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CaMKII Activation Is a Novel Effector of Alcohol's Neurotoxicity in Neural Crest Stem/Progenitor Cells

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Abstract

Prenatal ethanol exposure causes significant neurodevelopmental deficits through its induction of apoptosis in neuronal progenitors including the neural crest. Using an established chick embryo model, we previously showed that clinically relevant ethanol concentrations cause neural crest apoptosis through mobilization of an intracellular calcium transient. How the calcium transient initiates this cell death is unknown. Here we identify CaMKII as the calcium target responsible for ethanol-induced apoptosis. Immunostaining revealed selective enrichment of activated phosphoCaMKII(Thr286) within ethanol-treated neural crest. CaMKII activation in response to ethanol was rapid (<60 sec) and robust, and CaMKII activity was increased 300% over control levels. Treatment with CaMKII-selective inhibitors but not those directed against CaMKIV or PKC completely prevented the cell death. Forced expression of dominant-negative CaMKII prevented ethanol's activation of CaMKII and prevented the ethanol-induced death, whereas constitutively-active CaMKII in ethanol's absence significantly increased cell death to levels caused by ethanol treatment. In summary, CaMKII is the key signal that converts the ethanol-induced, short-lived Ca_i^{2+} transient into a long-lived cellular effector. This is the first identification of CaMKII as a critical mediator of ethanol-induced cell death. Because neural crest differentiates into several neuronal lineages, our findings offer novel insights into how ethanol disrupts early neurogenesis.

Keywords

ethanol; neural crest; CaMKII; apoptosis; intracellular calcium; fetal alcohol spectrum disorders

Introduction

Fetal Alcohol Spectrum Disorders (FASD) are a leading and preventable cause of neurodevelopmental disability. The most severe form, Fetal Alcohol Syndrome, affects 0.33 to 2.2 per 1000 live births with substantially higher rates (8 to 89.2 per 1000) in populations with appreciable alcohol use (Clarren et al. 2001; May et al. 2009). Clinically relevant ethanol concentrations disrupt multiple events of neuronal development including proliferation, survival, differentiation, migration, and synaptogenesis, the precise consequence being dictated by the cell's developmental state (reviewed in Miller 2006). At the molecular level, ethanol disrupts cellular function by displacing water from hydrophilic

pockets of proteins, thereby affecting the protein's activity and sensitivity to ligand-induced activation (Mihic et al. 1997); many but not all of ethanol's targets are G protein-coupled receptors (GPCR).

One target of prenatal ethanol exposure is an early neural progenitor population called the neural crest. The neural crest is a unique stem cell population that originates within the dorsal neuroectoderm (LeDouarin and Kalcheim, 1999). As the elevated neural folds fuse, neural crest cells emigrate into peripheral tissues and contribute to diverse structures including cranial ganglia, sympathetic and parasympathetic nervous system, and craniofacial bone and cartilage. Clinically relevant ethanol concentrations cause substantial apoptosis within neural crest progenitors as revealed by the neural crest cells' pyknotic appearance, detection by TUNEL, and dependence upon caspase activity (Kotch and Sulik 1992; Cartwright et al. 1995, 1998; Dunty et al. 2001; Sulik et al. 1981). Ethanol's disruption of neural crest development contributes to the deficits in cranial nerves, facial structure and other neurocristopathies that partly typify those with FASD.

Using a chick embryo FASD model we have shown that the ethanol-induced neural crest death uses an apoptotic mechanism because this cell death requires caspase activity and is visualized using TUNEL techniques (Smith and Cartwright 1997; Cartwright et al. 1998). We further showed that this neural crest cell death is caused by an intracellular Ca_i^{2+} transient that occurs within 1–2 sec of ethanol administration and in response to ethanol concentrations as low as 9 mM (Debelak-Kragtorp et al. 2003; Garic-Stankovic et al. 2006). This Ca_i^{2+} transient originates from ethanol's stimulation of a pertussis toxin-sensitive heterotrimeric G protein ($\text{G}\alpha_i$ and $\text{G}\beta\gamma$) coupled to an as-yet-unidentified GPCR, followed by activation of a phosphoinositidyl-phospholipase $\text{C}\beta$ (Garic-Stankovic et al. 2005). How these signals lead to neural crest apoptosis remains unknown. These progenitor cells are not excitatory and do not express NMDA or GABA receptors.

The calmodulin-dependent kinase CaMKII is a ubiquitous, multimeric holoenzyme (reviewed in Hanson et al. 1994, Wayman et al. 2008). Upon binding with calcium-activated calmodulin, CaMKII autophosphorylation at Thr286 releases it from autoinhibition and confers autonomous kinase activity to the enzyme. This autonomous pCaMKII maintains itself in an activated state and, in turn, phosphorylates diverse protein substrates to have a lasting effect on cell function. Here we report that CaMKII is a critical mediator of the ethanol apoptosis pathway. In the ethanol-exposed neural crest, CaMKII acts as a molecular switch to convert ethanol's short-lived Ca_i^{2+} transient into a long-term effect on cellular activity. The selective activation of CaMKII within this population is responsible for the neural crest losses and is a novel mechanism for alcohol's neurotoxicity.

Methods

Embryos

Fertile white leghorn eggs of the Hyline strain W98 (Hyline International, Spencer, IA) or DeKalb Special Black (Sunnyside Farms, Beaver Dam, WI) were incubated at 37.5°C to the desired developmental stage. Embryos were staged according to the criteria of Hamburger and Hamilton (HH, 1951). Although embryos did not develop past 48hr incubation, ARRIVE guidelines were followed.

Ethanol Exposure

For *in ovo* exposure, eggs having embryos of 3–5 somites (HH stage 8) were injected into the yolk with saline (0.9%) or ethanol (0.43 mmol/egg) in isotonic saline. This produces a peak embryonic ethanol concentration of ~60 mM within 15 min of injection and declines to 5 mM 3 hr thereafter (Debelak and Smith 2000; Cartwright et al., 1998). For *ex ovo*

exposure, HH8 embryos were dissected free of the egg yolk and directly incubated for 1 min in Dulbecco's minimal essential medium (DMEM) $\pm$ 52 mM ethanol; this ethanol concentration was previously shown to produce the half-maximal calcium release (Garic-Stankovic et al. 2005). Because HH8-9 embryos are no more than 10 cell diameters thick and the neural crest resides on the dorsal surface, their ethanol exposure is immediate.

Calcium Imaging

Ratiometric visualization and quantitation of intracellular calcium release was performed as described previously (Garic-Stankovic et al. 2009). In brief, Fura-2-loaded HH8 embryos were successively challenged at 60 sec intervals with Tyrode's buffer to ascertain baseline calcium content, 52 mM ethanol, 0.1 mM ionomycin as a positive control for embryo viability, and MnCl_2 to ascertain background fluorescence. Images were collected at 1-sec intervals and MetaFluor software calculated the fluorescence intensity at 500 nm emission following excitation at 340 nm and 380 nm. Calcium levels were calculated using the method of Grynkiewicz et al. (1985; Garic-Stankovic et al. 2009).

