Published in final edited form as:

Neurosci Lett. 2018 May 29; 676: 108–112. doi:10.1016/j.neulet.2018.04.026.

Activation of cyclic GMP-dependent protein kinase blocks alcohol-mediated cell death and calcium disruption in cerebellar granule neurons

Dimitrios E. Kouzoukas, PhD^{a,b,*}, Ramesh C. Bhalla, PhD, DVM^a, and Nicholas J. Pantazis, PhD^a

^aDepartment of Anatomy and Cell Biology, The University of Iowa Carver College of Medicine, Iowa City, IA, USA

^bDepartment of Molecular Pharmacology and Therapeutics, Loyola University Chicago Stritch School of Medicine, Maywood, IL USA

Abstract

Alcohol during brain development leads to the widespread neuronal death observed in fetal alcohol spectrum disorders (FASD). In comparison, the mature brain is less vulnerable to alcohol. Studies into maturation-acquired alcohol resistance uncovered a protective mechanism that reduces alcohol-induced neuronal death through nitric oxide-cGMP-cyclic GMP-dependent protein kinase (NO-cGMP-cGK) signaling. However, the downstream processes underlying this neuroprotection remain unclear. Alcohol can disrupt levels of intracellular calcium ($[Ca^{2+}]_i$) in vulnerable neuronal populations to trigger cell death in both *in vivo* and *in vitro* models of FASD. Since cGK has been demonstrated to regulate and inhibit intracellular Ca^{2+} release, we examined the hypothesis that cGK confers alcohol resistance by preventing $[Ca^{2+}]_i$ disruptions. Alcohol resistance, determined by neuronal survival after 24 hours of alcohol exposure, was examined in primary cerebellar granule neuron (CGN) cultures derived from 5 to 7 day-old neonatal mice with an activator, 8-Br-cGMP, and/or an inhibitor, Rp-8-pCPT-cGMPS, of cGK signaling. Intracellular Ca^{2+} responses to alcohol were measured by ratiometric Ca^{2+} imaging in Fura-2-loaded CGN cultures after 8-Br-cGMP treatment. Our results indicate that activating cGK with 8-Br-cGMP before alcohol administration provided neuroprotection, which the cGK inhibitor, Rp-8-pCPT-cGMPS, blocked. Alcohol exposure elevated $[Ca^{2+}]_i$, whereas 8-Br-cGMP pretreatment reduced both the level of the alcohol-induced rise in $[Ca^{2+}]_i$ as well as the number of cells that responded to alcohol by increasing $[Ca^{2+}]_i$. These findings associate alcohol resistance, mediated by cGK signaling, to reduction of the persistent and toxic increase in $[Ca^{2+}]_i$ from alcohol exposure.

*Correspondence & Present Address: Dimitrios E. Kouzoukas, PhD, Department of Molecular Pharmacology & Therapeutics, Loyola University Chicago Stritch School of Medicine, 2160 S. First Avenue, CTRE-415, Maywood, IL 60153, USA, Phone: +1-708-216-9021; Fax: +1-708-216-6596; dkouzoukas@luc.edu, Supported by NIH (AA011577 to Nicholas J. Pantazis, PhD) and The University of Iowa Graduate College.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of interest
None.

Keywords

alcohol; cGMP-dependent protein kinase; fetal alcohol spectrum disorder; intracellular calcium; neuroprotection

Introduction

Fetal Alcohol Spectrum Disorders (FASD) describes a continuum of permanent birth defects triggered by prenatal alcohol (ethanol) exposure and remains the single leading preventable cause of mental retardation [1]. In comparison, the adult brain is less prone to alcohol toxicity, suggesting that neuroprotective mechanisms exist in the mature brain to protect it from alcohol.

Maturation-acquired neuronal resistance has been demonstrated in rodent models of FASD. For example, alcohol exposure in mice and rats during postnatal days 4-5, but not later during postnatal days 8-9, severely depletes cerebellar granule and Purkinje neurons, reducing cerebellar size [2-5]. Since development of alcohol resistance coincides with expression of neuronal maturation markers, this suggests that susceptible neuronal populations in the cerebellum acquire an alcohol resistance as they mature [5]. Cerebellar granule neuron (CGN) cultures derived from neonatal mice also show an innate alcohol vulnerability, which diminishes as the neurons mature in culture [6]. Given the devastating CNS injury that fetal alcohol exposure produces, understanding the mechanisms behind this acquired neuronal alcohol resistance is crucial.

