Role of glycogen synthase kinase-3β in ketamine-induced developmental neuroapoptosis in rats

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Editor’s key points

- Ketamine increased neuroapoptosis in neonatal rat pups, which correlated with decreased GSK-3β phosphorylation.
- This suggests involvement of this cell survival signalling pathway in anaesthetic neurotoxicity.
- Co-administration of lithium mitigated ketamine-induced neurotoxicity and reduction in GSK-3β phosphorylation, providing a potential approach to neuroprotection.

Background. Ketamine-induced neuroapoptosis has been attributed to diverse stress-related mechanisms. Glycogen synthase kinase-3β (GSK-3β) is a multifunctional kinase that is active in neuronal development and linked to neurodegenerative disorders. We hypothesized that ketamine would enhance GSK-3β-induced neuroapoptosis, and that lithium, an inhibitor of GSK-3β, would attenuate this response in vivo.

Methods. Protein levels of cleaved caspase-3, protein kinase B (AKT), GSK-3β, and cyclin D1 were measured in post-natal day 7 rat pups after 1.5, 3, 4.5, and 6 h exposure to ketamine. A cohort of rat pups was randomized to a 6 h exposure to ketamine with and without lithium. Neuroapoptosis was measured by cleaved caspase-3 and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling staining by immunohistochemistry. Protein levels of cleaved caspase-3 and -9 and the total and phosphorylated forms of AKT, GSK-3β, and cyclin D1 (cell cycle protein) were also measured.

Results. Ketamine produced a duration-dependent increase in cleaved caspase-3 and cyclin D1, which corresponded to decreases in phosphorylated AKT and GSK-3β. Co-administration of lithium with ketamine attenuated this response.

Conclusions. Ketamine-induced neuroapoptosis is associated with a temporal decrease in GSK-3β phosphorylation, and simultaneous administration of lithium mitigated this response. These findings suggest that GSK-3β is activated during this ketamine-induced neuroapoptosis.

Keywords: AKT; apoptosis; glycogen synthase kinase-3β; ketamine; lithium; protein kinase B

Accepted for publication: 23 January 2013

Ketamine, a phencyclidine derivative, is a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) type glutamate receptor, and is commonly used for surgery and painful procedures in paediatric patients. Data from recent reports clearly show that ketamine induces neurotoxicity in animal models. Ketamine-induced developmental neurotoxicity is characterized by neuronal apoptosis, changes in dendritic morphology, and subsequent behavioural deficits. These diverse changes in cellular and behavioural features are also the hallmark of several neurodegenerative and mood disorders. Experimental models have implicated glycogen synthase kinase-3β (GSK-3β) signalling in these disorders. Given the similarities between ketamine-induced neurotoxicity and neurodegenerative and mood disorders, the role of GSK-3β in the former warranted investigation.

GSK-3 is a serine/threonine kinase, and is a regulator of cell metabolism in mammals. It regulates neurogenesis, neuronal polarization, and axon growth in the developing central nervous system. It is constitutively active in all tissues and peak expression occurs up to the 10th post-natal day in rats. In contrast to most protein kinases, GSK-3β activity is primarily inhibited by phosphorylation of the serine 9 residue by protein kinase B (AKT). Therefore, phosphorylation of the serine 9 residue renders GSK-3β non-functional such that increased levels of the phosphorylated GSK-3β at serine 9 (pGSK-3β) correspond to decreased GSK-3β activity. Ketamine increases apoptosis and GSK-3β activity in neuronal cell culture models. Selective inhibition of GSK-3β attenuates this apoptotic response. We previously reported that ketamine-induced neuroapoptosis leads to aberrant
cell cycle reentry by increasing cyclin D1. GSK-3β is a potent modulator of cyclin D1 transcription, and lithium, a selective inhibitor of GSK-3β, decreases cyclin D1 expression. Lithium also protects against ketamine-induced neuroapoptosis in neonatal mice. In the present study, we tested the hypothesis that ketamine increases GSK-3β activity and lithium counters this response. We measured the neuroapoptotic response to ketamine with and without lithium and the corresponding levels of phosphorylated AKT (pAKT) and pGSK-3β, and of cyclin D1.