Cell Death Studies

These were performed as described (Debelak-Kragtorp et al. 2003). In brief, pharmacological agonists or antagonists were transiently delivered to HH8 embryos *in ovo* on hydrophobic (SM2, 100 μm diameter) or anion-exchange beads (AG-50W, 75 μm , both from Bio-Rad, Hercules CA) that were preabsorbed with the agent and washed prior to implant. Pilot studies ascertained the appropriate concentration to be tested; because bead-mediated delivery is diffusion dependent, the bead soaking concentrations were generally 100- to 1000-fold greater than levels administered by direct delivery (Eichele et al. 1984). Pharmacological reagents were Bapta-AM (10 mM), CAIP (10 μM), cypermethrin (100 nM), Go-6983 (50 μM), KN92 (1 mM), KN93 (1 mM), Ro32-0432 (10 μM), STO-609 (5 μM), W7 (10 μM ; all from Calbiochem), myristoylated AIP (2.5 μM ; BioMol Research Laboratories), and calmidazolium (10 μM ; Alexis Biochemicals). All inhibitors were cell-permeable forms and were dissolved in DMSO, except myrAIP was in water. Controls received DMSO-treated beads and the final DMSO concentration was $<0.1\%$ and did not adversely affect development (Garic-Stankovic et al. 2005). Beads were placed immediately adjacent to the presumptive cranial neural crest at HH8. Eggs were reincubated and injected 2 hr later with saline or ethanol as above. Beads were removed via pipettor 3 hr after ethanol injection. Embryos were incubated to HH12/13- (17–19 somites), when cell death was visualized using the vital dye LysoTracker Red (0.5 μM , Molecular Probes) or acridine orange (5 μM ; Debelak and Smith 2000). We and others have shown elsewhere that these reagents identify apoptotic cells in the early embryo FAS model (Cartwright et al. 1998; Dunty et al. 2001; Giles et al. 2008; Smith and Cartwright 1997; Sulik et al. 1981, 1988; Wang and Bieberich, 2001; Zucker et al. 1999). In the small molecule screen, we enumerated the number of labeled cells within rhombomere 4, which normally lacks appreciable neural crest death. Each inhibitor was tested in at least triplicate with 8–15 embryos per treatment. For those compounds that prevented the ethanol-induced cell death, neural crest were visualized using immunostaining for *snail2* (formerly *slug*; #27568, 1:1000, AbCam, Cambridge MA) or *sox9* (#3697, 1:1000, AbCam) followed by Alexa488-conjugated secondary antibody (1:2000, Invitrogen). We counted the total number of neural crest and LTR⁺ neural crest cells that resided within the dorsal neural roof and adjacent mesenchyme of rhombomere 4, counting ten sequential transverse sections moving cranially from the rostral margin of the otic vesicle. We enumerated at least 150–200 cells per embryo and 3–4 embryos per treatment.

CaMKII Immunostaining

Ex ovo HH8- embryos were incubated in DMEM $\pm$ 52 mM ethanol for 1 min, then immediately fixed in 4% paraformaldehyde (45 min, 40°C) followed by Dent's fixative overnight. To specifically detect the activated form of CaMKII, phospho-CaMKII (pCaMKII), embryos were immunostained using antibody specific for the autoactivation phosphorylation site phosphothreonine-286 (anti-ACTIVE CaMKII, V1111, 1:10,000; Promega, Madison WI) followed by secondary antibody coupled to Alexa 488. Embryos were sectioned in the transverse plane and nuclei visualized with DAPI.

Western Analysis

Cranial regions from somite-matched embryos having 3–6 somites were directly incubated in DMEM $\pm$ 52 mM ethanol for 1 min and immediately flash-frozen. Tissue was solubilized in SDS-PAGE loading buffer and proteins were separated on a 7.5% SDS acrylamide gel, analyzing one dissected head per lane. Proteins were transferred using semi-dry electrophoresis to a PVDF membrane (Immobilon-P, Millipore) and blocked with 5% non-fat dry milk and 1% BSA. Total CaMKII was detected using antibody EP1829Y (1:3,000, Novus Biologicals), which is specific for the most abundant form CaMKII α . Activated pCaMKII was detected using Anti-ACTIVE pCaMKII antibody (V111, 1:10,000, Promega), which specifically detects the CaMKII autophosphorylation site on phosphothreonine-286. GAPDH was detected using antibody #9561 (1:50,000, Cell Signaling). For all antibody reagents the manufacturer confirmed that the epitope of interest was conserved in chick. Secondary antibodies were conjugated to horseradish peroxidase (1:50,000, Southern Biotech) and were detected using the SuperSignal West Pico chemillumination kit (Pierce, Rockford IL). Membranes were exposed to x-ray film and scanned densitometrically (OptiQuant Acquisition and Analysis v4.0, Packard Instruments) to quantify signal. Experiments analyzed either 3 individual crania or 3–6 pooled crania per treatment and were performed in triplicate.

CaMKII and PKC Activity Assays

Pooled crania dissected immediately above the level of somite 3 of HH8 embryos (11–13 per sample) were incubated in DMEM $\pm$ 52 mM ethanol for 1 minute and flash-frozen. Some samples were incubated 30 min in the CaMKII-selective inhibitor myrAIP or the PKC-selective inhibitor Go-6983 prior to the saline or ethanol challenge. CaMKII activity in the tissue homogenates (Branson) was quantified using the SignaTECT CaMKII Assay System (V8161; Promega) as described by the manufacturer. In brief, the assay quantifies [γ 32P]-labeling of a proprietary CaMKII-specific, biotinylated peptide substrate that is based on the T286 autophosphorylation site, followed by streptavidin capture; this recognition sequence is conserved in chick. Controls include calmodulin and substrate omission. The substrate is specific for CaMKII and does not detect CaMKIV, PKC or calcineurin (Goueli et al. 1995; Hanson et al. 1989). Protein Kinase C activity was measured using the parallel SignaTECT PKC Assay System (V7470; Promega), which quantified [γ 32P]-labeling of a biotinylated peptide derived from Neurogranin₍₂₈₋₄₃₎ and purified using the same capture technique. PKC controls omitted the phospholipid activation buffer. For both enzymes, samples were assayed in triplicate and protein concentration was determined using the BCA method. Results were expressed as pmol CaMKII activity/min- μ g protein and fmol PKC activity/min- μ g protein.

Targeted Misexpression Studies

cDNA encoding eGFP and the parent expression vector pCAGGS (Niwa et al. 1991) were kind gifts of T. Suzuki. The constitutively-active CaMKII construct (T286D) (Waldmann et al. 1990; Rich and Schulman 1998) and dominant-negative kinase-dead CaMKII construct

(K42M) (Hanson et al. 1994; Rich and Schulman 1998) were kind gifts of R.T. Moon (Kuhl et al. 2000). Constructs were used at a 3:1 ratio (1.2:0.4 $\mu\text{g}/\mu\text{l}$) of experimental-to-eGFP cDNA. DNA (2 μl in Fast Green) was injected into the lumen of the HH9 posterior hindbrain, and embryos were immediately electroporated (20V/100 msec on, 999.9 msec off, 5 pulses; CUY-21, Protech International) using 1 mm platinum electrodes with a fixed 4 mm gap. Eggs were injected 3hr later with saline or 0.43 mmol ethanol in isotonic saline.

Cell death within rhombomeres 3 and 4 was quantified 20 hr thereafter using LysoTracker Red as described above. Electroporation efficiency was simultaneously visualized using eGFP fluorescence; embryos lacking eGFP expression in rhombomeres 4/5/6 were discarded from analysis. Embryos were fixed and immunostained for eGFP (1:5000 antibody B-2, Santa Cruz), followed by secondary antibody (α -rabbit IgG-Alexa488, Molecular Probes), and sectioned in the transverse plane. Nuclei were visualized by DAPI counterstain. We counted the total number of eGFP⁺ and LTR⁺eGFP⁺ cells that resided within the dorsal neural roof and adjacent lateral quadrant of rhombomeres 4 and 3, counting ten sequential transverse sections moving cranially from the rostral margin of the otic vesicle. We enumerated 150–200 eGFP⁺ cells per embryo and 3–4 embryos per treatment group.

