is currently recognized as the only toxic effect evidenced in both animals and humans (LoPachin et al., 2004; Allam et al., 2011; Mehri et al., 2014). In this study, upon the exposure of NB-1 cells to ACR, cell viability followed a sustained dose- and time-dependent trend in which the cells gradually lumped and their protuberances shortened. Decreasing cell-cell connections resulted in fewer adherent cells, and a significant reduction of cell viability. Moreover, as the sites of release for neurotransmitters that maintain the body's normal physiological activities, synapses can undergo different degrees of functional and structural changes in the presence of harmful external factors (LoPachin et al., 2004; Lopachin and Gavin, 2008). Thus, this study also confirmed that ACR can damage synapses by affecting the key protein synapsin I, thereby impairing the function of neurons.

SCs initiate BDNF-related protective mechanisms after ACR injury

In the first phase of this study, we explored the possible repair mechanisms that occur following ACR-induced synaptic injury. We found that after ACR exposure, autocrine levels of BDNF increased to a certain extent, indicating that damage stimulated the repair feedback mechanism of neurons. However, decreases in total TrkB, Erk1/2, and synapsin I, and the neuron survival rate indicated that the protective effect of neurons was insufficient to completely repair the damage caused by ACR exposure.

Glial cells can promote synaptic connections and synaptic activity (Pfrieger and Barres, 1997). Recently, the concept of triple synapses was introduced, in which glial cells interact with presynaptic and postsynaptic neurons to form a triple synapse structure for signal transduction and integration (Xie,

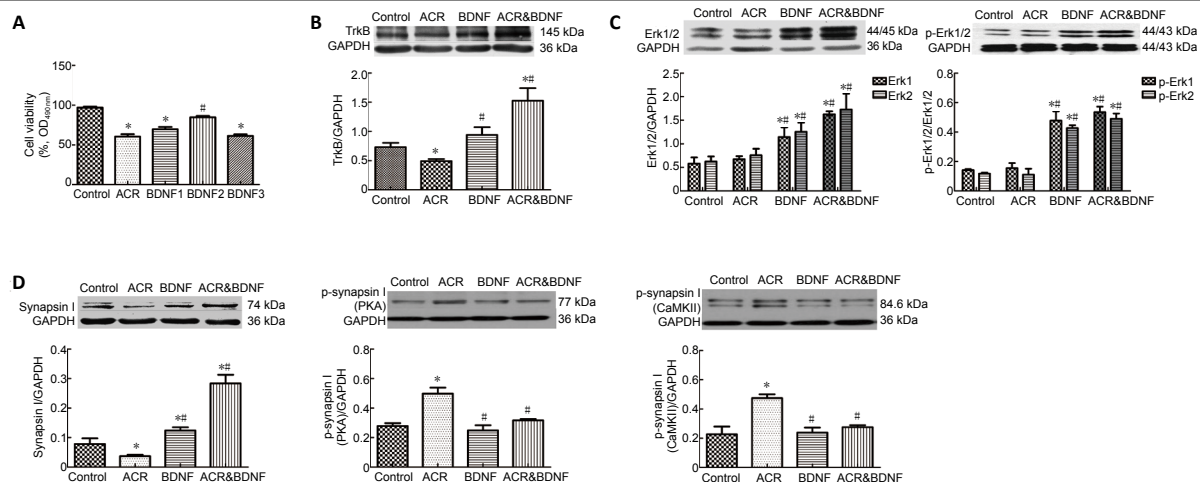


Figure 5 | Effects of BDNF on synapsin I, TrkB, and Erk1/2 protein expression of ACR-exposed NB-1 cells.

(A) Effects of BDNF on NB-1 cells induced by ACR. Intervention concentrations of BDNF were 50 ng/mL (BDNF1), 750 ng/mL (BDNF2), and 100 ng/mL (BDNF3). (B–D) NB-1 cells were treated with ACR and BDNF for 48 hours, and then protein expression of TrkB (B), Erk1/2 (C), and (phosphorylated, p-) synapsin I (D) was detected by western blot analysis. Control: Control group; ACR: 50 µg/mL ACR group; BDNF: 75 ng/mL BDNF group; ACR&BDNF: 50

µg/mL ACR + 75 ng/mL BDNF group. Relative expression of target protein expressed as OD ratio to GAPDH. Data are expressed as mean ± SEM ($n = 3$). * $P < 0.05$, vs. control group; # $P < 0.05$, vs. ACR group (one-way analysis of variance followed by the least significant difference test). ACR: Acrylamide; BDNF: brain-derived neurotrophic factor; Erk: extracellular signal-regulated kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; OD: optical density; TrkB: tropomyosin receptor kinase B.

