Despite Differences in Cytosolic Calcium Regulation, Lidocaine Toxicity Is Similar in Adult and Neonatal Rat Dorsal Root Ganglia *In Vitro*

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ABSTRACT

Background: Neuraxial local anesthetics may have neurological complications thought to be due to neurotoxicity. A primary site of action of local anesthetics is the dorsal root ganglia (DRG) neuron. Physiologic differences have been noted between young and adult DRG neurons; hence, the authors examined whether there were any differences in lidocaine-induced changes in calcium and lidocaine toxicity in neonatal and adult rat DRG neurons.

Methods: DRG neurons were cultured from postnatal day 7 (P7) and adult rats. Lidocaine-induced changes in cytosolic calcium were examined with the calcium indicator Fluo-4. Cells were incubated with varying concentrations of lidocaine and examined for viability using calcein AM and ethidium homodimer-1 staining. Live imaging of caspase-3/7 activation was performed after incubation with lidocaine.

Results: The mean KCl-induced calcium transient was greater in P7 neurons ($P < 0.05$), and lidocaine significantly inhibited KCl-induced calcium responses in both ages ($P < 0.05$). Frequency distribution histograms of KCl-evoked calcium increases were more heterogeneous in P7 than in adult neurons. With lidocaine, KCl-induced calcium transients in both ages became more homogeneous but remained different between the groups. Interestingly, cell viability was decreased by lidocaine in a dose-dependent manner similarly in both ages. Lidocaine treatment also activated caspase-3/7 in a dose- and time-dependent manner similarly in both ages.

Conclusions: Despite physiological differences in P7 and adult DRG neurons, lidocaine cytotoxicity is similar in P7 and adult DRG neurons *in vitro*. Differences in lidocaine- and KCl-evoked calcium responses suggest the similarity in lidocaine cytotoxicity involves other actions in addition to lidocaine-evoked effects on cytosolic calcium responses. **(ANESTHESIOLOGY 2014; 120:50-61)**

COMPLICATIONS such as transient neurologic symptoms and cauda equina syndrome have been described after spinal anesthesia and are thought to be due to local anesthetic neurotoxicity. The incidence of transient neurologic symptoms is higher when lidocaine is used compared with other local anesthetics such as bupivacaine, prilocaine, chloroprocaine, and ropivacaine.[1–4] Transient neurologic symptoms and cauda equina syndrome have most often been described in adult populations. The use of pediatric neuraxial anesthesia has increased in some centers over time.[5–7] In observational studies of neuraxial anesthesia in the pediatric population, neurologic complications are rare.[8–11] One study reported cauda equina syndrome with persistent neurological deficit at 1-yr follow-up in a 4-month-old after an inadvertently large bolus of local anesthetic through a caudal

What We Already Know about This Topic

- Local anesthetics exhibit neurotoxicity, which is likely to involve actions at the dorsal root ganglia, and may lead to neuronal complications
- Because postnatal and adult dorsal root ganglia neurons exhibit physiological differences, whether this toxicity may depend on age-related factors is unknown

What This Article Tells Us That Is New

- In a model of neuronal cultures of dorsal root ganglia neurons from postnatal day 7 and adult rats, lidocaine induced similar cytotoxic and apoptotic effects on neonatal and adult rat dorsal root ganglia neurons despite differences in their KCl-evoked calcium responses *in vitro*

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catheter.[12] The true incidence of neurologic complications may be difficult to ascertain in neonates and infants who are nonverbal or not yet ambulatory, underscoring the need for further studies of the complications associated with neuraxial anesthetics in this population.