Statistics

Unless indicated otherwise, experiments evaluated 8–15 embryos per treatment and were performed in at least triplicate. Results are presented as mean $\pm$ SEM unless otherwise indicated. Normally distributed data were subjected to either an unpaired, two-tailed *t*-test or one-way ANOVA employing the appropriate variance parameter using SigmaStat v.2.0 (Systat Software, Point Richmond, CA). $p < 0.05$ was the criterion for significance.

Results

A Ca_i^{2+} Transient Instigates Ethanol-Induced Apoptosis

At the 3–5 somite stages (HH8) the chick cranial region is comprised of the elevating neural folds; the premigratory neural crest resides within its dorsal margin (boxed region, Figure 1A). As we have shown previously, brief exposure to a clinically relevant ethanol concentration (52 mM) caused the rapid induction of a Ca_i^{2+} transient within the neural folds and including the premigratory neural crest (Figure 1C) (Debelak-Kragtorp et al. 2003; Garic-Stankovic et al. 2005, 2006); saline vehicle challenge was without effect (Figure 1B). The rise in calcium was calculated at 764 ± 55 nM, a value consistent with our prior work in this model (830 ± 59 nM, Garic-Stankovic et al. 2006) and representing a 5.54 ± 0.45 -fold increase in intracellular calcium levels (Dickens et al. 1990). Ca_i^{2+} levels returned to baseline by 1 minute thereafter (not shown). Significant cell death was observed 18 hr later at stage 12–13- (17–19 somites), which we showed previously is the peak time of ethanol-induced death (Cartwright et al. 1998). The dead cells predominantly resided within neural and mesenchymal regions that are also enriched in premigratory and migrating neural crest (compare signal at arrows, Figure 1D, E). Immunostain confirmed that many of the apoptotic cells in ethanol-treated hindbrain expressed the neural crest marker *snail2*, confirming their neural crest identity (Figure 1H). In contrast, few *snail2*⁺LTR⁺ cells were present in control hindbrain (Figure 1G). As we showed elsewhere (Debelak-Kragtorp et al. 2003), pretreatment with the Ca_i^{2+} chelator Bapta-AM prior to ethanol challenge prevented the ethanol-induced cell death (Figure 1F, I). Because the inhibitor-soaked bead was removed 3hr after ethanol challenge, Bapta treatment did not affect the endogenous cell death. Thus the ethanol-stimulated Ca_i^{2+} transient is the signal that initiates cell death within these cranial populations.

CaMKII Contributes to Ethanol-Induced Apoptosis

To identify the downstream target of the calcium transient, we performed a small molecule screen of Ca_i^{2+} -directed pharmacological agents and evaluated their ability to prevent the ethanol-induced death of neural crest progenitors. The hindbrain region spanning rhombomere 4 normally has few apoptotic neural crest cells (Figures 2A, 3A, 3B, 4). This number significantly increased following acute ethanol exposure ($p < 0.001$ vs. saline-only; Figures 2A), and immunostain revealed a significant decrease in the number of Sox9^+ neural crest cells, many of which were co-labeled with LTR (Figures 3C, 3D, 4). As expected, Bapta-AM pretreatment prevented the neural crest losses and normalized neural crest survival as measured by $\text{Sox9}^+/\text{LTR}^+$ double-labeling (compare Figure 3E, F vs. C, D; $p=0.002$ vs. ethanol, Figure 4).

Calmodulin is a Ca_i^{2+} sensor protein that converts the calcium transient into an effect on cell physiology through its calcium-dependent interactions with protein kinases and phosphatases. Pretreatment with two distinct calmodulin inhibitors, W7 (Hidaka et al. 1981) and calmidazolium (Gietzen et al. 1982) both abrogated the ethanol-induced cell death (Figure 2A; W7, $p < 0.001$; calmidazolium, (cazi) $p < 0.001$; compare signal at arrows, Figure 3G, H vs. 3C, D). Quantification of Sox9^+ populations confirmed that calmodulin inhibition significantly enhanced neural crest survival in ethanol-treated embryos ($p < 0.01$ vs. ethanol-only, Figure 4A, B).

An important target of activated calmodulin is the Calmodulin Kinase (CaMK) family of Ser/Thr protein kinases that are abundant in neuronal populations (Wayman et al. 2008). We found that KN93, which broadly inhibits members of the CaMK signaling cascade (Tokumitsu et al. 1990), prevented the ethanol-induced apoptosis (Figure 2B; $p < 0.001$); the structurally related inactive compound KN92 was ineffectual. To distinguish whether the signal utilized CaMKII or the CaMK kinase (CaMKK) cascade, we first tested myristolated AIP (myrAIP), a cell-permeable peptide highly selective for CaMKII that mimics its autoinhibitory domain and inhibits the enzyme (Ishida et al. 1995). MyrAIP completely prevented the ethanol-stimulated cell death (Figure 2B, 3I, J; $p < 0.001$). Quantification of Sox9^+ populations confirmed that myrAIP significantly prevented neural crest death and normalized their survival in ethanol-treated embryos (Figure 4A, B). In contrast a selective inhibitor of CaMKIV and the CaMKK cascade (Tokumitsu et al. 2002), STO-609, failed to protect neural crest from ethanol-induced cell death (Figure 2B, 3K, L; Figure 4A,B). These data suggested that CaMKII activation was necessary for alcohol's neurotoxicity.

In addition to activating CaMKII, calcium transients originating from G protein stimulation of phospholipase C can also activate Protein Kinase C (PKC). PKC can be stimulated by ethanol in some neuronal cell lines (Lee and Messing, 2008). We found that the PKC antagonists Go-6983 and Ro-32-0432 (Gschwendt et al. 1996; Wilkinson et al. 1993) did not prevent the ethanol-induced cell death (Figure 2C). Both compounds compete for the ATP site on conventional PKC isoenzymes. As PKC isoenzymes are highly conserved between chick and human (86%–94% amino acid identity), these data suggest that PKC is not involved in the cell death signal.

Ethanol Stimulates CaMKII Activity

Binding of Ca_i^{2+} /calmodulin to CaMKII initiates the rapid autophosphorylation of CaMKII at multiple sites including Thr286, Thr305, and Thr306; phosphorylation at Thr286 creates an autonomously active CaMKII that becomes independent of further interaction with Ca_i^{2+} /calmodulin (Rich and Schulman 1998; Wayman et al. 2008). This enables CaMKII to continue to modulate cellular activity long after the calcium transient has ended. The ability of CaMKII-directed small molecule inhibitors to prevent the ethanol-induced death

suggested that ethanol-treated neural crest should be enriched in active CaMKII. We first tested for the presence of activated CaMKII using antibodies directed against the Thr286 autophosphorylation site that is uniquely present in the autonomously active kinase. Exposure of HH8- embryos to 52 mM ethanol for only 1 min significantly increased the level of pCaMKII(T286) immunostaining as compared against saline-treated embryos (Figure 5A, B). Importantly, in ethanol-treated embryos the greatest pCaMKII(T286) immunoreactivity was present within the dorsal neural folds (arrows in Figure 5B), which is where the neural crest progenitors reside. Transverse sections through the neural folds revealed the presence of substantial pCaMKII(T286) throughout the neuroectoderm and especially within the dorsal folds that are enriched with neural crest progenitors (compare boxed regions in Figure 5C, D, and arrows in Fig. E, F). The pCaMKII(T286) immunostaining was selective and was not present, for example, in the ventral neural folds or in the underlying endoderm. Quantitation of immunofluorescence signal within the dorsal neural folds (boxed region, Figure 5) confirmed that this represented a significant increase in the pCaMKII signal (mean fluorescent intensity, Saline = 39.7 ± 3.2 (n=6), Ethanol = 68.5 ± 5.4 (n=6), $p < 0.001$ using Student's t-test).