Methods

Animal treatment

Experiments were approved by the Boston Children’s Hospital Institutional Review Board, and adhered to the Guide for the Care and Use of Laboratory Animals. We utilized pathogen-free Sprague–Dawley post-natal day 7 (P7) rat pups for all experimental procedures (Charles River Laboratories, Wilmington, MA, USA). Ketamine, pentobarbital, and LiCl were obtained from Sigma-Aldrich (St Louis, MO, USA). Rat pups were kept from their dam and visually monitored for respiratory effort and activity. Treatments were conducted in a temperature-controlled acrylic container maintained at 36.7°C. Similar conditions resulted in core body temperatures of 36.5–37.5°C. Exposure to ketamine increases neurodegeneration in P7 rat pups; we used a similar dosing regimen to determine the temporal effect of ketamine on neuroapoptosis and activation of GSK-3β. P7 rat pups were randomly divided into five groups (n=6 per group) receiving four intraperitoneal (i.p.) injections (10 ml kg⁻¹ of either ketamine (2 mg ml⁻¹) or vehicle (saline) at 90 min intervals over 6 h, with Group 1 receiving five consecutive doses of saline (control), Group 2 receiving two consecutive doses of ketamine followed by three injections of saline, Group 3 receiving three consecutive doses of ketamine followed by two injections of saline, Group 4 receiving four doses of ketamine followed by one injection of saline, and Group 5 receiving five consecutive doses of ketamine. These dosing regimens are similar to that used in previous investigations with a similar experimental paradigm that yielded a ketamine plasma concentration of (so) 5.80 (3.10) μg ml⁻¹ and brain concentration of 2.65 (1.60) μg g⁻¹.

A second cohort of P7 rat pups were randomly assigned to four groups (n=8 per group): (i) saline (control), (ii) lithium chloride (Li), (iii) lithium and ketamine (Li+KET), and (iv) ketamine (KET). The rats received five i.p. injections (10 ml kg⁻¹) of saline, lithium (120 mg kg⁻¹), lithium (120 mg kg⁻¹) and ketamine (20 mg kg⁻¹), or ketamine (20 mg kg⁻¹) alone, at 90 min intervals over 6 h. After the 6 h treatment, the rats were euthanized with pentobarbital (100 mg kg⁻¹, i.p.). The brains from each group (n=4 per group) were rapidly isolated, frozen in liquid nitrogen, and processed for protein analysis. Rat pups from a separate cohort (n=4 per group) were anaesthetized with pentobarbital (100 mg kg⁻¹ i.p.) and immediately perfused with saline followed by 4% paraformaldehyde. The brains were subsequently embedded in paraffin for histological and immunohistochemical processing.

Immunoblotting

Protein was extracted from flash-frozen brain tissue, separated by polyacrylamide gel electrophoresis, and transferred to membranes as described. Primary antibodies for cleaved caspase-3, cleaved caspase-9, AKT, phospho AKT (Ser 473), GSK-3β, phospho GSK-3β (Ser 9), cyclin D1, and β-actin (Cell Signaling Technology, Beverly, MA, USA) were incubated with the membrane overnight at 4°C with slow shaking, and horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies were incubated at room temperature for an additional 2 h. Immunoreactive bands in membranes were visualized by enhanced chemiluminescence (Thermol Scientific, Rockford, IL, USA) and densities were quantified with Image J 1.42 (NIH, Bethesda, MD, USA).

Immunohistochemical staining for cleaved caspase-3

Expression of cleaved caspase-3 was determined in brain tissue sections by immunohistochemistry. Sections of rat brain tissue were deparaffinized and rehydrated with distilled water. Endogenous peroxidases were inactivated by immersing the sections in hydrogen peroxide for 10 min, and then were incubated for 10 min with 10% normal goat serum to block non-specific binding. The sections were incubated at 4°C overnight with an anti-cleaved caspase-3 antibody and then with a biotinylated anti-rabbit IgG and peroxidase-conjugated streptavidin (ZYMED Laboratory, Inc., Carlsbad, CA, USA) followed by a chromogenic reaction by 3,3′-diaminobenzidine (DAB). All sections were counterstained with haematoxylin. The same protocol was applied to negative control slides with omission of the primary antibody. The number of positive cells was counted (magnification of ×400) in a double-blinded manner in seven adjacent fields in the somatosensory cortex in three brain sections from each animal.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling assay

