Cyanidin-3-Glucoside Reverses Ethanol-Induced Inhibition of Neurite Outgrowth: Role of Glycogen Synthase Kinase 3 Beta

Gang Chen · Kimberly A. Bower · Mei Xu · Min Ding · Xianglin Shi · Zun-Ji Ke · Jia Luo

Abstract Ethanol is a potent teratogen for the developing central nervous system (CNS), and fetal alcohol syndrome (FAS) is the most common nonhereditary cause of mental retardation. Ethanol disrupts neuronal differentiation and maturation. It is important to identify agents that provide neuroprotection against ethanol neurotoxicity. Using an in vitro neuronal model, mouse Neuro2a (N2a) neuroblastoma cells, we demonstrated that ethanol inhibited neurite outgrowth and the expression of neurofilament (NF) proteins. Glycogen synthase kinase 3β (GSK3β), a multifunctional serine/threonine kinase negatively regulated neurite outgrowth of N2a cells; inhibiting GSK3β activity by retinoic acid (RA) and lithium induced neurite outgrowth, while over-expression of a constitutively active S9A GSK3β mutant prevented neurite outgrowth. Ethanol inhibited neurite outgrowth by activating GSK3β through the dephosphorylation of GSK3β at serine 9. Cyanidin-3-glucoside (C3G), a member of the anthocyanin family rich in many edible berries and other pigmented fruits, enhanced neurite outgrowth by promoting p-GSK3β(Ser9). More importantly, C3G reversed ethanol-mediated activation of GSK3β and inhibition of neurite outgrowth as well as the expression of NF proteins. C3G also blocked ethanol-induced intracellular accumulation of reactive oxygen species (ROS). However, the antioxidant effect of C3G appeared minimally involved in its protection. Our study provides a potential avenue for preventing or ameliorating ethanol-induced damage to the developing CNS.

Keywords Alcohol · Development · Differentiation · Fetal alcohol syndrome · Neuroprotection

Abbreviations

C3G	Cyanidin-3-glucoside
FASD	Fetal alcohol spectrum disorders
FAS	Fetal alcohol syndrome
GSK3β	Glycogen synthase kinase 3β
ROS	Reactive oxygen species

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Introduction

Fetal alcohol spectrum disorders (FASD) are caused by maternal alcohol consumption during pregnancy (Riley and McGee 2005), and fetal alcohol syndrome (FAS), the most severe form of FASD, is associated with increased fetal demise, intrauterine growth restriction, central nervous system (CNS) malformations, mental retardation, and craniofacial and skeletal defects. Epidemiologic data indicate that in the US, FAS occurs in 0.2–1.5 per 1,000 live births, whereas alcohol-related birth defects and brain developmental disorders occur in approximately 9 per 1,000 live births (Stromland and Pinazo-Duran 2002). FAS is the most

common nonhereditary cause of mental retardation (Stratton et al. 1996; May and Gossage 2001). Moderate or even low levels of prenatal alcohol abuse can also be harmful and impair cognitive behavior and motor functions (Abel 1984; Mattson et al. 2001; O'Malley and Nanson 2002; O'Callaghan et al. 2003, 2007; Riley and McGee 2005). Prenatal exposure to alcohol disrupts many events of neuronal development, including neuronal survival, migration, neuromorphogenesis and synapse formation (Diamond and Gordon 1997; Luo and Miller 1998; Goodlett and Horn 2001; Olney 2004). These alcohol-induced alterations may underlie many of the behavioral deficits observed in FAS. Despite attempts to increase public awareness of the risks involved, increasing numbers of women are drinking during pregnancy (Ebrahim et al. 1999). Therefore, it is important to develop strategies that prevent or ameliorate alcohol-induced damages to the brain.

Anthocyanins are a group of natural phenolic compounds responsible for the coloring of many plants and fruits. Evidence suggests that anthocyanins may serve as a natural antioxidant (Prior 2003; Bagchi et al. 2004). Anthocyanins are reported to have many beneficial effects on human health, including reducing the risk of cardiovascular diseases, anti-inflammatory and anti-carcinogenic functions, involvement in diabetes prevention and vision improvement (Hou 2003; Bagchi et al. 2004; Ghosh and Konishi 2007; Zafra-Stone et al. 2007). Berry anthocyanins are beneficial in reducing age-associated oxidative stress, as well as in improving neuronal and cognitive brain function (Hou 2003; Bagchi et al. 2004). Cyanidin is the most common anthocyanin; it is present in 90% of fruits (Prior 2003). Cyanidin glucosides tend to have higher antioxidant capacity than peonidin or malvidin glucosides (Prior 2003). We purified cyanidin-3-glucoside (C3G) from blackberries and demonstrated that it has potent antioxidant and anti-tumor capacity (Ding et al. 2006). C3G inhibits neoplastic transformation and cancer cell metastasis (Ding et al. 2006). The present study is designed to determine whether C3G is beneficial in protecting ethanol neurotoxicity. We examined the effect of ethanol and C3G on the differentiation of mouse neuroblastoma Neuro2a (N2a) cells, a widely used in vitro model of neuronal differentiation. In response to serum starvation or treatment with retinoic acid (RA) or dibutyryl cAMP, N2a cells undergo neuronal differentiation characterized by cell cycle arrest, neurite outgrowth and up-regulation of neurofilament (NF) proteins (De Girolamo et al. 2000; Dasgupta and Milbrandt 2007).

We demonstrate here that ethanol inhibits neurite outgrowth and the expression of NFs in N2a cells. Treatment of C3G antagonizes the inhibitory effect of ethanol. Glycogen synthase kinase 3β (GSK3β), a multifunctional serine/threonine kinase, appears to play an important role in neuronal differentiation and mediates the action of C3G.

Materials and Methods

Materials

The antibodies directed against GSK3β and phospho-GSK3β (Ser9 and Tyr216) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). The antibodies directed against phospho-Tau (Ser396) and actin were purchased from Sigma Chemical Co. (St. Louis, MO). The antibody directed against light-chain NF was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antioxidants N-acetyl-L-cysteine (NAC) and glutathione monoethyl ester (GSH-MEE) were obtained from Calbiochem (La Jolla, CA). C3G was purified from blackberries using high performance liquid chromatography as previously described (Ding et al. 2006). The purity of C3G isolated by this method was 99%.