We quantified the increased pCaMKII(T286) content using western blot analysis. With respect to total CaMKII, its levels were low but detectable within the early chick cranial tissue. Ethanol treatment (1 min, 52 mM) did not affect the cranial content of total CaMKII (Figure 6A, B). However, the brief ethanol treatment caused a 2.50 ± 0.27 -fold increase in the levels of activated pCaMKII(T286) ($p < 0.005$ vs. saline), detected again using antibodies directed against phospho-Thr286. Because the neural crest progenitors comprise only 2–3% of the total cranial cell population, the rise in pCaMKII(T286) appeared modest by visual inspection but was significantly increased over the basal levels as quantified by densitometric scanning.

To further verify that the increased pCaMKII(T286) content represented an increase in the activated enzyme, we directly measured CaMKII activity within the HH8- crania using an established enzyme activity assay that is specific for CaMKII. Brief ethanol exposure (52 mM, 1 min) significantly increased cranial CaMKII activity to levels that were $305\% \pm 12\%$ greater than that in saline-treated crania ($p < 0.001$; Figure 7A), and a value that is in-line with the western blot analysis. The CaMKII-selective inhibitor myrAIP prevented the ethanol-induced rise in CaMKII activity, confirming that myrAIP had the expected inhibitory activity in these embryos and that the activity represented CaMKII. In contrast, endogenous cranial PKC activity was approximately fifty-fold lower in these same cranial extracts and PKC activity was not increased by ethanol treatment (Figure 7B); the PKC-selective inhibitor Go-6983 reduced the low level of PKC activity that was present within saline and ethanol-treated embryos, indicating it had the expected activity in this chick model. We conclude that brief ethanol treatment substantially and selectively enhanced CaMKII activity within cranial populations and particularly within those populations enriched in neural crest progenitors.

CaMKII Misexpression Alters Neural Crest Apoptosis

The small molecule assays described above showed that CaMKII inhibition *in vivo* prevented ethanol from causing cell death of neural crest and neuronal progenitors. We complemented the inhibitor studies with genetic approaches to further corroborate the CaMKII role in ethanol-induced cell death. Specifically, we tested whether forced expression of constitutively-active or dominant-negative CaMKII α forms would influence neural crest apoptosis in the presence or absence of ethanol challenge. We used two established CaMKII mutants whose cellular actions are well-documented in the literature. The CaMKII(T286D) mutant has an aspartate substitution at the autophosphorylation threonine 286 site, and this substitution acts as a phosphomimetic and confers constitutive

activity to the enzyme (Waldmann et al. 1990; Rich and Schulman 1998). The CaMKII(K42M) mutant has replaced the catalytic lysine with methionine and thus is kinase-dead. In addition, because CaMKII α operates in a heteromultimeric complex with CaMKII β , the K42M mutant can bind calcium/calmodulin but fails to phosphorylate T286 and thus it confers dominant-negative activity upon its incorporation into the CaMKII multimer (Hanson et al. 1994; Rich and Schulman 1998). We predicted that the constitutively-active and dominant-negative CaMKII forms would, respectively, enhance or reduce neural crest death in ethanol's presence. We also attempted to test the CaMKII role using morpholino knockdown. However those experiments were uninterpretable because the scrambled-control and CaMKII-directed morpholinos both led to substantial LTR signal in the transfected cells.

We first confirmed that the constructs conferred the expected CaMKII activities within chick cranial neural crest. Whereas electroporation with the parent eGFP vector did not affect CaMKII activity, CaMKII(T286D) overexpression significantly increased cranial CaMKII activity over the basal activity levels ($p < 0.05$; Figure 8A) and similar to the levels caused by ethanol challenge (Figures 7, 8A). In contrast, forced expression of the kinase-dead mutant CaMKII(K42M) overrode the activity of the endogenous CaMKII enzyme and prevented the ethanol-induced rise in CaMKII activity, an outcome consistent with its dominant-negative activity against endogenous CaMKII.

The CaMKII constructs also significantly altered neural crest survival in the presence and absence of ethanol. As a control, hindbrain neural crest electroporated with eGFP-only had many transfected cells, and a substantial number of the eGFP⁺ cells were observed exiting rhombomere 4 into the cranial mesenchyme, a behavior that is typical of migrating neural crest (Figure 8A). Only a small percentage of the eGFP⁺ cells were apoptotic (LTR⁺eGFP⁺/total eGFP⁺ = $3.1 \pm 0.6\%$; Figure 8B), indicating that the parent construct itself did not adversely affect cell survival. As expected, the ethanol-treated embryos had significantly more LTR⁺eGFP⁺ cells than did the control embryos ($18.7\% \pm 0.9\%$, $p < 0.001$; Figure 8B). Ethanol-treated embryos also had fewer migrating neural crest cells (Figure 9B), many of which were also LTR⁺ and thus were undergoing cell death. Expression of the constitutively-active CaMKII(T286D), in the absence of ethanol, significantly increased the frequency of LTR⁺eGFP⁺ cells ($24.0\% \pm 6.5\%$, $p < 0.001$) to a level not different from that caused by ethanol treatment ($p = 0.227$). In both the ethanol-treated (Figure 9B) and CaMKII(T286D)-treated embryos (Figure 9C), many of the LTR⁺eGFP⁺ cells resided within the dorsal roof of the hindbrain or within the stream of cells exiting rhombomere 4, suggesting a neural crest identity. In contrast, ethanol-treated embryos that expressed the dominant-negative CaMKII(K42M) contained significantly fewer LTR⁺eGFP⁺ cells ($5.1\% \pm 2.8\%$, $p < 0.001$; Figure 9D) as compared with ethanol-treated controls (Figure 8B), and this level did not differ from that in eGFP-saline controls. Thus the dominant-negative CaMKII(K42M) normalized cell survival and prevented the pro-death effect of ethanol, an outcome consistent with its inhibitory activity against CaMKII. In summary, the directed increase in CaMKII activity was sufficient to initiate cell death in neuronal progenitors including the neural crest, whereas CaMKII inhibition rescued these populations from ethanol-mediated cell death. These findings independently confirm the small molecule inhibition data and demonstrate that CaMKII is a central contributor to the ethanol-mediated loss of neural crest progenitors in the embryo.

