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# Neurotrophins protect against cytosine arabinoside-induced apoptosis of immature rat cerebellar neurons

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**Abstract**

Neurotrophin-induced neuroprotection against apoptosis was investigated using immature cultured cerebellar granule cells (CGC) from newborn rat pups. Apoptotic cell death induced by treatment with cytosine arabinoside (AraC) was confirmed by DNA fragmentation and quantified by cell survival assays. AraC was most effective in inducing apoptosis when added to CGC on the day of culture preparation, while less or no effect was observed when added at 24 or 48 h after plating, respectively. Pretreatment of CGC cultures for 24 h with brain-derived neurotrophic factor (BDNF) or neurotrophin-4 (NT-4), but not neurotrophin-3 (NT-3), robustly protected against AraC neurotoxicity. K252a, an inhibitor of the tropomyosin-related kinase (Trk) tyrosine kinase receptor family which showed no toxicity by itself, blocked BDNF protection of AraC-induced apoptosis in a concentration-dependent manner. Neither protein kinase C activation nor inhibition mimicked or affected BDNF protection against AraC neurotoxicity. BDNF, but not NT-3, treatment of immature CGC caused a marked, but transient activation of Akt through phosphatidylinositol (PI) 3-kinase. The neuroprotective effects of BDNF were suppressed by pretreatment with LY 294002 (a PI 3-kinase inhibitor). BDNF neuroprotection was also preceded by activation of mitogen activated protein kinase (MAPK) and suppressed by two MAPK/ERK (MEK)-selective inhibitors, PD 98059 and U-0126. Moreover, inhibitors of PI 3-kinase and MEK potentiated AraC-induced neurotoxicity. These results show that neurotrophins protect against AraC-induced apoptosis, at least in part, through TrkB-mediated activation of the PI 3-kinase/Akt and MEK signaling pathways.
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*Keywords:* Neurotrophins; Cerebellar granule cells; AraC neurotoxicity; PI 3-kinase/Akt; Neurotoxicity; MAPK; Neuroprotection

## 1. Introduction

Apoptosis, also known as programmed cell death, is a physiological event used by organisms to remove cells during normal development or repair, and to maintain adult tissue homeostasis (for a review see Thompson, 1995; Meier et al., 2000). This form of programmed cell death is typified by specific morphological changes, internucleosomal DNA cleavage, the requirement of RNA and protein synthesis and a rapid and organized phagocytotic clearance of cellular debris (Margolis et al., 1994; Thompson, 1995; Savill and Fadok, 2000). Intensive investigations have recently begun to increase the understanding of the molecular mechanisms underlying certain types of induced neuronal apoptosis including that of cytosine β-D-arabinofuranoside (AraC) toxicity.

AraC at high concentrations has been shown to induce apoptosis of cerebellar granule cells (CGC) (Dessi et al., 1995). A pyrimidine anti-metabolite used in the therapy of haematological tumours, AraC is thought to exert toxicity through the inhibition of topoisomerase-II-mediated DNA

*Abbreviations:* AraC, cytosine β-D-arabino-furanoside; BDNF, brain-derived neurotrophic factor; Cal C, calphostin C; DIV, days in vitro; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAPK, mitogen activated protein kinase; MEK, MAPK/ERK kinase; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide); NT-3, neurotrophin-3; NT-4, neurotrophin-4; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; Trk, tropomyosin receptor kinase

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repair or DNA ligase, either of which leads to DNA strand breaks (Tomkins et al., 1994). This compound, which gains access to the cell via a specific nucleoside transporter, may also produce toxic effects, once phosphorylated by deoxycytidine kinase, by blocking DNA repair through inhibition of ribonucleotide reductase and DNA polymerase-α (Robertson et al., 1993). AraC-induced death has been shown to increase cyclin E-associated cyclin-dependent kinase activity in HL60 cells (Ping Dou et al., 1995), and to require the expression of p53 in CGC (Enokido et al., 1996; Chen et al., 1999) as well as in superior cervical ganglion neurons (Anderson and Tolkovsky, 1999). Moreover, AraC induces oxidative stress-induced DNA damage and Bax-dependent apoptosis in neurons (Geller et al., 2001; Besirli et al., 2003). In chronic lymphocytic leukaemia cells, AraC has been shown to cause a decrease in mRNA levels of anti-apoptotic Bcl-2 (Peterson et al., 1996), which is of interest in that elevated Bcl-2 levels correlate with resistance to AraC toxicity (Campos et al., 1993; Guedez et al., 1996). Involvement of dephosphorylation of Rel A (p65) of the NF-κB complex in AraC-induced apoptosis of Jurkat cells has also been reported (Sreenivasan et al., 2003).