Cell apoptosis was also determined using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) assay (Millipore, Serological Corporation, Norcross, GA, USA). Brain sections were deparaffinized and rehydrated. After treatment with proteinase K (20 μg ml⁻¹) (Roche Applied Science, Indianapolis, IN, USA) and quenching with 3.0% hydrogen peroxide, sections were incubated in a terminal deoxynucleotidyl transferase (TdT) reaction mix for 1 h at 37°C. Sections were washed, then incubated for 30 min in a solution of anti-digoxigenin conjugate, and colorized with DAB. All sections were counterstained with haematoxylin. The TdT reaction mix was omitted for control sections. TUNEL-positive cells were counted in a double-blinded manner from randomly selected sampling areas.
Statistical analysis
Changes in cleaved caspase-3 and -9 and cyclin D1 levels are presented as percentage of the control value. AKT and GSK-3β ratios of phosphorylated to total forms are reported. Cleaved caspase-3 and TUNEL-positive cells are presented as absolute values per microscopic field. Data are expressed as mean [standard error of the mean (SEM)]. Differences in TUNEL, cleaved caspase-3, AKT, GSK-3β, and cyclin D1 levels were analysed by one-way analysis of variance, followed by a post hoc Dunnett’s test or Newman–Keuls test for individual comparisons. Data analyses were generated using SPSS for Windows version 19.0 (SPSS Inc., Chicago, IL, USA) and Prism 5 (GraphPad Software, La Jolla, CA, USA). Statistical significance was set at $P<0.05$, and all $P$-values were unadjusted for multiple comparisons.

Results
Ketamine temporally increased caspase-3 activation and decreased phosphorylation of GSK-3β
Analysis of cleaved caspase-3, -9, and total and phosphorylated AKT and GSK-3β in brain protein lysates from the five time-dependent groups is shown in Figure 1. Ketamine significantly increased cleaved caspase-3 in a time-dependent manner. Cleaved caspase-9 protein also significantly increased at 6 h treatment. After 3 h treatment, ketamine also significantly decreased pAKT and pGSK-3β in comparison with control.

Lithium attenuated ketamine-induced neuroapoptosis
Apoptosis was determined in brain slices by cleaved caspase-3 immunohistochemistry (Fig. 2). Cleaved caspase-3-positive cell counts were 33-fold higher in the cortices of ketamine-treated rat pups. Lithium in combination with ketamine significantly decreased four-fold cleaved caspase-3 compared with the ketamine only group. The presence of apoptotic cells in brain slices was confirmed with a TUNEL assay (Fig. 3). Ketamine significantly increased the number of TUNEL-positive cells by 45.5-fold compared with control. Lithium in combination with ketamine significantly decreased the number of TUNEL-positive cells in comparison with the ketamine alone group. Lithium alone did not significantly alter the number of TUNEL-positive cells.

Ketamine at a dose of 20 mg kg⁻¹ significantly increased cleaved caspase-3, -9 levels, thereby confirming activation of the intrinsic apoptotic pathway (Fig. 4). There were 1.5- and 1.8-fold increases in cleaved caspase-3 and -9, respectively, in the ketamine alone group in comparison with the control group. When compared with the ketamine only group, addition of lithium significantly decreased cleaved caspase-3 and -9.

Lithium attenuated ketamine-induced decreases in phosphorylation of AKT and GSK-3β
pAKT expression in the ketamine group was less than in control (Fig. 5). When P7 rats were treated with lithium or ketamine and lithium, pAKT was significantly increased compared with the ketamine only group. pAKT was increased in
the lithium plus ketamine and lithium alone groups compared with control. Ketamine significantly decreased expression of pGSK-3β. Lithium in combination with ketamine significantly increased pGSK-3β compared with the ketamine alone group.

**Lithium attenuated ketamine-induced increases in cyclin D1 expression**

Ketamine significantly increased the expression of cyclin D1 1.8-fold when compared with control (Fig. 6). Lithium in combination with ketamine significantly decreased the expression of cyclin D1 compared with the ketamine alone group, while lithium alone increased cyclin D1 expression.

**Discussion**

Ketamine treatment of P7 rat pups increased neuroapoptosis and decreased phosphorylation of GSK-3β. Consistent with an increase in GSK-3β activity. Ketamine combined with lithium significantly attenuated neuroapoptosis and dephosphorylation of GSK-3β. GSK-3β is one of a few protein kinases that is inactivated by phosphorylation. Aberrant activation of GSK-3β has also been linked to neurological and psychiatric disorders such as schizophrenia and bipolar disorder.