Cell Culture and Ethanol Exposure Protocol

Neuro2a cells obtained from ATCC were grown in Eagle's MEM containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 25 μg/ml gentamicin, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. A method utilizing sealed containers was used to maintain ethanol concentrations in the culture medium. With this method, ethanol concentrations in the culture medium can be accurately maintained (Luo et al. 2001). A pharmacologically relevant concentration of 400 mg/dl was used in this study. In general, the concentration for in vitro studies is higher than that required to produce a similar effect in vivo (Luo et al. 2001).

Measurement of Neurite Outgrowth

Cells were viewed by phase-contrast microscopy using an inverted microscope. Neurite length was measured as the distance between the center of the cell soma and the tip of its longest neurite. The neurite had to meet the following requirements: it must emerge from an isolated cell (not a clump of cells), it must not contact other cells or neurites, and it must be longer than the diameter of the cell body. For each experiment, 30 cells per well were randomly measured with MetaMorph software (Molecular Devices, Sunnyvale, CA). The measurements were performed in triplicates (3 wells).

Cell Transfection

V5-tagged GSK3β S9A construct (substitution of serine 9 to alanine) carried by vector pcDNA3 was previously

described (Ma et al. 2007). Cell transfection was carried out with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instructions.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS, pH 7.4) and lysed with RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (NP-40), 0.1% sodium dodecylsulfate (SDS), 0.5% deoxycholic acid sodium, 0.1 mg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, and 3% aprotinin] on ice for 10 min, solubilized cells were centrifuged, and the supernatant was collected.

The procedures for immunoblotting and quantitative analysis of proteins imaged in the blots have been previously described (Chen et al. 2004). Briefly, after the protein concentrations were determined, aliquots of the protein samples (20–40 μg) were loaded into the lanes of a SDS-polyacrylamide gel. The protein samples were separated by electrophoresis, and the separated proteins were transferred to nitrocellulose membranes. The membranes were blocked with either 5% BSA or 5% nonfat milk in 0.010 M PBS (pH 7.4) and 0.05% Tween-20 (TPBS) at room temperature for 1 h. Subsequently, the membranes were probed with primary antibodies directed against target proteins for 2 h at room temperature or overnight at 4°C. After three quick washes in TPBS, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (Amersham, Arlington Hts. IL) diluted at 1:2,000 in TPBS for 1 h. The immune complexes were detected by the enhanced chemiluminescence method (Amersham). In some cases, the blots were stripped and re-probed with an anti-actin antibody. The density of immunoblotting was quantified with the software Quantity One (Bio-Rad Laboratories, Hercules, CA). The expression of target proteins was normalized to the levels of actin.

GSK3β Small Interfering RNA (siRNA)

GSK3β siRNA (Catalog # 6301; Cell Signaling Tech., Inc) was transfected into SK-N-MC cells with oligofectamine reagent (Invitrogen) according to the manufacturer's protocol. At 48 h after transfection, the cells were subjected to immunoblotting analysis for GSK3β expression.

Detection of Intracellular Reactive Oxygen Species

Intracellular reactive oxygen species (ROS) levels were measured using the fluorescent dye carboxy-H$_2$DCFDA

staining method as previously described (Chen et al. 2008). Once incorporated into cells, H$_2$DCFDA is converted into a nonfluorescent polar derivative (H$_2$DCF) by cellular esterases. H$_2$DCF is rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein (DCF) by intracellular ROS, mainly H$_2$O$_2$ (LeBel et al. 1992). Therefore, the intensity of the DCF signal reflects the quantity of intracellular ROS. N2a cells were treated with ethanol in the presence or absence of C3G or other antioxidants for the indicated times. After treatment, cells were transferred to luminometer cuvettes containing 10 μM carboxy-H$_2$DCFDA (C400) (Molecular Probes, Inc., Eugene, OR) in pre-warmed PBS buffer for 30 min. Intracellular ROS levels (DCF signals) were measured with a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA) at an emission wavelength of 525 nm.

Statistical Analysis

Differences among treatment groups were tested using analysis of variance (ANOVA). Differences in which P was less than 0.05 were considered statistically significant. In cases where significant differences were detected, specific post-hoc comparisons between treatment groups were examined with Student–Newman–Keuls tests.

Results

GSK3β Mediates Neurite Outgrowth of N2a Cells

Neuronal differentiation of N2a cells was induced by serum starvation or treatment of RA or C3G in the absence of serum (Fig. 1). The percentage of cells extending neurites under these treatment conditions was similar, approximately 70–80% (Fig. 1b). RA or C3G further increased the average length of neurites (Fig. 1c), compared to serum starvation only. The intermediate filaments of neuronal cells NFs are important components of the cytoskeletal network in neurons. High levels of NFs are detected along axons of neurons. NFs consist of three subunits, NF-H, NF-M, and NF-L, corresponding to heavy, medium, and light chains, respectively. Consistent with morphological differentiation, light-chain neurofilament (NF-L, 68 kDa) was up-regulated by serum starvation or treatment with RA or C3G (Fig. 1d and e). It was noted that RA- or C3G-mediated upregulation of NF-L expression was more than that induced by serum starvation.