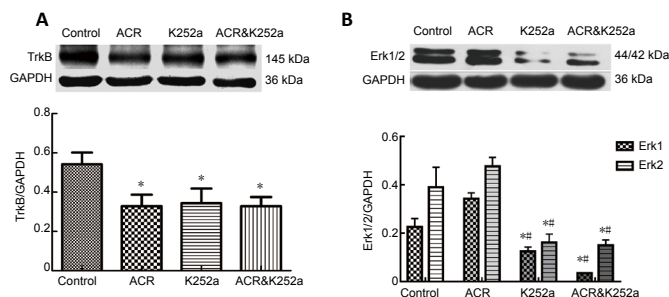


Figure 6 | Effect of K252a on expression of TrkB (A) and Erk1/2 (B) in NB-1 cells 48 hours after ACR exposure detected by western blot analysis.

Control: Control group; ACR: 50 µg/mL ACR group; K252a: 1 nmol/mL K252a group; K252a&ACR: 1 nmol/mL K252a + 50 µg/mL ACR group. Data are expressed as mean ± SEM ($n = 3$). * $P < 0.05$, vs. control group; # $P < 0.05$, vs. ACR group (one-way analysis of variance followed by the least significant difference test). ACR: Acrylamide; Erk: extracellular signal-regulated kinase; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; K252a: a TrkB inhibitor; OD: optical density; TrkB: tropomyosin receptor kinase B.

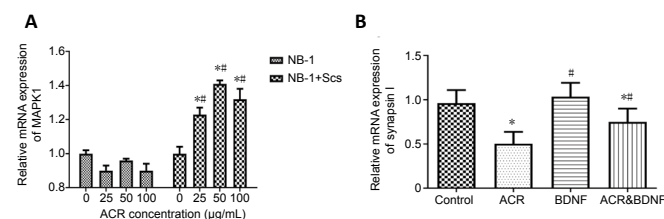


Figure 7 | Effect of BDNF on mRNA expression of MAPK1 (A) and synapsin I (B) in NB-1 cells after ACR exposure detected by reverse transcription polymerase chain reaction analysis.

(A) MAPK1 mRNA expression. NB-1 cells were pretreated with ACR with or without SCs for 48 hours. (B) Synapsin I mRNA expression. Control: Control group; ACR: 50 µg/mL ACR group; BDNF: 75 ng/mL BDNF group; ACR&BDNF: 50 µg/mL ACR + 75 ng/mL BDNF group. Data are expressed as the mean ± SEM ($n = 3$). * $P < 0.05$, vs. control group; # $P < 0.05$, vs. ACR group (one-way analysis of variance followed by the least significant difference test). ACR: Acrylamide; BDNF: brain-derived neurotrophic factor; MAPK: mitogen-activated protein kinase; SCs: Schwann cells.

2004; Faissner et al., 2010; Oliveira, 2010; Danjo et al., 2011; Takata et al., 2011). In the triple synapse, glial cells construct a bridge between neurons, and the functional protein (BDNF receptor) they express is involved in synaptic plasticity via BDNF binding (Woodhall et al., 2001; Djalali et al., 2005). Therefore, in the second phase of this study, we added SCs to simulate the actual *in vivo* protection mechanism in cocultures of glial cells and neurons to further understand the damage repair effect of BDNF. SCs inhibited ACR-induced damage and reduced the viability of NB-1 cells by increasing protein expression of BDNF, TrkB, and Erk1/2, as well as the binding of BDNF and TrkB to glial cell-regulated synaptic functional proteins. These results provide an important basis for the protective effect of BDNF in the synaptic microenvironment.

Exogenous BDNF intervention effectively alleviates neuronal damage induced by ACR

BDNF acts on both neurons and glial cells. Glial cells, which express BDNF receptors, can promote the migration and infiltration of glial cells around damaged neurons after injury, which coordinate with intrinsic glial cells around neurons to reduce damage or promote repair. Upon binding to the TrkB receptor, BDNF can activate the phospholipase C signal transduction pathway to continuously increase the

intracellular calcium concentration and activate related protective functions. It can also activate further secretion of functional proteins, which in turn activates the function of glial cells in the injured area (Bagayogo and Dreyfus, 2009). Indeed, neurotrophic factors (e.g. BDNF and its receptors) released by glial cells have become important molecular markers for repair processes performed by glial cells.