The exact mechanisms of local anesthetic neurotoxicity have not been wholly elucidated. Local anesthetics act to block conduction primarily through inhibition of voltage-gated sodium channels, but they also act on other channels, such as potassium and calcium channels.[13,14] However, with *in vivo* and *in vitro* models, it has been shown that local anesthetic neurotoxicity is not mediated through blockade of voltage-gated sodium channels.[15–17] With *in vitro* models using neuronal cells, local anesthetics have been shown to be cytotoxic.[16–18] Local anesthetics have been shown to cause necrosis and apoptosis through mechanisms that include increases in intracellular calcium, release of mitochondrial cytochrome c, and inhibition of mitochondrial respiration.[18–20]

Many of these studies used neuronal cell lines or primary cultures of adult dorsal root ganglia (DRG) neurons. A primary site of action of local anesthetics administered neuraxially is the DRG neuron, where blockade inhibits sensation. DRG neurons are heterogeneous and can be classified on the basis of morphology and biochemical markers.[21–23] Differences have been noted between neonatal and adult DRG neurons in culture. Calcium currents differ between neonatal and adult DRG neurons.[24] Microarray analysis of adult and neonatal rat DRG neurons showed differences in ion channel and signaling molecule messenger RNA expression.[25] As local anesthetics act on several different sites, physiological differences between neonatal and adult rat DRG neurons suggest that there may also be an age-related difference in lidocaine toxicity.

The aim of this study was to determine whether there are differences in lidocaine toxicity between young and adult rat DRG neurons. Previously, lidocaine cytotoxicity has been linked to lidocaine-induced increases in cytosolic calcium;[16,18] hence in this study, we first examined whether cytosolic calcium regulation, specifically KCl-evoked and lidocaine-evoked cytosolic calcium increases, was different in postnatal day 7 (P7) and adult rat DRG neurons and whether differences in those responses could account for possible differences in lidocaine cytotoxicity. We compared the cytotoxicity of a 10-min exposure to lidocaine at varying concentrations. In addition, in order to determine whether lidocaine initiated apoptosis, we examined caspase-3/7 activation.

Materials and Methods

Animals

Adult (weighing approximately 450 g) and P7 Sprague–Dawley rats (weighing 13–18 g) were used following the guidelines approved by the Institutional Animal Care and Use Committee of New York University Langone Medical Center.

DRG Cultures

Each rat was anesthetized with 4–5% isoflurane in oxygen for at least 5 min and transcardially perfused with cold artificial cerebrospinal fluid. The rat was then decapitated, and the spinal cord was removed by hydraulic extrusion. The vertebral column was dissected and placed into cold Hibernate A media (Brain Bits, Springfield, IL) supplemented with 2% B27, 0.5 mM Glutamax, penicillin (100 U/ml), and streptomycin (100 μg/ml). DRG were dissected and incubated in supplemented Hibernate A containing trypsin (2.5 mg/ml; Sigma-Aldrich, St. Louis, MO) and collagenase type I (200 U/ml) for 75 min in a rotating incubator at 35°C. The suspension was centrifuged at 2,560g_{max} for 5 min at 4°C. Tissues were further dissociated mechanically by trituration by glass pipettes of decreasing diameter. The suspension was centrifuged at 480g_{max} for 5 min at 4°C. The pellet was resuspended in supplemented Hibernate A and placed on top of an OptiPrep gradient (4.8, 6, 7.4, 9, and 12%; Axis-Shield, Oslo, Norway). The gradient was centrifuged at 900g_{avg} for 15 min at 4°C. Fractions 7.4, 9, and 12% were collected, washed in supplemented Hibernate A, and centrifuged at 480g_{max} for 5 min at 4°C. The pellet was resuspended in Neurobasal A supplemented with 2% B27, 0.5 mM Glutamax, penicillin (100 U/ml), and streptomycin (100 μg/ml). Cells were plated on coverslips precoated with poly-D-lysine (25 μg/ml; Sigma-Aldrich) and allowed to attach for 60 min in a tissue culture incubator with 5% CO_2 and an average humidity of 95% at 37°C. Supplemented Neurobasal A media was then added. Cells were fed every other day and maintained in the tissue culture incubator until use. Each culture is defined as cells prepared from one isolation. All culture media and additives were obtained from Gibco (Carlsbad, CA) unless otherwise stated.