Previous studies suggested that GSK3β may negatively regulate the differentiation of N2a cells (García-Pérez et al. 1999; Orme et al. 2003). We confirmed this and showed an inhibitor of GSK3β, lithium, promoted neurite outgrowth
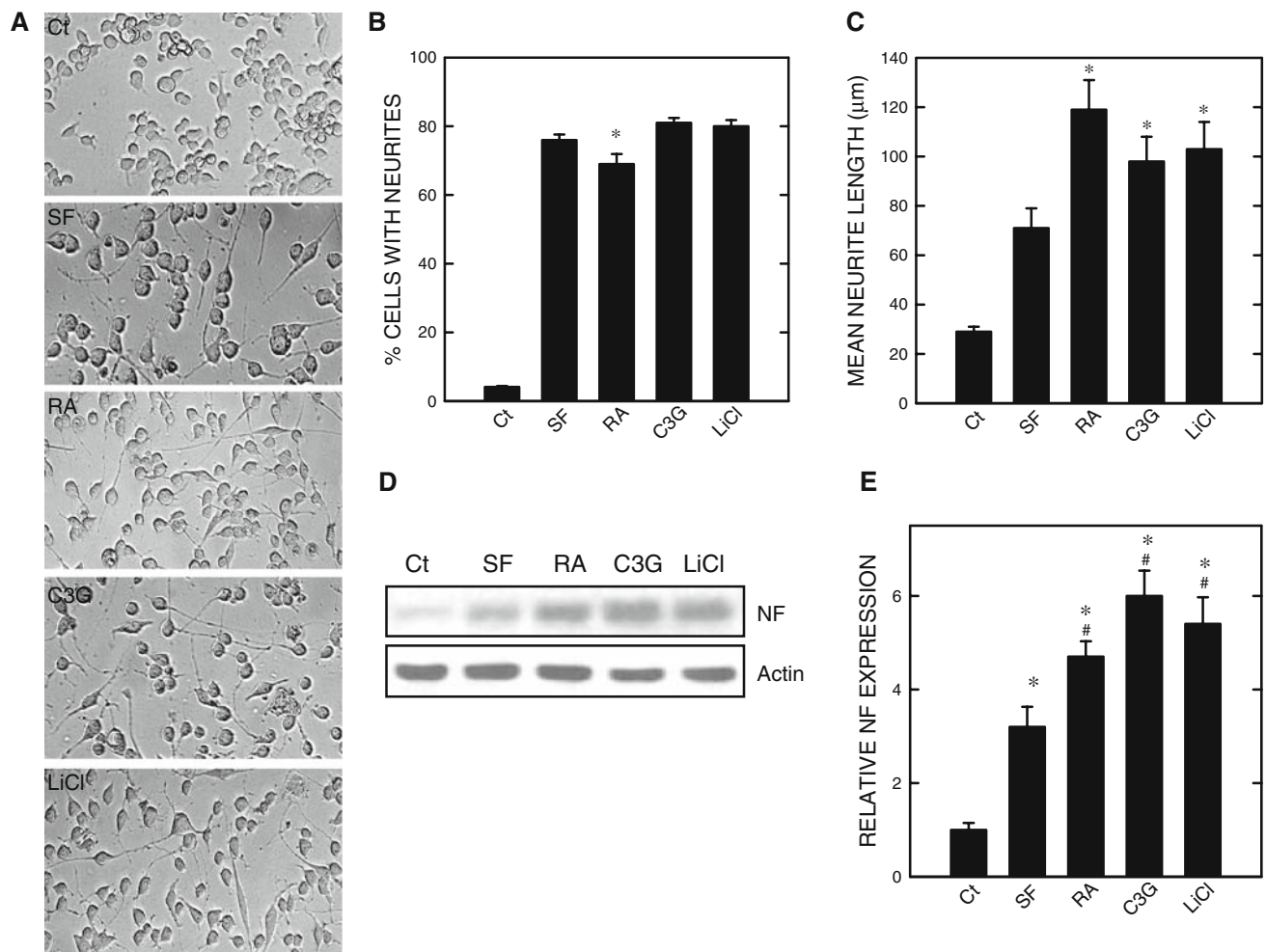
Fig. 1 Regulation of neurite outgrowth of N2a cells. N2a cells were maintained in a culture medium containing 10% serum. To induce differentiation, the culture medium was removed and replaced with a new medium containing 0% serum (SF) supplemented with/without retinoic acid (RA, 5 μM), cyanidin-3-glucoside (C3G, 5 μM), or LiCl (10 mM). **a**. At 48 h after serum starvation (SF) and treatment of RA, C3G, or LiCl, cell morphology was examined under an inverted light microscope. **b**. The percentage of cells baring neurites in 100 cells was calculated as described under the section "Materials and Methods". **c**. The average length of neurites was determined. For each experiment, the result was the mean of 100 cells. * denotes significant difference from control or SF-treated cultures. The experiment was replicated three times. **d**. Cell lysates were collected and the expression of light-chain neurofilament (NF) was determined by immunoblotting. The experiment was replicated three times. **e**. The relative amounts of NF were measured by densitometry and normalized to the expression of actin. * denotes significant difference from SF-treated cultures

and expression of NF-L (Fig. 1). We sought to determine whether treatment of RA or C3G inhibited $GSK3\beta$ activity. The activity of $GSK3\beta$ is regulated by its phosphorylation status; phosphorylation at serine 9 inhibits $GSK3\beta$ activity while phosphorylation at tyrosine 216 (Tyr216) activates $GSK3\beta$. Both RA and C3G induced phosphorylation of $GSK3\beta$ at Ser9 [p-$GSK3\beta$(Ser9)] without affecting p-$GSK3\beta$(Tyr216) (Fig. 2). However, the time sequences of p-$GSK3\beta$(Ser9) induced by RA and C3G treatment were different. RA-induced p-$GSK3\beta$(Ser9) expression was not evident until 6 h of exposure. C3G produced a much more rapid and stronger increase in p-$GSK3\beta$(Ser9) expression; C3G-induced p-$GSK3\beta$(Ser9) occurred at 0.5 h after