The AKT-GSK-3β pathway is a major cell signaling pathway that plays an important role in neuronal development and survival. GSK-3β is widely expressed in all organs, and is particularly abundant in the brain. It also plays a prominent role during neural development because it controls neuronal progenitor cell proliferation and the establishment of neuronal polarity. Both of these processes are disrupted during ketamine exposure in developing neuronal cell culture models. Pharmacological antagonism of NMDA receptors alters the AKT-GSK-3β signalling pathway. In the present study, ketamine produced a duration-dependent increase in cleaved caspase-3 and -9 expression, which points to activation of the intrinsic apoptotic pathway (Fig. 1). This concurs with our previous report indicating that the duration of ketamine exposure correlates with increased neuronal degeneration. However, the predicted plasma levels obtained...
from the dosing regimens used in our current and previous reports significantly exceed the peak levels measured in pediatric patients after a single bolus of ketamine.33 The temporal increases in apoptosis detected in our study mirror decreased phosphorylation of AKT and GSK-3β, which implies increased GSK-3β activity and is consistent with previous reports in neuronal cell cultures.13 14 We previously reported that ketamine decreased phosphorylation of AKT and GSK-3β in a model of ketamine administration with concurrent noxious stimulation. However, these changes do not definitively account for the significant alterations in cleaved caspase-3 that were detected in this experimental paradigm, which included an inflammatory noxious stimulation that might affect AKT signalling.23 These observations clearly demonstrate that prolonged ketamine exposure leads to increased GSK-3β activity.

Increased GSK-3β activity has also been related to several chronic neurological and psychiatric disorders such as Alzheimer’s disease, schizophrenia, and mood disorders.8 24 25 GSK-3β might induce neurodegeneration by activating pro-apoptotic mitochondrial derangements and the intrinsic apoptotic pathway.34 Ketamine is associated with schizophrenic and dissociative states in humans.35 Furthermore, specific inhibitors of GSK-3β attenuate the psychotomimetic effects of ketamine in mice.36 However, these are acute reactions to low doses of ketamine and are unlikely due to activation of apoptotic pathways. Lithium, a selective inhibitor of GSK-3β and commonly prescribed drug for mood disorders, protects against ketamine-induced neuroapoptosis in neonatal mice.18 These findings make a compelling argument for attenuating GSK-3β activity in mitigation of the neurotoxic effect of ketamine. We examined the effect of inhibiting GSK-3β activity by administering lithium concurrently with ketamine in rat pups. Consistent with the study in mice,8 we found that lithium reduced the number of apoptotic cells when combined with ketamine. This finding...
corresponded with decreases in cleaved caspase -3 and -9 levels. In order to determine the effect of lithium on the AKT-GSK-3β pathway, we measured the levels of pAKT and pGSK-3β in the four treatment groups. Lithium increased pAKT and pGSK-3β levels when compared with the control and ketamine only groups. This is consistent with the modulatory role of lithium in enhancing phosphorylation of AKT and GSK-3β in neurons. However, it is unclear if the protective effect of lithium in our study is solely due to increased phosphorylation of AKT or attenuation of ketamine-induced dephosphorylation of AKT and GSK-3β. Taken together, the protective effect of lithium against ketamine-induced neuroapoptosis stems from maintenance or augmentation of phosphorylation of AKT and GSK-β, presumably leading to a reduction in GSK-β activity.

We previously reported that ketamine increases cyclin D1 expression, which leads to an aberrant cell cycle reentry and neuronal apoptosis. GSK-3β regulates cyclin D1 activity and lithium disrupts this process. The concurrent administration of lithium to ketamine-treated rats pups resulted in decreased expression of cyclin D1, thereby substantiating the role of the GSK-3β-cyclin D1 pathway in ketamine-induced neuroapoptosis. Lithium increased pAKT, which in turn maintained levels of cyclin D1. These findings indicate that lithium protects against ketamine-induced neuroapoptosis by regulating the AKT-GSK-3β signalling and aberrant cell reentry pathways. However, this putative GSK-β pathway should be considered as one of many parallel neurodegenerative processes that are activated by ketamine exposure during a vulnerable developmental period. Several lines of investigation have implicated other neuronal cell death mechanisms such as excitotoxicity, intracellular calcium dysregulation, and other parallel neurodegenerative pathways in the neurotoxic effect of ketamine on the developing brain.

In summary, our findings showed that ketamine induced neuroapoptosis in the developing rat brain, and lithium significantly attenuated ketamine-induced neuroapoptosis. Inhibition of neuroapoptosis might due to activation of GSK-3β and aberrant cell cycle reentry. These results indicate that ketamine-induced neuroapoptosis regulates the AKT-GSK-3β pathway and shares several established mechanisms associated with other neurodegenerative and mood disorders.

**Declaration of interest**

None declared.

**Funding**

This work was supported by the CHMC Anesthesia Foundation and the Boston Children’s Hospital Endowed Chair in Neuroanesthesia (S.G.S.).

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Handling editor: H. C. Hemnings