Calcium Imaging

Cells on coverslips were loaded with 5 μM Fluo-4 AM (Molecular Probes, Eugene, OR) for 40 min at 37°C in the original culture medium. The cells were then washed twice in an incubation buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 2 mM $CaCl_2$. Cells were incubated in incubation buffer for 30 min at 37°C to allow deesterification of intracellular AM esters. The Petri dish containing each coverslip was placed in a stage-top incubator containing a heated microscope stage (Heating Insert P S1; Zeiss, Jena, Germany) connected to a temperature regulator (Tempcontrol 73-2 Digital; Zeiss). Cells were maintained at 37°C in humidified room air. After baseline images were taken, lidocaine was added. After 10 min, 100 mM KCl was added to the lidocaine solution, and additional images were taken for 5 min. Images were taken with a Zeiss microscope (Axiovert 200M) using a ×20 EC Plan-Neofluar and the filter set 450–490

(Ex)/515–565 (Em) in a continuous time-based mode. The change in intracellular calcium was expressed as change in peak fluorescence intensity in calcium transient (*i.e.*, the difference between peak and baseline level) relative to the cross-sectional area of each cell.[26,27] Approximately 50% of all DRG neurons were successfully loaded with Fluo-4 AM, which is within the usual loading efficiency range using similar products.* Of these, greater than 90% were responsive to 100 mM KCl stimulation and were included in the analysis.

Fluorescence Imaging of Cell Viability

Cells were maintained in culture for at least 2 days before exposure to different concentrations of lidocaine for 10 min at 37°C. Solutions of lidocaine were prepared from 5% lidocaine in 7.5% dextrose (Hospira, Lake Forest, IL) mixed with supplemented Neurobasal A media. Control coverslips were incubated in lidocaine-free supplemented Neurobasal A media for 10 min at 37°C. Coverslips that served as dead controls were incubated with 70% isopropanol for 30 min at 37°C. After 10-min exposure, all coverslips were washed twice with warm Dulbecco phosphate-buffered saline (Gibco) (37°C). The buffer containing detached cells was collected from each well into microcentrifuge tubes. These were centrifuged at 2,044*g* for 1 min at room temperature. The pellet was resuspended in Dulbecco phosphate-buffered saline, and the detached cells were plated on coverslips precoated with poly-D-lysine (25 μg/ml) and allowed to attach for at least 30 min in a tissue culture incubator. Both adherent (still remaining on coverslips after lidocaine treatment) and detached cells were stained with the LIVE/DEAD assay reagents (calcein AM and ethidium homodimer-1; Invitrogen, Carlsbad, CA) for 30 min at room temperature following the protocols provided by Invitrogen. Cells were counterstained with the nuclear stain Hoechst 33342 (6 μg/ml; Sigma-Aldrich) in the last 15 min of the incubation. Coverslips with adherent cells were rinsed three times with Dulbecco phosphate-buffered saline for 3 min. Coverslips with detached cells, which had been reattached, were gently rinsed once with Dulbecco phosphate-buffered saline for 3 min. The coverslips were mounted on slides and allowed to dry for at least 40 min at room temperature. Living cells contain esterases that convert the nonfluorescent cell-permeant calcein AM to the fluorescent green dye calcein which is retained in intact cells. Cell-impermeant ethidium homodimer-1 enters cells with damaged membranes and emits enhanced red fluorescence upon binding to nucleic acids. All slides were imaged on the same experimental day using a Zeiss microscope (Axiovert 200M) coupled to an X-Cite 120 illuminator with a 120W metal halide lamp (EXFO Life Sciences and Industrial Division, Mississauga, Canada). Images in fluorescence mode were taken using a ×10 EC Plan-Neofluar objective (Zeiss). The green fluorescence of calcein was visualized using the filter set 450–490 (Ex)/515–565 (Em). The red fluorescence of ethidium homodimer-1 was visualized using the filter set 546/12 (Ex)/590 (Em). Blue Hoechst staining was visualized with the filter set 310–390 (Ex)/420 (Em). Four images per coverslip were acquired with a ×10 objective before lidocaine incubation. After staining with the LIVE/DEAD reagents, 20 images per coverslip were acquired with a ×10 objective. Four of these 20 images were chosen randomly to determine the percentage of adherent DRG neurons remaining on the coverslips after lidocaine treatment. The exposure time of each channel was determined using the control samples. Images were analyzed using AxioVision (Zeiss). The fluorescent background of each control was determined based on the histogram. Levels of fluorescence greater than this background were considered positive. DRG neurons were distinguished from nonneuronal cells by their characteristic appearance: large, spherical cell bodies with a birefringent outline. This was confirmed by immunofluorescent staining with the neuronal marker microtubule-associated protein 2 in separate coverslips. DRG neurons with only green calcein staining were counted as living, and neurons with only red ethidium staining were counted as dead. DRG neurons labeled with both green calcein staining and red ethidium staining were classified as compromised cells.