exposure. Inhibition of $GSK3\beta$ activity by these treatments was confirmed by a dephosphorylation of its substrate Tau at serine 396 [p-Tau(Ser396)] (Fig. 2). Generally, the expression levels of p-$GSK3\beta$(Ser9) inversely correlated to p-Tau(Ser396). LiCl directly inhibits $GSK3\beta$ activity and also stimulates $GSK3\beta$ phosphorylation at serine 9 (Zhang et al. 2003); its inhibition on $GSK3\beta$ activity in N2a cells was verified (Fig. 2). To further establish the role of $GSK3\beta$ and the effect of p-$GSK3\beta$(Ser9) on neurite outgrowth, we transfected N2a cells with a constitutively active S9A $GSK3\beta$ mutant construct. This S9A construct functions as a dominant active protein (Ma et al. 2007). As shown in Fig. 3, over-expression of the S9A $GSK3\beta$
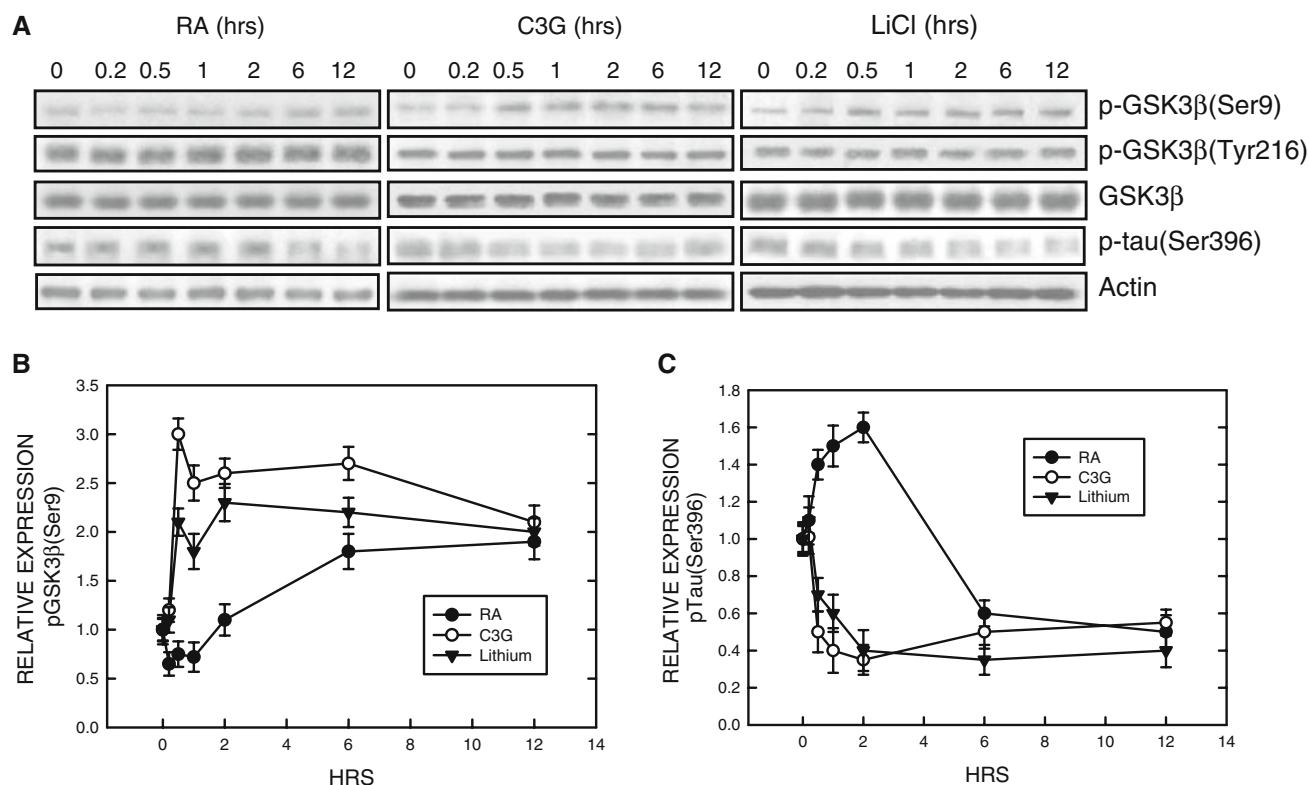
Fig. 2 Regulation of GSK3β phosphorylation. **a**. N2a cells grown in a medium containing 0% serum were treated with retinoic acid (RA, 5 μM), cyanidin-3-glucoside (C3G, 5 μM), or LiCl (10 mM) for specified times. Cell lysates were collected and the expression of phosphorylated GSK3β [p-GSK3β(Ser9) and p-GSK3β(Tyr216)], p-Tau (Ser396) and total GSK3β was examined with immunoblotting.

The blots were stripped and re-probed with an anti-actin antibody. The experiment was replicated three times. **b**. The relative amounts of phosphorylated GSK3β(Ser9) were measured by densitometry and normalized to the expression of total GSK3β. Each data point (mean $\pm$ SEM) is the mean of three independent experiments. **c**. The relative amounts of p-Tau(Ser396) were measured as described above

mutant inhibited neurite outgrowth induced by all treatments (serum starvation, RA or C3G) (Fig. 3). Together, these results indicated that inhibition of GSK3β by stimulating p-GSK3β(Ser9) promoted N2a cell differentiation.

C3G Protects N2a Cells Against Ethanol Inhibition of Neurite Outgrowth by Modulating GSK3β Activity

Ethanol inhibited serum starvation and RA-induced neurite outgrowth as well as the expression of NF-L (Fig. 4). Ethanol decreased both the percentage of cells baring neurites and the average length of neurites. Consistent with its inhibition of neurite outgrowth, ethanol also reduced the expression of NF-L (Fig. 4d). However, when C3G was included, ethanol-mediated inhibition of neuronal differentiation was reversed. C3G not only completely blocked the ethanol-induced decrease in the percentage of cells baring neurites (Fig. 4b), but it significantly inhibited ethanol-mediated reduction in neurite length (Fig. 4c).

Consistent with the results on neurite outgrowth, C3G blocked ethanol-mediated down-regulation of NF-L expression (Fig. 4d).

Serum starvation caused an initial and brief dephosphorylation of p-GSK3β(Ser9), followed by an increase in p-GSK3β(Ser9) at 2 h and 6 h (Fig. 5a). As expected, the levels of p-Tau(Ser396) inversely correlated to that of p-GSK3β(Ser9). Serum starvation did not affect p-GSK3β(Tyr216) and the expression of GSK3β. Ethanol exposure antagonized serum starvation- and RA-induced up-regulation of p-GSK3β(Ser9) (Fig. 5b). However, C3G-treated cells were resistant to ethanol-caused dephosphorylation of p-GSK3β(Ser9). Although ethanol antagonized RA-induced up-regulation of p-GSK3β(Ser9), inclusion of C3G completely reversed ethanol antagonism.