We previously established [7-9] that the alcohol resistance of CGN cultures can be enhanced by activating neuronal nitric oxide synthase (nNOS) to increase intracellular nitric oxide (NO) levels, which in turn stimulates soluble guanylyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP)-dependent protein kinase (cGK) downstream. Activation of either sGC or cGK diminishes alcohol-mediated cell death in CGN cultures, while inhibition of either blocks neuroprotection from NO [7-9]. Deficient nNOS expression in neonatal mice [10, 11] exacerbates the alcohol-induced neuronal loss. Since the acquired alcohol resistance in the rat cerebellum also coincides with a developmental increase of nNOS expression [5, 12, 13], these studies indicate that the NO pathway has a physiological role in providing resistance to alcohol.

Recently, our attention has focused on downstream cGK targets that can transmit the neuroprotective signal provided by the NO pathway. Although one downstream effector of neuroprotection has been revealed to be nuclear factor- κ B (NF- κ B) [14, 15], cGK can influence multiple downstream targets to offer alcohol resistance. Alcohol has been observed in both *in vitro* [16-19] and *in vivo* models of FASD [20, 21] to trigger neuronal death by disrupting intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$). Conversely, cGK has been demonstrated to regulate and inhibit $[\text{Ca}^{2+}]_i$ release in multiple contexts [22, 23], including cerebellar tissue [24].

This evidence has led us to examine the relationship between cGK modulation of $[\text{Ca}^{2+}]_i$ and alcohol-induced neuronal death. In this study, we tested the hypothesis that cGMP activation of cGK can prevent the alcohol-induced $[\text{Ca}^{2+}]_i$ elevations and resulting neuronal

death. Experiments using alcohol-vulnerable CGN cultures examined 1) neuronal survival and 2) $[Ca^{2+}]_i$ following alcohol exposure in the presence of activators and/or inhibitors of cGK signaling. Our findings suggest that cGK mediates alcohol resistance by moderating alcohol's disruption of $[Ca^{2+}]_i$.

Materials and methods

Animals

A breeding colony of C57BL/6 mice (initially obtained from Jackson Laboratories, Bar Harbor, ME) was housed in The University of Iowa Animal Care Facility. All animal experiments were approved by The University of Iowa Institutional Animal Care and Use Committee and performed according to the guidelines of the National Institutes of Health.

Cell culture

Primary CGN cultures offer a well-defined and manageable biological system widely used to investigate the direct neuronal effects of alcohol [25]. These cultures do not proliferate and any cell number reduction is due to decreased neuronal survival [6].

CGN cultures were established from neonatal mice (5–7 days old) as previously described [18]. For all treatment conditions in a single experiment, cultures derived from each mouse litter were established in duplicate or triplicate and constituted one replicate. CGN were plated into poly-D-lysine (PDL, 50 mg/ml)-coated 96-well culture trays (1.5×10^6 cells/ml; 300 μ l/well) for cell survival experiments, and into six-well culture trays (1.0×10^6 cells/ml; 2.0 ml/well) which contained PDL-coated (100 mg/ml) glass coverslips (25 mm diameter) for Ca^{2+} imaging experiments. CGN cultures were incubated overnight at 37°C in humidified air (5% CO_2) prior to use.

Pharmacological treatments and alcohol exposure

Alcohol exposure at 400 mg/dl (87 mM ethanol) was chosen for most experiments which began 24 hours after plating. Although chronic alcoholics can achieve similar blood alcohol concentrations [26], it is a high physiological dose. We previously demonstrated that alcohol elicits Ca^{2+} -dependent cell death in a concentration-dependent manner in CGN cultures and that lower alcohol concentrations (100 and 200 mg/dl) produced cell death to a smaller degree [6, 18]. Since this indicates that higher alcohol concentrations are eliciting the same effect with greater magnitude, 400 mg/dl was used to more readily achieve statistical significance. This alcohol concentration also matches blood alcohol levels often attained in rodent studies of alcohol neurotoxicology [10, 12, 27–29] and our previous *in vitro* studies [8, 9, 18, 30].

Cultures were also treated with one or more of the following: PBS (vehicle control); 8-Br-cGMP, a cGMP analogue that activates cGK, or Rp-8-pCPT-cGMPS, a specific inhibitor of cGK. Exposure occurred 30 minutes before adding alcohol as described. Culture trays in cell survival experiments were placed in sealed containers containing alcohol baths of equal concentration (0 or 400 mg/dl) to that of culture media, 5% CO_2 , and incubated at 37°C.

Cell survival experiments

Twenty-four hours after adding alcohol, viable cells were counted using a dye-exclusion method (trypan blue is excluded by viable cells). CGN cultures suspended in 0.2% trypan blue, were viewed under an inverted phase-contrast microscope (Diaphot; Nikon Instruments Inc., Melville, NY) and counted on a hemocytometer as previously described [18].