In consideration of the experimental results described above and corresponding theoretical basis, BDNF intervention seems to have a high probability of mitigating ACR toxicity. Therefore, to further confirm the feasibility of BDNF intervention, we further verified changes in the expression of proteins in this pathway after application of exogenous BDNF. In this study, 75 ng/mL was selected as the intervention concentration because it had the greatest effect, and BDNF treatment was carried out alone or alongside cells incubated with ACR, which causes synaptic injury. It can be observed from the results that during the first phase, increased concentrations of ACR reduced expression of TrkB; however, expression of TrkB and Erk1/2 was significantly increased by exposure to 75 ng/mL BDNF and 50 µg/mL ACR during this phase. These results indicate that when neuronal cells are exposed to ACR, particularly at the early stage of injury (when the damage is

not severe), activation of protective mechanisms is sensitive to BDNF, which increases BDNF levels by upregulating TrkB expression, thereby initiating the downstream pathway. Our results indicate that increasing BDNF levels were beneficial in initiating damage repair mechanisms of the body. The inhibitor test also confirmed this assumption. First, K252a blocked the increase of TrkB expression. Second, as TrkB was no longer working, we observed a significant downstream decline in Erk1/2 levels. Thus, the consistency of changes in TrkB and MAPK, as well as damage observed during the three stages of the study, suggested that increased TrkB expression after treatment with BDNF may be a protective mechanism, consistent with other related studies. Binding of BDNF to its specific receptor, TrkB (which may undergo self-dimerization), further activates intracellular signaling pathways such as the MAPK-Erk1/2 pathway, as described in previous studies (Yoshii and Constantine-Paton, 2010; Zhang et al., 2012). In addition, an important role for TrkB as a high-affinity receptor for BDNF has also been reported in many studies. For example, Martínez et al. (1998) found that axonal branching and synaptic density were significantly decreased in hippocampal synapses of TrkB-knockout mice, indicating that TrkB has a regulatory effect on synaptic growth. Sun et al. (2007) reported that the thoracic spinal cords of adult rhesus monkeys expressed BDNF and TrkB, and these molecules may be involved in the normal physiological function of monkey spinal cord and post-injury repair.

Previous studies have demonstrated that the neuroprotective effects of BDNF may be achieved via MAPK and PI3K pathways (Almeida et al., 2005; You et al., 2010), which can promote neuronal survival by altering gene expression and participating in synaptic remodeling. Further comparisons of these two pathways revealed that MAPK inhibitors partially block the neuroprotective effect of BDNF, while PI3K inhibitors do not affect the protection elicited by BDNF (Hua et al., 2016); this conclusion is consistent with our findings. Therefore, we infer that the dominant protective pathway of BDNF may be MAPK. According to the results of this study, we propose that the possible mode of action of this pathway in BDNF-mediated neuroprotection is as follows: ACR stimulation leads to activation of the BDNF autocrine cycle, which upregulates BDNF expression leading to increased activation of TrkB, which results in activation of Erk1/2, which elicits changes in key functional proteins (e.g. synapsin I) that result in synaptic plasticity changes (Carvalho et al., 2008; Xie and Yung, 2012). In this process, we concluded that regulation of TrkB seems to be the key point of subsequent intervention, based on the consistency of TrkB and Erk1/2 changes.

This study has certain limitations. For example, recent reports suggest that BDNF has a relatively short half-life. Thus, elucidating whether it can play an important role through the MAPK-Erk1/2 pathway in actual applications requires more *in vivo* study and even clinical trials (Angelova et al., 2013; Géral et al., 2013).

In conclusion, this study started with the phenomenon and mechanism of ACR injury to explore the protective pathways and effect of BDNF on neurosynaptic injury. Our results indicate that synapsin I may act as a downstream effector of the BDNF tyrosine kinase cascade to protect against ACR-induced nerve damage by maintaining the integrity of synaptic structure and function. Understanding the mechanism of BDNF-mediated reversal of neuronal damage will provide evidence for its use in the prevention and treatment of a range of neurodegenerative diseases.

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