We further investigated the role of GSK3β in ethanol-induced repression of neurite outgrowth using a GSK3β siRNA and lithium. As shown in Fig. 6a, GSK3β siRNA effectively knocked down the expression of GSK3β. More importantly, both GSK3β siRNA and lithium significantly
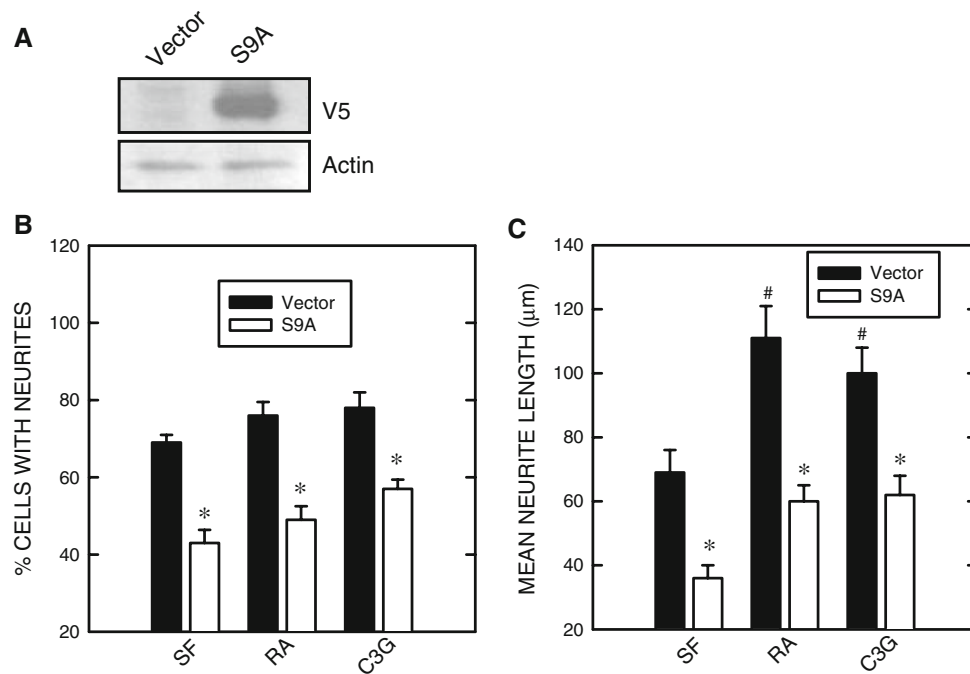
Fig. 3 Effect of GSK3β mutant (S9A) on neurite outgrowth. **a**. N2a cells were transfected with either a construct of GSK3β mutant (S9A) carrying a V5 tag or an empty vector. Forty-eight hours after transfection, cell lysates were collected and the expression of S9A GSK3β was determined by immunoblotting using an anti-V5 antibody. **b**. N2a cells were transfected with either a GSK3β mutant S9A construct or an empty vector. Twenty-four hours after transfection, cells were induced to differentiate as described under Fig. 1. The percentage of cells baring neurites was calculated. **c**. The average length of neurites was determined. For each experiment, the result was the mean of 100 cells. The experiment was replicated three times. * denotes significant difference from vector-transfected cultures. # denotes significant difference from vector-transfected and SF-treated cultures

ameliorated ethanol-mediated inhibition of neurite outgrowth in N2a cells (Fig. 6b).

The Antioxidant Property of C3G is Minimally Involved in Its Neuroprotective Effect

We have previously shown that ethanol causes intracellular accumulation of ROS and induces oxidative stress (Chen et al. 2008). Since C3G is a potent antioxidant (Ding et al. 2006), we sought to determine whether the neuroprotective effect of C3G was mediated by blocking ethanol-induced ROS production. As shown in Fig. 7a, both C3G and antioxidant NAC effectively inhibited ethanol-induced intracellular ROS accumulation. Unlike C3G, however, NAC did not promote neurite outgrowth (data not shown). NAC only modestly mitigated ethanol-induced reduction in a percentage of cells baring neurites, but did not alter ethanol's inhibition on the growth of neurites (average neurite length) (Fig. 7b). Furthermore, NAC had little effect on ethanol-induced dephosphorylation of p-GSK3β(Ser9) (Fig. 7c). These results indicated that the neuroprotective effect of C3G was minimally mediated by its antioxidant property.