Ca²⁺ measurements

Intracellular [Ca²⁺]_i was determined using the ratiometric fluorescent indicator, Fura-2 [31] and a digital imaging microscope system (Deltascan-1; Photon Technology International, Birmingham, NJ) as previously described [18].

Statistical analyses

For cell survival experiments, data were expressed as “cell number/well” and as percent alcohol-induced cell loss [18, 30]. For each replicate, the difference in cell number between alcohol-treated and non-alcohol control cultures is expressed as a percent of the non-alcohol control. This accounts for variability in the natural responses of CGN cultures derived from different litters (replicates) and plating conditions. Statistical differences in cell numbers and percent cell loss were analyzed with repeated measures ANOVAs, with alcohol and test agent concentrations considered as variables with repeated measures within each replicate. Post hoc tests consisted of Bonferroni-corrected paired t-tests and pairwise comparisons in cell number and percent cell loss analyses, respectively.

Given variability in Ca²⁺ measurement experiments, some cells (2.2%) display an exceedingly low or high *baseline* [Ca²⁺]_i (below 10 nM or above 200 nM) prior to alcohol addition and were removed from analyses. A mean [Ca²⁺]_i was calculated at each 5 second time point for each cell in the view-field (39.9 ± 2.1 cells, range of 18 to 50 cells) per coverslip. Cells from experimental replicates were pooled to increase the accuracy of [Ca²⁺]_i calculations. Planned comparisons (Student's t-test) between treatments were made using the maximum [Ca²⁺]_i level (peak [Ca²⁺]_i) each cell attained during the 9 minute testing period [18]. Percentages of cells with large [Ca²⁺]_i responses (attaining peaks of 200 nM or greater) per treatment were analyzed by chi-square (χ^2).

All data are presented as mean $\pm$ SEM, with statistical differences of $p < 0.05$ considered significant, except when Bonferroni-corrected thresholds were employed for multiple comparisons. All statistical analyses were performed using SPSS Statistics (IBM, New York, NY).

Results

Activation of cGK ameliorated alcohol-mediated cell loss

We examined the role of cGK in preventing alcohol-induced neuronal death in CGN cultures. CGN cultures were pretreated with 8-Br-cGMP, a non-hydrolyzable cGMP analogue that activates cGK. 8-Br-cGMP addition prevented alcohol-induced cell loss (Figs. 1A–B), reducing cell loss from $21.0 \pm 2.9\%$ to $4.1 \pm 2.3\%$ at the highest dose, 40 μ M. Subsequent experiments used an 8-Br-cGMP concentration of 40 μ M for two reasons. First,

the results in Figs. 1A–B indicate that this concentration significantly reduced alcohol-induced cell loss. Second, 8-Br-cGMP concentrations greater than 40 μ M were toxic.

In addition to activating cGK, 8-Br-cGMP also stimulates cGMP-dependent ion channels [32] and cGMP phosphodiesterases [33], which are both expressed in the cerebellum [32, 33]. To assess whether the neuroprotective effect of 8-Br-cGMP requires cGK, we utilized Rp-8-pCPT-cGMPS, a specific inhibitor of cGK. Results shown in Figs. 1C–D reveal that Rp-8-pCPT-cGMPS (31.3 nM) addition completely eliminated the neuroprotective effect of 8-Br-cGMP, suggesting that the cGMP derivative acts by stimulating the cGMP kinase. Rp-8-pCPT-cGMPS alone produced no neuronal loss.

Cyclic GK activation prevented the alcohol-induced rise in $[Ca^{2+}]_i$

We previously demonstrated that alcohol concentrations of 200, 400, and 800 mg/dl causes a sustained rise in $[Ca^{2+}]_i$ linked to cell death in CGN cultures [18]. In the current study, we tested the hypothesis that cGK activation prevents alcohol-induced neuronal death by reducing the associated rise in $[Ca^{2+}]_i$. Thus, we measured $[Ca^{2+}]_i$ in Fura-2-loaded CGN cultures receiving cGK-activator, 8-Br-cGMP (0 or 40 μ M), prior to alcohol exposure. Since we desired a more robust visual effect in the color rendition of $[Ca^{2+}]_i$, we raised the alcohol concentration to 800 mg/dl only for Fig. 3A (three replicates), but reduced it back to 400 mg/ml for subsequent $[Ca^{2+}]_i$ experiments (Figs. 2B–D). Fig. 2A (top row) shows cells before (baseline), and the same cells 60 seconds after alcohol exposure (bottom row). Cells in the top view-fields (Figs. 2Ai–2Aii) are mostly blue, indicating low $[Ca^{2+}]_i$ at baseline. In the bottom identical view-fields, more yellow and red cells, indicating high $[Ca^{2+}]_i$, were observed with alcohol treatment (Fig. 2Aiii), than in the group that received 8-Br-cGMP and alcohol (Fig. 2Aiv). Cells in Fig 2Aiv are mostly green and blue indicating low $[Ca^{2+}]_i$. In summary, 8-Br-cGMP greatly reduced the capability of alcohol to raise $[Ca^{2+}]_i$ in these cultures.