Discussion

The major finding of this paper is our identification of CaMKII as a novel and direct target of ethanol in neural progenitors. Ethanol exposure causes the rapid activation of CaMKII. Importantly, we show that activated CaMKII is the molecular switch that converts ethanol's

calcium transient into a lasting regulator of neural crest progenitor survival and apoptosis. The molecular signals that mediate ethanol's neurotoxicity are unclear. We showed previously that the apoptosis studied here is caused by a calcium transient originating from activated G $\beta\gamma$ /G α_i and phospholipase C β (Figure 10; Debelak-Kragtorp et al. 2003; Garic-Stankovic et al. 2005, 2006). We thus anticipated that the calcium sensor governing this apoptosis switch might be the well known ethanol target PKC (Lee and Messing, 2008). While ethanol's calcium transient may have activated PKC in these cells, we found no evidence that PKC contributed to the apoptosis. Instead, we unexpectedly found that CaMKII was responsible for converting the short-lived calcium transient into a long-lived effector of apoptotic signaling. This is a novel function for CaMKII because it has not been previously implicated in ethanol-mediated neuronal death. Moreover, although the ethanol-induced calcium transient occurs throughout the neural progenitors (Debelak-Kragtorp et al. 2003), pCaMKII activation was largely restricted to neural crest. The differential activation of CaMKII between these cell populations explains why the neural crest is much more sensitive to ethanol-induced cell death as compared with neighboring populations at this time of development.

Although CaMKII is known to modulate various forms of neuronal apoptosis, its contributions are complex and at times contradictory. Whereas CaMKII offers neuroprotection under certain situations (e.g. Hansen et al. 2003), more often CaMKII is a positive effector of neuronal death including that induced by beta-amyloid (Lin et al. 2004) and glutamate excitotoxicity (Hajimohammadreza et al. 1995; Takano et al. 2003; Waxham et al. 1996). These latter reports have special significance for ethanol given the adverse consequences of ethanol and ethanol withdrawal upon *N*-methyl-D-aspartate receptors (NMDAR), cellular calcium, and neural survival (Bhave and Hoffman, 1997; Hu and Ticku 1995; Ikonomidou et al. 2000). It is therefore surprising that CaMKII dysregulation has not been linked with ethanol's neurotoxicity until now. Ethanol alters the activity of numerous proteins and signals that influence neuronal survival. These include, for example, NMDAR, nitric oxide synthase, GSK3 β , oxidative stress modifiers, neuroinflammatory signals, and trophic factors such as TGF β and IGFs (Bonthius et al. 2008; de la Monte et al. 2005; Ikonomidou et al. 2000; Luo 2010; Maffi et al. 2008; Powrozek and Miller 2009; Zou and Crews 2010). While some of these mechanisms are calcium-independent, several others also intersect with calcium signaling pathways. This suggests the possibility that CaMKII may contribute to other models of ethanol-mediated neurotoxicity during development, and perhaps in the adult alcoholic.

This is the first study to investigate if acute ethanol exposure affects CaMKII in neural populations. To our knowledge, CaMKII has been largely ignored as a direct target of ethanol's action. A few reports note elevated CaMKII in brain regions receiving chronic alcohol exposure including the cerebral cortex, nucleus accumbens and astrocytes (Mahadev et al. 2001; McBride et al. 2009; Smith and Navratilova 1999) but not in cultured neurons (Xu et al. 2008). Whether those changes represent primary or compensatory alterations to chronic alcohol is unknown. However, in studies with unexpected similarities to our work, acute ethanol exposure strongly and rapidly stimulates CaMKII during oocyte activation (Tatone et al. 1999; Winston and Maro 1995). As with our model, ethanol activates the oocyte CaMKII through phosphoinositidyl-phospholipase C signaling. The subsequent activation of cyclins releases the oocyte from its metaphase block. The function of CaMKII in neural crest progenitors is unknown. However these pluripotent cells are undergoing rapid expansion and are similarly poised to differentiate into diverse cell types. The developmental pluripotency of both cell types suggests that CaMKII is a key switch that governs their survival. Ethanol's dysregulation of that switch causes that population's inappropriate apoptosis.

What might be the CaMKII target that controls the apoptotic switch in neural crest? One possibility is the dysregulation of trophic signals that provide critical support to neural crest. In support of this, we recently showed that acute ethanol exposure quickly represses the trophic signal β -catenin and its transcriptional activity in these neural crest populations (Flentke, Garic, Hernandez and Smith, submitted). Of potential relevance to that finding and the work presented here is the recent report that CaMKII activation is essential for zebrafish neural crest to undergo epithelial-mesenchymal transformation (EMT) and commence migration toward the dorsal fin (Garriock and Krieg, 2007). We have shown that neural crest progenitors have greatest sensitivity to alcohol-induced apoptosis prior to their EMT; migratory neural crest requires much higher ethanol concentrations to perturb migration and survival (Cartwright and Smith 1995). Ethanol's precocious activation of EMT signals such as CaMKII could induce failsafe mechanisms that cause the apoptotic removal of those aberrantly transformed cells. In support of this view, our separate studies find that CaMKII also contributes to the endogenous apoptosis of neural crest progenitors (Garic-Stankovic A, Flentke GR, Smith SM, unpublished). CaMKII thus acts as a novel master switch that governs neural crest survival. Ethanol's inappropriate and selective activation of this gatekeeper via its calcium transient explains the unique sensitivity of neural crest to ethanol's toxicity at these early developmental stages. Our findings inform other models in which ethanol dysregulates calcium-dependent signals including those governing neuronal migration, proliferation and differentiation.

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References

- Bhave SV, Hoffman PL. Ethanol promotes apoptosis in cerebellar granule cells by inhibiting the trophic effect of NMDA. *J Neurochem.* 1997; 68:578–586. [PubMed: 9003043]
- 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.* 2008; 29:1080–1091. [PubMed: 18824032]
- Cartwright MM, Smith SM. Stage-dependent effects of ethanol on cranial neural crest cell development: partial basis for the phenotypic variations observed in fetal alcohol syndrome. *Alcohol Clin Exp Res.* 1995; 19:1454–1462. [PubMed: 8749810]
- Cartwright MM, Tessmer LL, Smith SM. Ethanol-induced neural crest apoptosis is coincident with their endogenous death, but is mechanistically distinct. *Alcohol Clin Exp Res.* 1998; 22:142–149. [PubMed: 9514299]
- Clarren SK, Randels SP, Sanderson M, Fineman RM. Screening for fetal alcohol syndrome in primary schools: a feasibility study. *Teratology.* 2001; 63:3–10. [PubMed: 11169548]
- Debelak KA, Smith SM. Avian genetic background modulates the neural crest apoptosis induced by ethanol exposure. *Alcohol Clin Exp Res.* 2000; 24:307–314. [PubMed: 10776667]
- Debelak-Kragtorp KA, Armant DR, Smith SM. Ethanol-induced cephalic apoptosis requires phospholipase C-dependent intracellular calcium signaling. *Alcohol Clin Exp Res.* 2003; 27:515–523. [PubMed: 12658119]
- de la Monte SM, Tong M, Carlson RI, Carter JJ, Longato L, Silbermann E, Wands JR. Ethanol inhibition of aspartyl-asparaginyl-beta-hydroxylase in fetal alcohol spectrum disorder: potential link to the impairments in central nervous system neuronal migration. *Alcohol.* 2009; 43:225–240. [PubMed: 19393862]
- Dickens CJ, Gillespie JJ, Greenwell JR. Measurement of intracellular calcium and pH in avian neural crest cells. *J Physiol.* 1990; 428:531–544. [PubMed: 2231423]
- Dunty WC, Chen SY, Zucker RM, Dehart DB, Sulik KK. Selective vulnerability of embryonic cell populations to ethanol-induced apoptosis: implications for alcohol-related birth defects and neurodevelopmental disorder. *Alcohol Clin Exp Res.* 2001; 25:1523–1535. [PubMed: 11696674]