Discussion

We demonstrate that GSK3β is a negative regulator of N2a cell differentiation. GSK3β activity is inhibited during serum starvation- or RA-induced differentiation. GSK3β, a multifunctional serine/threonine kinase, regulates diverse biological processes, including glycogen metabolism, insulin signaling, cytoskeleton modification, transcription activation, and cell cycle/survival regulation. The activity of GSK3β is regulated by site-specific phosphorylation. Full activity of GSK3β generally requires phosphorylation at Tyr216, and conversely, phosphorylation at serine 9 (Ser9) inhibits GSK3β activity. Ser9 phosphorylation is probably the most important regulatory mechanism of GSK3β activation. Our results indicate that serum starvation or RA treatment inhibits GSK3β activity by promoting p-GSK3β(Ser9). Inhibition of GSK3β activity by lithium also induces neurite outgrowth in N2a cells. Lithium-mediated promotion of neurite outgrowth in N2a cells has been previously reported (García-Pérez et al. 1999; Orme et al. 2003). Contrarily, activation of GSK3β by over-expressing a constitutively active GSK3β mutant (S9A) significantly inhibits serum starvation- and RA-induced
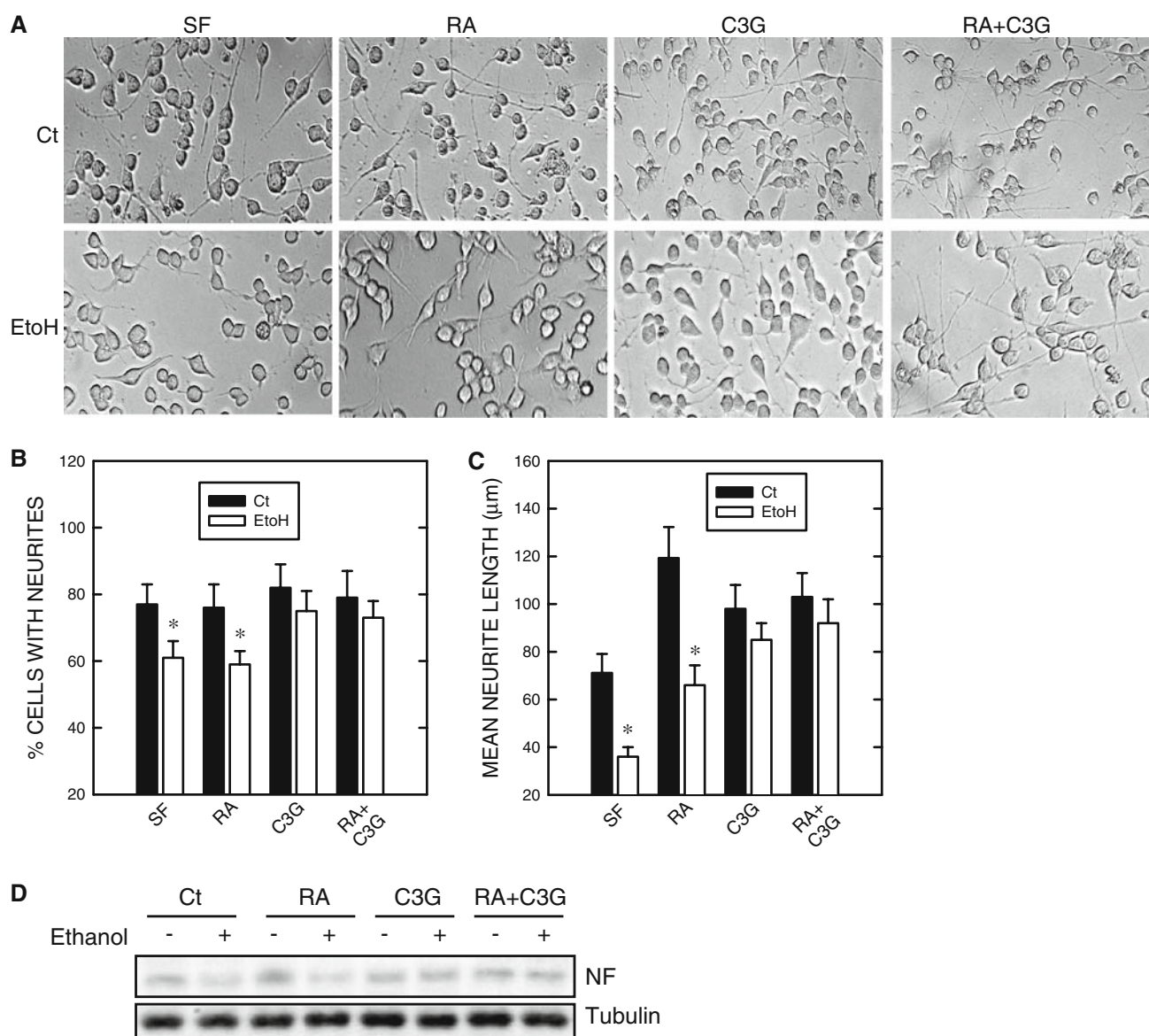
Fig. 4 Effect of ethanol on neurite outgrowth. N2a cells grown in a medium containing 0% serum were treated with retinoic acid (RA, 5 μM), cyanidin-3-glucoside (C3G, 5 μM), or both (5 μM of each) in the absence or presence of ethanol (400 mg/dl) for 48 h. **a** Cell morphology was examined under an inverted light microscope. **b** The percentage of cells baring neurites was calculated. **c** The average length of neurites was determined. * denotes significant difference from non-ethanol-treated controls. **d** Cell lysates were collected and the expression of light-chain neurofilament (NF) was determined by immunoblotting. The experiment was replicated three times

neurite outgrowth (Fig. 3). Therefore, GSK3β negatively regulates neurite outgrowth of N2a cells. Studies using primary neurons also indicate the involvement of GSK3β in the regulation of neurite outgrowth and neuronal differentiation. For example, lithium is shown to enhance neurite outgrowth of cultured cerebellar granule neurons, while expression of constitutively active GSK3β blocks neurite outgrowth (Muñoz-Montaño et al. 1999). Inhibition of GSK3β by selective inhibitors, a dominant-negative form of GSK3β or genetic knockdown promotes dendrite initiation and growth in cultured cortical neurons and

hippocampal neurons in slice cultures (Naska et al. 2006). However, one report suggests that GSK3β may be a positive regulator of neuronal differentiation under certain circumstances because lithium is shown to inhibit neurite outgrowth in cultured hippocampal neurons generated from 18-day rat embryos (Takahashi et al. 1999).