In Figs. 2B–D, five replicate cultures were exposed to alcohol (400 mg/dl). Mean $[Ca^{2+}]_i$ was calculated from the $[Ca^{2+}]_i$ values of all cells in the CGN cultures and plotted in 5 second intervals (Fig. 2B). Average baseline $[Ca^{2+}]_i$, determined for 30 seconds before alcohol exposure, was 70.3 ± 1.2 nM (882 cells). Following alcohol addition, intracellular $[Ca^{2+}]_i$ climbed over the course of experiment (9 minutes). In contrast, the alcohol-induced $[Ca^{2+}]_i$ rise was much smaller in 8-Br-cGMP-pretreated cultures. One difference from the 800 mg/dl alcohol-treated cultures (Fig 2A) is that 400 mg/dl produced more gradual $[Ca^{2+}]_i$ elevations (Fig 2B), in agreement with our previous study [18]. To evaluate $[Ca^{2+}]_i$ responses to alcohol, we compared the maximum “peak” $[Ca^{2+}]_i$ attained during the evaluation period (Fig. 2C) [18]. Alcohol alone increased $[Ca^{2+}]_i$, reaching a peak of 163.3 ± 4.8 nM, and never returned to baseline. In comparison, the peak $[Ca^{2+}]_i$ elicited by alcohol in cells pretreated with 8-Br-cGMP was substantially lower (117.4 ± 2.4 nM; t-test, $p = 0.05$). 8-Br-cGMP addition alone did not change basal $[Ca^{2+}]_i$ levels (data not shown). Analyzing peak $[Ca^{2+}]_i$, grouped by replicates instead of by individual cells, provided similar results (174.0 ± 25.0 vs. 120.9 ± 12.8 nM; paired t-test, $p = 0.05$).

Alcohol elicits substantial $[Ca^{2+}]_i$ increases for a sizable minority of cells in susceptible CGN cultures [18]. Thus, we tested whether 8-Br-cGMP pretreatment decreased the

proportion of cells with large $[Ca^{2+}]_i$ responses to alcohol. The histograms in Fig 2D reveal the cellular distribution of $[Ca^{2+}]_i$ in response to alcohol exposure (400 mg/dl) and the change in this distribution with 8-Br-cGMP pretreatment (40 μ M). Peak $[Ca^{2+}]_i$ values for individual cells were grouped into 20 nM increments (Fig. 2D) and the number of cells in each increment was determined [18]. As shown in the top histogram in Fig 2D, alcohol had a robust effect in raising $[Ca^{2+}]_i$ to a high level (above 200 nM, highlighted by the vertical line) in a considerable proportion (27.4%) of cells. In contrast, 8-Br-cGMP pretreatment reduced the proportion of cells with $[Ca^{2+}]_i$ above 200 nM to 4.1% (bottom histogram in Fig 2D). In summary, 8-Br-cGMP modulates the response of CGN to alcohol by greatly reducing the number of cells reaching high levels of $[Ca^{2+}]_i$ (χ^2 , $p = 0.05$).

Discussion

Alcohol-induced neuronal death has been previously linked to $[Ca^{2+}]_i$ disruption. Chelation or preventing Ca^{2+} efflux from intracellular stores is neuroprotective [18-21]. Given the connection between alcohol-induced neuronal death and disruption of $[Ca^{2+}]_i$ homeostasis, preventing this disruption is a valid neuroprotective strategy. Our findings here associate NO-cGMP-cGK-mediated neuroprotection to moderating the $[Ca^{2+}]_i$ disruption induced by alcohol exposure. Like previous reported [8, 9], cGK activation with 8-Br-cGMP protected CGN cultures from alcohol toxicity, an effect that the cGK inhibitor, Rp-8-pCPT-cGMPS, blocked. Alcohol elevated $[Ca^{2+}]_i$, a key event triggering alcohol-induced neuronal death [18], which cGK activation prevented.

We previously observed that alcohol concentrations of 200, 400, 800 mg/dl produce dose-dependent increases in $[Ca^{2+}]_i$ and neuronal death in CGN cultures [18]. Herein, 8-Br-cGMP treatment before alcohol exposure (400 mg/dl) 1) attenuated the rise in mean $[Ca^{2+}]_i$ levels; 2) reduced the peak $[Ca^{2+}]_i$ that was produced; and 3) decreased the number of cells that attained high $[Ca^{2+}]_i$ (> 200 nM) levels. These data suggest that cGK activation conferred alcohol resistance by moderating alcohol-induced $[Ca^{2+}]_i$ elevations.