- Eichele G, Tickle C, Alberts BM. Microcontrolled release of biologically active compounds in chick embryos: beads of 200-microns diameter for the local release of retinoids. *Anal Biochem.* 1984; 142:542–555. [PubMed: 6543292]
- Garic-Stankovic A, Hernandez M, Chiang PJ, Armant DR, Debelak-Kragtorp KA, Smith SM. Ethanol selectively triggers neural crest apoptosis thru its activation of a pertussis toxin-sensitive G-protein and a phospholipase C β -dependent Ca²⁺ transient. *Alcohol Clin Exp Res.* 2005; 29:1237–1246. [PubMed: 16046880]
- Garriock RJ, Krieg PA. Wnt11-R signaling regulates a calcium sensitive EMT event essential for dorsal fin development of *Xenopus*. *Dev Biol.* 2007; 304:127–140. [PubMed: 17240368]
- Gietzen K, Sadorf I, Bader H. A model for the regulation of the calmodulin-dependent enzymes erythrocyte Ca²⁺-transport ATPase and brain phosphodiesterase by activators and inhibitors. *Biochem J.* 1982; 207:541–548. [PubMed: 6299272]
- Giles S, Boehm P, Brogan C, Bannigan J. The effects of ethanol on CNS development in the chick embryo. *Reprod Toxicol.* 2008; 25:224–230. [PubMed: 18242952]
- Goueli BS, Hsiao K, Tereba A, Goueli SA. A novel and simple method to assay the activity of individual protein kinases in a crude tissue extract. *Anal Biochem.* 1995; 225:10–17. [PubMed: 7778758]
- Gryniewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem.* 1985; 260:3440–3450. [PubMed: 3838314]
- Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ, Johannes FJ. Inhibition of protein kinase C μ by various inhibitors. Differentiation from protein kinase C isoenzymes. *FEBS Lett.* 1996; 392:77–80. [PubMed: 8772178]
- Hajimohammadreza I, Probert AW, Coughenour LL, Borosky SA, Marcoux FW, Boxer PA, Wang KK. A specific inhibitor of calcium/calmodulin-dependent protein kinase II provides neuroprotection against NMDA- and hypoxia/hypoglycemia-induced cell death. *J Neurosci.* 1995; 15:4093–4101. [PubMed: 7538570]
- Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. *J Morphol.* 1951; 88:49–92.
- Hansen MR, Bok J, Devaiah AK, Zha XM, Green SH. Ca²⁺/Calmodulin-dependent protein kinases II and IV both promote survival but differ in their effects on axon growth in spiral ganglion neurons. *J Neurosci Res.* 2003; 72:169–184. [PubMed: 12671991]
- Hanson PI, Kapiloff MS, Lou LL, Rosenfeld MG, Schulman H. Expression of a multifunctional Ca²⁺/calmodulin-dependent protein kinase and mutational analysis of its autoregulation. *Neuron.* 1989; 3:59–70. [PubMed: 2619995]
- Hanson PI, Meyer T, Stryer L, Schulman H. Dual role of calmodulin in autophosphorylation of multifunctional CaM kinase may underlie decoding of calcium signals. *Neuron.* 1994; 12:943–56. [PubMed: 8185953]
- Hidaka H, Sasaki Y, Tanaka T, Endo T, Ohno S, Fujii Y, Nagata T. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc Natl Acad Sci USA.* 1981; 78:4354–4357. [PubMed: 6945588]
- Hu XJ, Ticku MK. Chronic ethanol treatment up-regulates the NMDA receptor function and binding in mammalian cortical neurons. *Brain Res Mol Brain Res.* 1995; 30:347–56. [PubMed: 7637584]
- Ikonomidou C, Bittigau P, Ishimaru MJ, Wozniak DF, Koch C, Genz K, Price MT, Stefovsk V, Hörster F, Tenkova T, Dikranian K, Olney JW. Ethanol-induced apoptotic neurodegeneration and fetal alcohol syndrome. *Science.* 2000; 287:1056–1060. [PubMed: 10669420]
- Ishida A, Fujisawa H. Stabilization of calmodulin-dependent protein kinase II through the autoinhibitory domain. *J Biol Chem.* 1995; 270:2163–2170. [PubMed: 7836445]
- Kotch LE, Sulik KK. Patterns of ethanol-induced cell death in the developing nervous system of mice: neural fold states through the time of anterior neural tube closure. *Int J Devl Neurosci.* 1992; 10:273–279.
- Kuhl M, Sheldahl LC, Malbon CC, Moon RT. Ca²⁺/calmodulin-dependent protein kinase II is stimulated by Wnt and frizzled homologs and promotes ventral cell fates in *Xenopus*. *J Biol Chem.* 2000; 275:12701–12711. [PubMed: 10777564]
- Le Douarin, NM.; Kalcheim, C. *The Neural Crest.* 2. Cambridge: Cambridge University Press; 1999.

- Lee AM, Messing RO. Protein kinases and addiction. *Ann NY Acad Sci.* 2008; 1141:22–57. [PubMed: 18991950]
- Lin KF, Change RC, Suen KC, So KF, Hugon J. Modulation of calcium/calmodulin kinase-II provides partial neuroprotection against beta-amyloid peptide toxicity. *Eur J Neurosci.* 2004; 19:2047–2055. [PubMed: 15090032]
- Luo J. Lithium-mediated protection against ethanol neurotoxicity. *Front Neurosci.* 2010; 4:41. [PubMed: 20661453]
- Maffi SK, Rathinam ML, Cherian PP, Pate W, Hamby-Mason R, Schenker S, Henderson GI. Glutathione content as a potential mediator of the vulnerability of cultured fetal cortical neurons to ethanol-induced apoptosis. *J Neurosci Res.* 2008; 86:1064–1076. [PubMed: 18058941]
- Mahadev K, Chetty CS, Vemuri MC. Effect of prenatal and postnatal ethanol exposure on Ca²⁺/calmodulin-dependent protein kinase II in rat cerebral cortex. *Alcohol.* 2001; 23:183–188. [PubMed: 11435029]
- May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev.* 2009; 15:176–192. [PubMed: 19731384]
- McBride WJ, Schultz JA, Kimpel MW, McClintick JN, Wang M, You J, Rodd ZA. Differential effects of ethanol in the nucleus accumbens shell of alcohol-preferring (P), alcohol-non-preferring (NP) and Wistar rats: a proteomics study. *Pharmacol Biochem Behav.* 2009; 92:304–313. [PubMed: 19166871]
- Mihic SJ, Ye Q, Wick MJ, Koltchine VV, Krasowski MD, Finn SE, Mascia MP, Valenzuela CF, Hanson KK, Grennblatt EP, Harris RA, Harrison NL. Sites of alcohol and volatile anaesthetic action on GABA_A and glycine receptors. *Nature.* 1997; 389:385–389. [PubMed: 9311780]
- Miller, MW. *Brain Development: Normal Processes and the Effects of Alcohol and Nicotine.* New York: Oxford University Press; 2006.
- Niwa H, Yamamura K, Miyazaki J. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene.* 1991; 108:193–200. [PubMed: 1660837]
- Powrozek TA, Miller MW. Ethanol affects transforming growth factor beta1-initiated signals: cross-talking pathways in the developing rat cerebral wall. *J Neurosci.* 2009; 29:9521–33. [PubMed: 19641115]
- Rich RC, Schulman H. Substrate-directed function of calmodulin in autophosphorylation of Ca²⁺/calmodulin-dependent protein kinase II. *J Biol Chem.* 1998; 273:28424–28429. [PubMed: 9774470]
- Smith SM, Cartwright MM. Spatial visualization of apoptosis using a whole mount in situ DNA end-labeling technique. *Biotechniques.* 1997; 22:832–834. [PubMed: 9149856]
- Smith, SM.; Debelak-Kragtorp, KA. Neural crest and alcohol exposure. In: Miller, MW., editor. *The Developing Brain: Lessons Learned from Alcohol and Nicotine Exposures.* New York: Oxford University Press; 2006. p. 279-294.
- Smith TL, Navratilova E. Increased calcium/calmodulin protein kinase activity in astrocytes chronically exposed to ethanol: influences on glutamate transport. *Neurosci Lett.* 1999; 269:145–148. [PubMed: 10454153]
- Sulik KK, Johnston MC, Webb MA. Fetal alcohol syndrome: embryogenesis in a mouse model. *Science.* 1981; 214:936–938. [PubMed: 6795717]
- Sulik KK, Cook CS, Webster WS. Teratogens and craniofacial malformations: relationships to cell death. *Development.* 1988; 103(Suppl):213–231. [PubMed: 3074910]
- Takano H, Fukushi H, Morishima Y, Shirasaki Y. Calmodulin and calmodulin-dependent kinase II mediate neuronal cell death induced by depolarization. *Brain Res.* 2003; 962:41–47. [PubMed: 12543454]
- Tatone C, Iorio R, Francione A, Gioia L, Colonna R. Biochemical and biological effects of KN-93, an inhibitor of calmodulin-dependent protein kinase II, on the initial events of mouse egg activation induced by ethanol. *J Reprod Fertil.* 1999; 115:151–157. [PubMed: 10341733]
- Tokumitsu H, Chijiwa T, Hagiwara M, Mizutani A, Terasawa M, Hidaka H. KN-62, 1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine, a specific inhibitor of Ca²⁺/calmodulin-dependent protein kinase II. *J Biol Chem.* 1990; 265:4315–4320. [PubMed: 2155222]

- Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, Kobayashi R. STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase. *J Biol Chem.* 2002; 277:15813–15818. [PubMed: 11867640]
- Waldmann R, Hanson PI, Schulman H. Multifunctional Ca²⁺/calmodulin-dependent protein kinase made Ca²⁺ independent for functional studies. *Biochemistry.* 1990; 29:1679–1684. [PubMed: 2158811]
- Wang G, Bieberich E. Prenatal alcohol exposure triggers ceramide-induced apoptosis in neural crest-derived tissues concurrent with defective cranial development. *Cell Death Dis.* 2010; 1:e46. [PubMed: 21364652]
- Waxman MN, Grotta JC, Silva AJ, Strong R, Aronowski J. Ischemia-induced neuronal damage: a role for calcium/calmodulin-dependent protein kinase II. *J Cereb Blood Flow Metab.* 1996; 16:1–6. [PubMed: 8530541]
- Wayan GA, Lee YS, Tokumitsu H, Silva A, Soderling TR. Calmodulin-kinases: modulators of neuronal development and plasticity. *Neuron.* 2008; 59:914–931. [PubMed: 18817731]
- Wilkinson SE, Parker PJ, Nixon JS. Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. *Biochem J.* 1993; 94:335–337. [PubMed: 8373348]
- Winston NJ, Maro B. Calmodulin-dependent protein kinase II is activated transiently in ethanol-stimulated mouse oocytes. *Dev Biol.* 1995; 170:350–352. [PubMed: 7649368]
- Xu M, Chandler LJ, Woodward JJ. Ethanol inhibition of recombinant NMDA receptors is not altered by coexpression of CaMKII- α or CaMKII- β . *Alcohol.* 2008; 42:425–432. [PubMed: 18562151]
- Zou J, Crews F. Induction of innate immune gene expression cascades in brain slice cultures by ethanol: key role of NF-kappaB and proinflammatory cytokines. *Alcohol Clin Exp Res.* 2010; 34:777–789. [PubMed: 20201932]
- Zucker RM, Hunter ES 3rd, Rogers JM. Apoptosis and morphology in mouse embryos by confocal laser scanning microscopy. *Methods.* 1999; 18:473–480. [PubMed: 10491277]

Figure 1.

Ethanol mobilization of Ca_i^{2+} causes neural crest apoptosis. (A) Dorsal-view diagram of HH8- embryo showing the cranial neural folds imaged for Ca_i^{2+} (boxed region). (B, C) Fura-2 ratiometric image of boxed region in (A), where blue indicates low Ca_i^{2+} signal and green indicates elevated Ca_i^{2+} signal. Scale bar indicates 100 μm . (B) Saline challenge does not evoke a Ca_i^{2+} transient in neural crest progenitors (*), shown in dorsal view. (C) 52 mM ethanol evokes a Ca_i^{2+} transient in neural folds including regions populated by neural crest (*). (D–F) Apoptosis visualized using LysoTracker Red (LTR, white dots) in somite-matched stage 12+ embryos, dorsal view. Scale bar indicates 200 μm . (D) An embryo treated with saline (Sal) at HH8 displays the typical pattern of programmed cell death within the midbrain and hindbrain rhombomeres 1/2, 3 and 5 (arrows). (E) Acute ethanol treatment

substantially elevates the number of apoptotic cells within dorsal midline populations of the hindbrain and midbrain including neural crest progenitors (compare signal at arrows). Ethanol occasionally causes cephalic developmental delay, as shown here. **(F)** Pretreatment with the Ca_i^{2+} chelator Bapta-AM (10 mM) prior to ethanol challenge substantially reduced cell death within the dorsal hindbrain and midbrain (compare signal at arrows). Shown are representative images of 8–10 embryos per treatment. OV, otic vesicle. **(G–I)** Transverse sections through hindbrain at rhombomere 4 visualizing cell death (LTR, red) and neural crest (*snail2*, green); I is cut obliquely across rhombomere 4 and 5. **(G)** Saline-treated hindbrain contains significant numbers of *snail2*⁺ neural crest and few are co-labeled with LTR. **(H)** Ethanol-treated hindbrain contains many *snail2*⁺ cells that are co-labeled with LTR. **(I)** Fewer LTR⁺*slug*⁺ cells are observed in ethanol-exposed hindbrain pretreated with Bapta-AM.

Figure 2.

CaMKII and calmodulin inhibitors prevent ethanol-induced cell death. Shown is the number of LTR+ cells residing with rhombomere 4, a hindbrain region normally containing few apoptotic cells (saline treatment, open bars) but has substantial numbers following ethanol treatment (closed bars). **(A)** Cell death numbers in embryos treated with DMSO control bead or inhibitors of Ca_i^{2+} (Bapta-AM, 10 mM) or calmodulin (10 μM W7, 10 μM calmidazolium [cazi]). **(B)** Cell death numbers following treatment with pan-CaMK inhibitor KN93 (1 mM), CaMKII-selective myrAIP (2.5 μM), CaMIV-specific STO-609 (5 μM) or the inactive analog KN92 (1 mM). **(C)** Cell death numbers following treatment with Protein Kinase C inhibitors Go-6983 (50 μM) or Ro32-0432 (10 μM). Shown is mean $\pm$ SEM of 3–4 replicate experiments, each having 8–12 embryos per treatment. Significantly differs from (*)

ethanol-DMSO or (+) saline-DMSO at $p < 0.001$ as determined by ANOVA followed by Student-Newman-Keuls pairwise multiple comparison.

Figure 3.