Brain-derived neurotrophic factor (BDNF) and its related family member, neurotrophin-4 (NT-4) are growth factors involved in the regulation of neuronal survival, differentiation and maturation (Finkbeiner, 2000; Poo, 2001; Lu, 2003). Their actions are mediated via the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors (for review, Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). The TrkB receptor, through which BDNF and NT-4 exert their actions, has an insulin receptor tyrosine-autophosphorylation NPXY motif. This motif recognizes the phosphotyrosine-binding domain of SNT-1/2 (*suc*1-associated neurotrophic factor target, also known as FRS2α/β, fibroblast growth factor receptor substrate) (for review, Dhalluin et al., 2000), and causes activation through stimulation of tyrosine phosphorylation. Then, through sequential recruitments of three adaptor proteins, shc, Grb-2 and Gab-1, phosphatidylinositol 3-kinase (PI 3-kinase) can be activated (for review, Friedman and Greene, 1999; Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001). Grb-2 also constitutively complexes with SOS, a guanine nucleotide exchange factor for Ras, a small GTP binding protein, which then through Ras can bind to and also activate PI 3-kinase. Activation of PI 3-kinase then leads to activation of Akt and neuroprotection. Akt, which is also known as PKB or RAC, is a serine/threonine kinase and downstream target of PI 3-kinase. Akt is activated by the binding of PI 3-kinase products, PI 3,4,5-trisphosphate (PI 3,4,5-$P_3$) and/or PI 3,4,-bisphosphate (PI 3,4-$P_2$), to its pleckstrin homology domain (Stokoe et al., 1997; Franke et al., 1997). This then allows PI-dependent kinases (PDK-1, PDK-2) to phophorylate Ser473 and Thr308 (for review, Chan et al., 1999; Datta et al., 1999; Kaplan and Miller, 2000).

Not only can Ras directly interact with PI 3-kinase to cause its activation and in turn promote neurotrophin-induced neuronal survival, Ras additionally can mediate neurotrophin-induced activation of the mitogen-activated protein kinase (MAPK)/ERK/extracellular signal-regulated kinase (MEK/ERK) mitogen-activated protein kinase signaling pathway (for review, Kaplan and Miller, 2000; Patapoutian and Reichardt, 2001; Ying et al., 2002; Barnabe-Heider and Miller, 2003). This Ras/MEK–ERK–MAPK pathway has a prominent role in synaptic plasticity, long-term potentiation, neuronal development and cell survival (for review, Grewal et al., 1999; Finkbeiner, 2000; Poo, 2001; Vicario-Abejon et al., 2002; Lu, 2003). However, the role of this pathway in neurotrophin-induced survival of CGC is unclear. While some studies showed that the activation of the MEK/ERK pathway is not required for BDNF-dependent neuroprotection of CGC (Gunn-Moore et al., 1997) and neuroblastoma cells (Encinas et al., 1999), other investigators reported that this MAPK activation is a contributor of the BDNF-induced survival in the CGC culture (Skaper et al., 1998; Bonni et al., 1999). BDNF-induced activation of MAPK has also been shown to promote survival of postnatal rat retinal ganglion cells (Meyer-Franke et al., 1998; Shen et al., 1999), neurogenesis in cultured embryonic cortical progenitor cells (Barnabe-Heider and Miller, 2003) and cortical and hippocampal neurons in an in vivo model of hypoxic ischemia (Han and Holtzman, 2000). The present study was undertaken to characterize AraC-induced apoptosis of CGC and to explore the effects of neurotrophins on this form of death. CGC, which have a purity of greater than 95% in culture, have proven to be a useful model for the study of the molecular events involved in neuronal apoptosis and the role of the PI 3-kinase/Akt and MEK/MAPK pathways in growth factor-induced survival.

## 2. Experimental procedures

### 2.1. Neuronal cultures

All procedures employing experimental rats were performed in compliance with the National Institutes of Health Guidelines for the care and use of laboratory animals, and were designed to minimize the number of animals used and their suffering. CGC were prepared from 8-day-old Sprague–Dawley rats (Taconic Farms, Germantown, NY) as previously described (Chen et al., 1999). Briefly, cerebella were chopped into 400-μm cubes followed by trypsin dissociation and trituration. Cells were suspended in basal modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, 50 (g/ml gentamicin and 25 mM KCl, and then plated at a density of $2.7 \times 10^5$ cells/cm$^2$ in poly-L-lysine precoated 24-well plates for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) analysis and 60 mm dishes for DNA fragmentation and

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