Growing evidence connects alcohol-induced neuronal death to $[Ca^{2+}]_i$ dysregulation and to endoplasmic reticulum (ER)-related processes [18-21, 34]. IP₃R-dependent ER Ca^{2+} depletion can cause ER stress, excessive mitochondrial Ca^{2+} uptake, and in turn promotes ROS generation and apoptosis [34]. Given this link, preventing disruption of $[Ca^{2+}]_i$ homeostasis is a valid neuroprotective strategy.

Cyclic GK phosphorylates multiple substrates that affect $[Ca^{2+}]_i$ homeostasis to confer neuroprotective benefits. For instance, alcohol elicits inositol trisphosphate receptor (IP₃R)-mediated release of Ca^{2+} from intracellular stores to trigger cell death in primary neuronal culture [18], rat hippocampal explants [19], and avian embryo models of FASD [21]. IP₃Rs contain serine phosphorylation sites for cGK [24] and multiple isoforms of both proteins are highly expressed in the cerebellum [35, 36]. Yet, both decreased [23, 36, 37] and increased [38, 39] intracellular Ca^{2+} release has been observed after IP₃R phosphorylation. Given these seemingly conflicting literature reports, how cGK affects IP₃R activity likely depends on additional undetermined regulatory factors. One such factor is IP₃R-associated cGK substrate (IRAG), which, when phosphorylated, reduces Ca^{2+} release from all IP₃R isoforms

[40]. However, brain IRAG expression is limited to the thalamus [41] and therefore cannot explain the maturation-acquired alcohol resistance that develops in other brain regions. Further investigation is necessary to determine if IP₃Rs play a role in cGK-mediated neuroprotection.

In conclusion, cGK activation via 8-Br-cGMP, prevented both neuronal death and reduced the associated rise in [Ca²⁺]_i from alcohol in CGN cultures. Inhibition of cGK by Rp-8-pCPT-cGMPS prevented cGMP-mediated neuroprotection to alcohol. These findings associate alcohol resistance, mediated by the NO-cGMP-cGK pathway, to mitigating the persistent and toxic alcohol-induced increase in [Ca²⁺]_i. Future studies aimed at the neuroprotective substrates of cGK may provide exciting new insights into alcohol resistance and their connection to alleviating the alcohol-induced disruption of [Ca²⁺]_i homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Mr. Maysam Takepoo and Dr. Guiying Li of The University of Iowa provided exceptional technical assistance. Drs. Michael A. Collins, John J. Callaci, and Saverio Gentile of Loyola University Chicago gave an invaluable informal review. This work was supported by funding from National Institutes of Health (AA011577 awarded to Dr. Nicholas J. Pantazis) and The University of Iowa Graduate College.

References

- [1]. May PA, Keaster C, Bozeman R, Goodover J, Blankenship J, Kalberg WO, Buckley D, Brooks M, Hasken J, Gossage JP, Robinson LK, Manning M, Hoyme HE, Prevalence and characteristics of fetal alcohol syndrome and partial fetal alcohol syndrome in a Rocky Mountain Region City, *Drug Alcohol Depend* 155 (2015) 118–127. [PubMed: 26321671]
- [2]. West JR, Goodlett CR, Bonthius DJ, Hamre KM, Marcussen BL, Cell population depletion associated with fetal alcohol brain damage: mechanisms of BAC-dependent cell loss, *Alcohol Clin Exp Res* 14 (1990) 813–818. [PubMed: 2088116]
- [3]. West JR, Acute and long-term changes in the cerebellum following developmental exposure to ethanol, *Alcohol Alcohol Suppl* 2 (1993) 199–202. [PubMed: 7748300]
- [4]. Pierce DR, Williams DK, Light KE, Purkinje cell vulnerability to developmental ethanol exposure in the rat cerebellum, *Alcohol Clin Exp Res* 23 (1999) 1650–1659. [PubMed: 10549998]
- [5]. Karacay B, Li S, Bonthius DJ, Maturation-dependent alcohol resistance in the developing mouse: cerebellar neuronal loss and gene expression during alcohol-vulnerable and -resistant periods, *Alcohol Clin Exp Res* 32 (2008) 1439–1450. [PubMed: 18565154]
- [6]. Pantazis NJ, Dohrman DP, Goodlett CR, Cook RT, West JR, Vulnerability of cerebellar granule cells to alcohol-induced cell death diminishes with time in culture, *Alcohol Clin Exp Res* 17 (1993) 1014–1021. [PubMed: 8279660]
- [7]. Pantazis NJ, West JR, Dai D, The nitric oxide-cyclic GMP pathway plays an essential role in both promoting cell survival of cerebellar granule cells in culture and protecting the cells against ethanol neurotoxicity, *J Neurochem* 70 (1998) 1826–1838. [PubMed: 9572266]
- [8]. Bonthius DJ, Karacay B, Dai D, Hutton A, Pantazis NJ, The NO-cGMP-PKG pathway plays an essential role in the acquisition of ethanol resistance by cerebellar granule neurons, *Neurotoxicol Teratol* 26 (2004) 47–57. [PubMed: 15001213]
- [9]. Bonthius DJ, Karacay B, Dai D, Pantazis NJ, FGF-2, NGF and IGF-1, but not BDNF, utilize a nitric oxide pathway to signal neurotrophic and neuroprotective effects against alcohol toxicity in