We are the first to demonstrate that GSK3β mediates the effect of ethanol on neurite outgrowth. Ethanol exposure not only decreases the percentage of cells baring neurites, but also reduces the length of those neurites. Ethanol-induced inhibition of neurite outgrowth is likely mediated by its
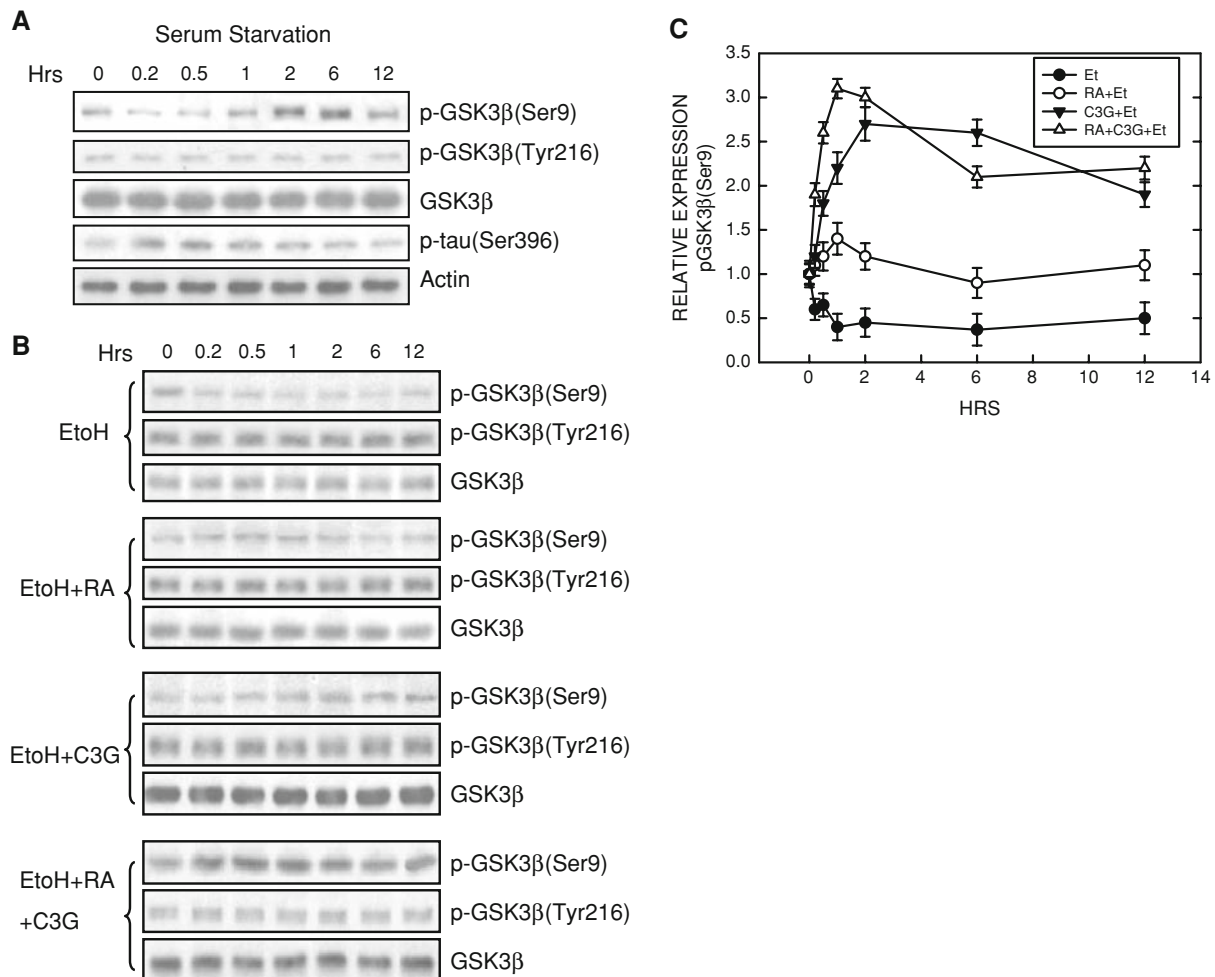
Fig. 5 Effect of ethanol on GSK3β phosphorylation. **a**. Serum starvation was initiated. At specified times after serum starvation, cell lysates were collected and the expression of phosphorylated GSK3β [p-GSK3β(Ser9) and p-GSK3β(Tyr216)], p-Tau (Ser396) and total GSK3β was examined with immunoblotting. **b**. N2a cells grown in a medium containing 0% serum were treated with retinoic acid (RA, 5 μM), cyanidin-3-glucoside (C3G, 5 μM), or both in the absence or presence of ethanol (400 mg/dl) for specified times. Cell lysates were collected and the expression of phosphorylated GSK3β [p-GSK3β(Ser9) and p-GSK3β(Tyr216)] and total GSK3β was examined with immunoblotting. The experiment was replicated three times. **c**. The relative amounts of p-GSK3β(Ser9) were measured by densitometry and normalized to the expression of total GSK3β. Each data point (mean ± SEM) is the mean of three independent experiments

modulation of GSK3β activity. Ethanol dephosphorylates GSK3β at serine 9 without affecting p-GSK3β(Tyr216). Although it remains unclear whether GSK3β is a direct target of ethanol action, evidence from both in vitro and in vivo studies suggest that it may mediate ethanol neurotoxicity. Ethanol exposure during development activates GSK3β in cerebellar neurons probably by impairing insulin signaling (de la Monte and Wands 2002). Furthermore, lithium is shown to reduce ethanol-induced caspase-3 activation in the developing brain and apoptosis in cultured neurons (Zhong et al. 2006; Chakraborty et al. 2008).

We revealed that C3G reverses ethanol-induced inhibition of neurite outgrowth. C3G treatment is sufficient to promote N2a cell differentiation; this is consistent with its inhibition of GSK3β activity. C3G drastically up-regulates

p-GSK3β(Ser9), and over-expressing a constitutively active GSK3β mutant (S9A) significantly inhibits C3G-induced neurite outgrowth (Fig. 3). These results indicate that C3G-induced promotion of neurite outgrowth is also mediated by its inhibition on GSK3β. More importantly, C3G-treated cells are more resistant to ethanol-induced inhibition of neurite outgrowth as well as NF expression (Fig. 4). Ethanol blocks RA-induced up-regulation of p-GSK3β(Ser9) but fails to inhibit C3G-mediated promotion of p-GSK3β(Ser9). Thus, the resistance is likely due to C3G-mediated p-GSK3β(Ser9) up-regulation being unaffected by ethanol (Fig. 5). It is unclear why ethanol inhibits p-GSK3β(Ser9) regulated by RA but not that regulated by C3G. Several up-stream kinases are capable of regulating p-GSK3β(Ser9), including p70 S6 kinase, extracellular

signal-regulated kinases (ERKs), p90Rsk (also called
MAPKAP kinase-1), protein kinase B (also called Akt),
certain isoforms of protein kinase C (PKC), and cyclic
AMP-dependent protein kinase (protein kinase A) (Grimes
and Jope 2001). In addition, p-GSK3β(Ser9) is subject to
regulation by protein phosphatases, such as PP2A and
PP2B (Lee et al. 2005). It is likely that the mechanism
underlying C3G regulation of p-GSK3β(Ser9) is different
from that by RA or serum starvation. This possibility is
currently under investigation.