CaMKII activity is essential for ethanol-induced cell death in neural crest. Shown is hindbrain cell death in representative HH12/13- embryos, visualized in whole mount using acridine orange (A, C, E, G, I, K) and in rhombomere 4 cross-section using *Sox9* and LTR (B, D, F, H, J, L). (A, B) Saline control embryo displays the endogenous cell death pattern in r1/2, r3 and r5 (A) and little cell death in r4 (B). (C, D) Cell death is significantly expanded throughout the hindbrain and cranial mesenchyme of an ethanol-treated embryo. (E, F) The intracellular calcium chelator Bapta prevents ethanol-induced cell death in the hindbrain (E) including r4 (F). It also caused minor hindbrain dilation. (G, H) The calmodulin inhibitor Calmidazolium (cazi, 10 μ M) prevents ethanol-induced but not endogenous cell death. (I, J) The CaMKII-specific inhibitor MyrAIP (2.5 μ M) prevents ethanol-induced but not endogenous cell death. (K, L) The CaMKIV-selective inhibitor STO-609 (5 μ M) fails to prevent ethanol-induced cell death. * indicates the otic vesicle. In the whole hindbrain views, the upper arrow indicates the endogenous cell death within r3, and the lower arrow indicates the paucity of endogenous apoptosis within r4. Note that both ethanol treatment and several of the calcium inhibitors may also alter hindbrain structure.

Figure 4.

Quantitation of ethanol-induced neural crest death. The mean number of Sox9⁺ neural crest per section (**A**) and the percentage of Sox9⁺ neural crest cells labeled with the cell death indicator LTR (**B**) was determined for rhombomere 4. Embryos were treated with saline (S), ethanol (E), or ethanol plus the indicated antagonist of calcium signaling pathways. (*) $p < 0.01$ vs. saline-only, (+) $p < 0.02$ vs. ethanol-only using ANOVA and Student-Newman-Keuls post hoc analysis, N=3–4 embryos per group and counting 150–200 Sox9⁺ cells/embryo.

Figure 5.

Ethanol rapidly increases pCaMKII in dorsal neural folds. pCaMKII was visualized using antibody directed against the autophosphorylation site Thr-286 (green); nuclei are visualized using DAPI (blue). (A, B) Dorsal view of HH8- (3 somite) embryos challenged for 1 min with saline (A) or 52 mM ethanol (B). Ethanol-treated embryos had significant pCaMKII signal in the dorsal neural folds that are enriched in neural crest (compare green signal at arrows). Scale bar in (A) indicates 100 μ m. (C, D) Transverse sections through the HH8-neural folds. Increased pCaMKII content occurs within ethanol-treated dorsal neural fold populations including neural crest progenitors (boxed region). Compare intensity of green signal in boxes (C vs. D). (E, F) High magnification of boxed region in C, D shows increased pCaMKII signal following brief ethanol treatment. Scale bar indicates 50 μ m (C) and 137 μ m (E).

Figure 6.

Brief ethanol exposure rapidly induces pCaMKII. Western blot analysis was performed on isolated crania exposed to saline or 52 mM ethanol for 1 min. **(A)** Quantitation of total CaMKII and pCaMKII, the latter using antibody directed against the Thr-286 autophosphorylation site, and normalized to Gapdh content. Acute ethanol treatment rapidly elevated pCaMKII levels and did not affect total CaMKII, relative to Gapdh. Shown is mean $\pm$ SEM of three independent experiments each analyzing four individual saline- or ethanol-treated crania with one crania per lane. * indicates significantly different from saline-treatment at $p < 0.005$ as determined by unpaired Student's t-test. **(B)** Representative western blot for total CaMKII (upper panel), pCaMKII (middle panel) and Gapdh (lower panel). S, saline-treated; E, ethanol-treated.

Figure 7.

Ethanol induced CaMKII but not PKC activity. Isolated HH8 crania were treated with the indicated inhibitor and then challenged with saline or 52 mM ethanol for 1 min prior to enzyme assay. **(A)** Acute ethanol treatment selectively increases CaMKII activity in cranial populations and is prevented by pretreating embryos with the CaMKII-selective inhibitor myrAIP. * $p < 0.001$ vs. saline; + $p < 0.001$ vs. ethanol. **(B)** Protein Kinase C activity is very low in HH8 chick crania and is not further increased by 52 mM ethanol. Pretreatment with the PKC-selective inhibitor Go-6893 attenuates the low PKC activity in this cell population. * $p = 0.01$ vs. saline; + $p = 0.039$ vs. ethanol. Shown is the mean $\pm$ SD of 11–13 HH8 cranial

assayed in triplicate. Significance determined by one-way ANOVA followed by Holm-Sidak post hoc comparison.

Figure 8.

Forced expression of CaMKII mutants alters neural crest apoptosis. **(A)** CaMKII enzyme activity is substantially increased in HH12⁺/13⁻ crania that express constitutively active CaMKII(T286D), but not in crania expressing kinase-dead CaMKII(K42M) or eGFP-only. Shown is mean $\pm$ SD of 7 pooled crania assayed in duplicate. (*) $p < 0.05$ vs. no electroporation, (+) $p < 0.05$ vs. ethanol, using ANOVA with a Holm-Sidak post hoc analysis. **(B)** CaMKII(T286D) increases the percentage of eGFP⁺ cells undergoing apoptosis (LTR⁺eGFP⁺/eGFP⁺) to levels comparable to ethanol treatment. (*) $p < 0.001$ vs. eGFP-only, (+) $p < 0.001$ vs. ethanol-eGFP using ANOVA with a Holm-Sidak post hoc analysis, N=3–4 embryos per group and counting 150–200 eGFP⁺ cells/embryo.

Figure 9.

CaMKII affects neural crest apoptosis. Embryos were transfected with the indicated constructs $\pm$ ethanol at HH9 and imaged at HH12+ for eGFP (green), apoptosis (red) and nuclei (blue). Shown are representative transverse sections through rhombomere 4 with dorsal upward. **(A)** Expression of eGFP-only; arrows indicate eGFP⁺ cells, very few of which are also labeled by LTR. Their mesenchymal position identifies them as migrating neural crest. **(B)** eGFP expression in embryo challenged with ethanol (0.43 mmol/egg). Ethanol significantly increases the number of eGFP⁺LTR⁺ cells, indicated by arrows, within neural crest-enriched regions of the dorsal neural tube and migrating into the mesenchyme. **(C)** Forced expression of constitutively active CaMKII(T286D) significantly increased the number of eGFP⁺LTR⁺ cells, indicated by arrows. The majority reside in regions enriched in neural crest, both within the dorsal neural tube and migrating into the mesenchyme. **(D)** Overexpression of kinase-dead CaMKII(K42M) prevented ethanol-induced apoptosis; arrows indicate eGFP⁺ cells, very few of which are LTR⁺. Scale bar in (A) indicates 50 μ m.

Figure 10.

Ethanol signaling pathway that initiates neural crest apoptosis. Shown is a summary of published studies (Debelak-Kragtorp et al. 2003; Garic-Stankovic et al. 2005, 2006) and results herein. Ethanol interacts with a G-protein-coupled receptor (GPCR) of unknown identity to activate Gαi2/3 and Gβγ. Within seconds, the latter stimulates Phospholipase Cβ-mediated synthesis of inositol-1,4,5-trisphosphate (Ins(1,4,5)P3) and calcium release predominantly from intracellular stores. As shown here, the mobilized calcium interacts with calmodulin to activate pCaMKII and thereby initiate the cell death of neural crest and neuronal precursors. We found no evidence for PKC activation or contributions to this ethanol-induced cell death. “?” indicates that the contribution of this signal to ethanol-mediated cell death is unknown at this time.