cerebellar granule cell cultures, *Brain Res Dev Brain Res* 140 (2003) 15–28. [PubMed: 12524173]

- [10]. Bonthius DJ, Tzouras G, Karacay B, Mahoney J, Hutton A, McKim R, Pantazis NJ, Deficiency of neuronal nitric oxide synthase (nNOS) worsens alcohol-induced microencephaly and neuronal loss in developing mice, *Brain Res Dev Brain Res* 138 (2002) 45–59. [PubMed: 12234657]
- [11]. Bonthius DJ, McKim RA, Koele L, Harb H, Kehrberg AH, Mahoney J, Karacay B, Pantazis NJ, Severe alcohol-induced neuronal deficits in the hippocampus and neocortex of neonatal mice genetically deficient for neuronal nitric oxide synthase (nNOS), *J Comp Neurol* 499 (2006) 290–305. [PubMed: 16977619]
- [12]. Goodlett CR, Eilers AT, Alcohol-induced Purkinje cell loss with a single binge exposure in neonatal rats: a stereological study of temporal windows of vulnerability, *Alcohol Clin Exp Res* 21 (1997) 738–744. [PubMed: 9194933]
- [13]. Wang W, Nakayama T, Inoue N, Kato T, Quantitative analysis of nitric oxide synthase expressed in developing and differentiating rat cerebellum, *Brain Res Dev Brain Res* 111 (1998) 65–75. [PubMed: 9804896]
- [14]. Bonthius DJ, Bonthius NE, Li S, Karacay B, The protective effect of neuronal nitric oxide synthase (nNOS) against alcohol toxicity depends upon the NO-cGMP-PKG pathway and NF-kappaB, *Neurotoxicology* 29 (2008) 1080–1091. [PubMed: 18824032]
- [15]. Bonthius DJ, Luong T, Bonthius NE, Hostager BS, Karacay B, Nitric oxide utilizes NF-kappaB to signal its neuroprotective effect against alcohol toxicity, *Neuropharmacology* 56 (2009) 716–731. [PubMed: 19135070]
- [16]. Holownia A, Ledig M, Menez JF, Ethanol-induced cell death in cultured rat astroglia, *Neurotoxicol Teratol* 19 (1997) 141–146. [PubMed: 9136130]
- [17]. Hirata H, Machado LS, Okuno CS, Brasolin A, Lopes GS, Smaili SS, Apoptotic effect of ethanol is potentiated by caffeine-induced calcium release in rat astrocytes, *Neurosci Lett* 393 (2006) 136–140. [PubMed: 16257121]
- [18]. Kouzoukas DE, Li G, Takapoo M, Moninger T, Bhalla RC, Pantazis NJ, Intracellular calcium plays a critical role in the alcohol-mediated death of cerebellar granule neurons, *J Neurochem* 124 (2013) 323–335. [PubMed: 23121601]
- [19]. Reynolds AR, Saunders MA, Prendergast MA, Ethanol Stimulates Endoplasmic Reticulum Inositol Triphosphate and Sigma Receptors to Promote Withdrawal-Associated Loss of Neuron-Specific Nuclear Protein/Fox-3, *Alcohol Clin Exp Res* 40 (2016) 1454–1461. [PubMed: 27177604]
- [20]. Debelak-Kragtorp KA, Armant DR, Smith SM, Ethanol-induced cephalic apoptosis requires phospholipase C-dependent intracellular calcium signaling, *Alcohol Clin Exp Res* 27 (2003) 515–523. [PubMed: 12658119]
- [21]. Garic-Stankovic A, Hernandez MR, Chiang PJ, Debelak-Kragtorp KA, Flentke GR, Armant DR, Smith SM, Ethanol triggers neural crest apoptosis through the selective activation of a pertussis toxin-sensitive G protein and a phospholipase Cbeta-dependent Ca^{2+} transient, *Alcohol Clin Exp Res* 29 (2005) 1237–1246. [PubMed: 16046880]
- [22]. Felbel J, Trockur B, Ecker T, Landgraf W, Hofmann F, Regulation of cytosolic calcium by cAMP and cGMP in freshly isolated smooth muscle cells from bovine trachea, *J Biol Chem* 263 (1988) 16764–16771. [PubMed: 2846548]
- [23]. Fernandez SF, Huang MH, Davidson BA, Knight PR, 3rd, Izzo JL, Jr., Mechanisms of angiotensin II-mediated decreases in intraneuronal Ca^{2+} in calcium-loaded stellate ganglion neurons, *Hypertension* 45 (2005) 276–282. [PubMed: 15642775]
- [24]. Haug LS, Jensen V, Hvalby O, Walaas SI, Ostvold AC, Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic nucleotide-dependent kinases in vitro and in rat cerebellar slices in situ, *J Biol Chem* 274 (1999) 7467–7473. [PubMed: 10066812]
- [25]. Luo J, Mechanisms of ethanol-induced death of cerebellar granule cells, *Cerebellum* 11 (2012) 145–154. [PubMed: 20927663]
- [26]. Minion GE, Slovis CM, Boutiette L, Severe alcohol intoxication: a study of 204 consecutive patients, *J Toxicol Clin Toxicol* 27 (1989) 375–384. [PubMed: 2628586]