It has been demonstrated that ethanol produces ROS
(Chen et al. 2008), and C3G is a potent antioxidant (Ding
et al. 2006). Like other commonly used antioxidants, such
as NAC or GSH-MEE, C3G is effective at scavenging
ethanol-induced ROS production (Fig. 6). NAC or GSH-
MEE, however, fail to promote p-GSK3β(Ser9) and induce
neurite outgrowth of N2a cells (data not shown). Unlike
C3G, these antioxidants do not override ethanol-induced
dephosphorylation of GSK3β at serine 9 (Fig. 6). As a
result, these antioxidants do not offer protection against
ethanol-induced inhibition of neurite outgrowth (Fig. 6).
These results suggest that antioxidant properties of C3G
are minimally involved in its modulation of neurite
outgrowth.

Research suggests anthocyanins may exert their benefi-
cial effects either through their ability to lower oxidative
stress and inflammation or by directly altering the signaling
involved in neuronal communication, calcium buffering

ability, neuroprotective stress shock proteins, plasticity,
and stress signaling pathways (Shukitt-Hale et al. 2008).
Regardless of the mechanisms of C3G's action, our study
supports that C3G is beneficial in ameliorating alcohol
neurotoxicity in vitro. Anthocyanins found in berry fruits
have been implicated in improving aged-related neuronal
and behavioral deficits in animal models (Galli et al. 2002;
Andres-Lacueva et al. 2005; Shukitt-Hale et al. 2007,
2008). Anthocyanins are also shown to protect neurons
against focal cerebral ischemic injury in rat brains (Swee-
ney et al. 2002; Shin et al. 2006).

Anthocyanins are able to cross the blood–brain barrier
and localize in various brain regions important for motor,
learning and memory functions. In a study where rats are
fed a blueberry supplementation or a control diet for
8–10 weeks, several anthocyanins (cyanidin-3-O-beta-
galactoside, cyanidin-3-O-beta-glucoside, cyanidin-3-O-
beta-arabinose, malvidin-3-O-beta-galactoside, malvidin-3-
O-beta-glucoside, malvidin-3-O-beta-arabinose, peonidin-
3-O-beta-arabinose, and delphinidin-3-O-beta-galactoside)
are found in the cerebellum, cortex, hippocampus or stria-
tum of rats, but not in the controls (Andres-Lacueva et al.
2005). In another study where rats are fed a blackberry
anthocyanin-enriched diet for 15 days, C3G is the predom-
inant form of anthocyanins detected in the brain
homogenate, and C3G content is higher in the brain than in
the plasma (Talavéra et al. 2005). Peripheral C3G is able to
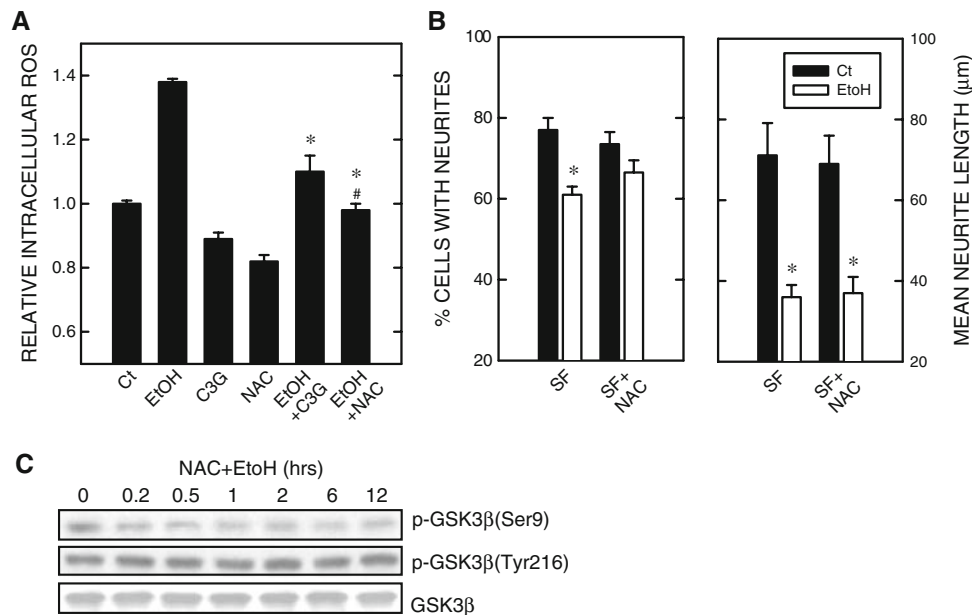cross the blood–brain barrier and distribute in brain regions

Fig. 7 Effect of antioxidants on ethanol-induced inhibition of neurite outgrowth. **a** N2a cells were pretreated with C3G (5 μM) or antioxidant NAC (10 mM) for 30 min, then exposed to ethanol (0 or 400 mg/dl) for 6 h. Intracellular reactive oxygen species (ROS) were measured as described under the section "Materials and Methods". ROS levels were expressed relative to untreated controls. Each data point represents the mean of three independent experiments. * denotes significant difference from ethanol-treated cultures. # denotes significant difference from ethanol + C3G group. **b** N2a cells were pretreated with antioxidant NAC (10 mM) for 30 min, then induced to differentiate by serum starvation (SF) in the presence or absence of ethanol (400 mg/dl) for 48 h. The percentage of cells baring neurites (panel on the left) and the average length of neurites (panel on the right) were determined as described under Fig. 1. The experiment was replicated three times. * denotes significant difference from non-ethanol-treated controls. **c** N2a cells were pretreated with antioxidant NAC (10 mM) for 30 min, then subject to serum starvation (SF) and ethanol exposure (400 mg/dl) for specified times. Cell lysates were collected and the expression of phosphorylated GSK3β [p-GSK3β(Ser9) and p-GSK3β(Tyr216)] and total GSK3β was examined with immunoblotting. The experiment was replicated three times

that are affected by ethanol. It is therefore a feasible candidate with therapeutic potential for the treatment of alcohol neurotoxicity.

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