- [27]. Maier SE, Miller JA, Blackwell JM, West JR, Fetal alcohol exposure and temporal vulnerability: regional differences in cell loss as a function of the timing of binge-like alcohol exposure during brain development, *Alcohol Clin Exp Res* 23 (1999) 726–734. [PubMed: 10235310]
- [28]. Ke Z, Wang X, Liu Y, Fan Z, Chen G, Xu M, Bower KA, Frank JA, Li M, Fang S, Shi X, Luo J, Ethanol induces endoplasmic reticulum stress in the developing brain, *Alcohol Clin Exp Res* 35 (2011) 1574–1583. [PubMed: 21599712]
- [29]. Huebner SM, Blohowiak SE, Kling PJ, Smith SM, Prenatal Alcohol Exposure Alters Fetal Iron Distribution and Elevates Hepatic Hecpudin in a Rat Model of Fetal Alcohol Spectrum Disorders, *J Nutr* 146 (2016) 1180–1188. [PubMed: 27146918]
- [30]. Pantazis NJ, Dohrman DP, Luo J, Thomas JD, Goodlett CR, West JR, NMDA prevents alcohol-induced neuronal cell death of cerebellar granule cells in culture, *Alcohol Clin Exp Res* 19 (1995) 846–853. [PubMed: 7485829]
- [31]. Grynkiewicz G, Poenie M, Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties, *J Biol Chem* 260 (1985) 3440–3450. [PubMed: 3838314]
- [32]. Kingston PA, Zufall F, Barnstable CJ, Widespread expression of olfactory cyclic nucleotide-gated channel genes in rat brain: implications for neuronal signalling, *Synapse* 32 (1999) 1–12. [PubMed: 10188632]
- [33]. Seeger TF, Bartlett B, Coskran TM, Culp JS, James LC, Krull DL, Lanfear J, Ryan AM, Schmidt CJ, Strick CA, Varghese AH, Williams RD, Wylie PG, Menniti FS, Immunohistochemical localization of PDE10A in the rat brain, *Brain Res* 985 (2003) 113–126. [PubMed: 12967715]
- [34]. Yang F, Luo J, Endoplasmic reticulum stress and ethanol neurotoxicity, *Biomolecules* 5 (2015) 2538–2553. [PubMed: 26473940]
- [35]. Lohmann SM, Walter U, Miller PE, Greengard P, De Camilli P, Immunohistochemical localization of cyclic GMP-dependent protein kinase in mammalian brain, *Proc Natl Acad Sci U S A* 78 (1981) 653–657. [PubMed: 7017721]
- [36]. Snyder SH, Supattapone S, Isolation and functional characterization of an inositol trisphosphate receptor from brain, *Cell Calcium* 10 (1989) 337–342. [PubMed: 2548727]
- [37]. Supattapone S, Danoff SK, Theibert A, Joseph SK, Steiner J, Snyder SH, Cyclic AMP-dependent phosphorylation of a brain inositol trisphosphate receptor decreases its release of calcium, *Proc Natl Acad Sci U S A* 85 (1988) 8747–8750. [PubMed: 2847175]
- [38]. Wojcikiewicz RJ, Luo SG, Phosphorylation of inositol 1,4,5-trisphosphate receptors by cAMP-dependent protein kinase. Type I, II, and III receptors are differentially susceptible to phosphorylation and are phosphorylated in intact cells, *J Biol Chem* 273 (1998) 5670–5677. [PubMed: 9488697]
- [39]. Kim S, Titcombe RF, Zhang H, Khatri L, Girma HK, Hofmann F, Arancio O, Ziff EB, Network compensation of cyclic GMP-dependent protein kinase II knockout in the hippocampus by Ca^{2+} -permeable AMPA receptors, *Proc Natl Acad Sci U S A* 112 (2015) 3122–3127. [PubMed: 25713349]
- [40]. Masuda W, Betzenhauser MJ, Yule DI, InsP3R-associated cGMP kinase substrate determines inositol 1,4,5-trisphosphate receptor susceptibility to phosphoregulation by cyclic nucleotide-dependent kinases, *J Biol Chem* 285 (2010) 37927–37938. [PubMed: 20876535]
- [41]. Geiselhoring A, Gaisa M, Hofmann F, Schlossmann J, Distribution of IRAG and cGKI-isoforms in murine tissues, *FEBS Lett* 575 (2004) 19–22. [PubMed: 15388327]

Highlights

- Cyclic GK activation improved neuronal survival in alcohol-exposed CGN cultures, which a cGK inhibitor blocked.
- Cyclic GK activation reduced both the level of alcohol-induced rise in $[Ca^{2+}]_i$ and number of cells that responded to alcohol by increasing $[Ca^{2+}]_i$.

Fig. 1. Cyclic GK activation decreased alcohol-induced neuronal loss.

CGN cultures were pretreated with the cGK-activator, 8-Br-cGMP (six replicates) 30 minutes before alcohol exposure (0 or 400 mg/dl). Cultures in panels C and D also received the cGK inhibitor, Rp-8-pCPT-cGMPS. Left panels (A and C) show cell numbers assessed after 24 hours of alcohol exposure. Alcohol significantly reduced cell numbers (paired t-tests with Bonferroni correction; * $p = 0.013$). Right panels (B and D) show cell number reductions as percent cell loss. Addition of 8-Br-cGMP at the two highest concentrations provided a dose-dependent neuroprotective effect, reducing the alcohol-induced neuronal loss (repeated measures ANOVA with pairwise comparisons; # $p = 0.05$; paired t-tests, & $p = 0.025$ after Bonferroni correction). The cGK inhibitor, Rp-8-pCPT-cGMPS, eliminated the neuroprotective effect of 8-Br-cGMP.

Fig. 2. Pretreatment with 8-Br-cGMP reduced the alcohol-induced rise in [Ca²⁺]_i.

Panel A shows pseudocolor 340/380 ratio images of CGN cultures (representative for three replicates) before (Ai–Aii) and identical view-fields 60 seconds after adding alcohol (800 mg/dl) (Aiii–Aiv). The color scale bar maps [Ca²⁺]_i; blue and red respectively depict low and high [Ca²⁺]_i. Arrows (Aiii) highlight cells with increased [Ca²⁺]_i after alcohol exposure, shifting in color from blue (low [Ca²⁺]_i) to red/yellow (higher [Ca²⁺]_i). Fewer cells (less arrows) with raised [Ca²⁺]_i are evident in the culture (Aii, Aiv) that received 8-Br-cGMP 30 minutes before alcohol. Panel B: Lines show mean ± SEM [Ca²⁺]_i in cells (combined from six replicates) after adding alcohol (400 mg/dl) over the duration of the experiment (9 minutes). Alcohol initiated a rise in [Ca²⁺]_i which continued for the experiment duration. 8-Br-cGMP pretreatment reduced the alcohol-induced rise of [Ca²⁺]_i. Panel C: Utilizing the data from Panel B, the highest [Ca²⁺]_i attained for each cell (Peak [Ca²⁺]_i) over the duration of the experiment was derived and a mean was calculated. The dashed line indicates the baseline [Ca²⁺]_i level, measured 30 seconds after adding alcohol. Treatment with 8-Br-cGMP decreased the peak [Ca²⁺]_i level elicited by alcohol (* *p* < 0.05). Panel D: To ascertain the cell distribution across the range of Peak [Ca²⁺]_i, a histogram was derived from the Peak [Ca²⁺]_i data in Panel C. The X-axis (Peak [Ca²⁺]_i) in Fig 3D was divided into 20 nM segments and the percent of cells in each segment was determined. A vertical dashed line is drawn at an [Ca²⁺]_i of 200 nM, and the percentage of cells with more than 200 nM [Ca²⁺]_i was determined. In the absence of 8-Br-cGMP, 27.4% of the cells attained an [Ca²⁺]_i greater than 200 nM, after alcohol addition. Treatment with 8-Br-cGMP substantially lowered this percentage to 4.1% (χ^2 , *p* < 0.05).