Live Fluorescent Imaging of Caspase-3/7 Activation

Imaging of cells was done at least 2 days after seeding. Cells on coverslips were loaded with 5 μM CellEvent caspase-3/7 reagent (Invitrogen) for 20 min at 37°C. The CellEvent caspase-3/7 detection reagent is a peptide sequence conjugated to a nucleic acid–binding dye. The sequence is recognized and cleaved by activated caspase-3 and -7, releasing the dye to bind nucleic acids, undergoing a fluorogenic response. The Petri dish containing each coverslip was then placed in a stage-top incubator containing a heated microscope stage (Heating Insert P S1; Zeiss) connected to a temperature regulator (Tempcontrol 37-2 Digital; Zeiss). Cells were maintained at 37°C in humidified room air. Lidocaine was added after baseline images were taken, and the cells were imaged at 5-min intervals over a 60-min time period with a Zeiss microscope (Axiovert 200M) using a ×20 EC Plan-Neofluar and the filter set 450–490 (Ex)/515–565 (Em). The fluorescent background of each control was determined based on the histogram. Levels of fluorescence greater than this background were considered positive. The number of newly positive DRG neurons after the addition of lidocaine was quantified for analysis.

Statistical Analysis

Data are expressed as mean ± SD. The KCl-induced calcium transient between adult and P7 DRG neurons was tested using unpaired *t* test. Differences in the calcium transient

* The Molecular Probes Handbook, 11th edition. Available at: http://www.invitrogen.com/site/us/en/home/References/Molecular-Probes-The-Handbook/Technical-Notes-and-Product-Highlights/Loading-and-Calibration-of-Intracellular-Ion-Indicators.html. Accessed May 15, 2013.

within and between age groups were tested with two-way ANOVA test. Otherwise, differences between untreated control and lidocaine-treated groups within each age were tested using one-way ANOVA with Dunnett posttest, and differences between ages were tested with two-way ANOVA with Bonferroni posttest. The statistical test used is indicated in the text or figure legends. Data analysis was done with GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). Analyses were two-tailed. Differences between groups with a P value less than 0.05 were considered statistically significant.

Results

Cytosolic Calcium Imaging

The KCl-evoked cytosolic calcium transient per area was higher in P7 than in adult rat DRG neurons in a statistically significant manner (fig. 1A; $P < 0.05$). This difference cannot be accounted for by differences in cell volume as a frequency distribution histogram showed that the cross-sectional area sizes of P7 DRG neurons were on average smaller than those of adult DRG neurons (fig. 1B). In the DRG cultures, adherent cells with larger diameter and thus larger cross-sectional area are also statistically taller, corresponding to a larger volume (unpublished observations).

Figure 2A shows representative traces for P7 and adult rat DRG neurons of cytosolic calcium responses with exposure to lidocaine for 10 min alone followed by the addition of 100 mM KCl. Lidocaine itself caused increases in cytosolic calcium in some of the P7 and adult DRG neurons at the concentrations tested (fig. 2B). As a population, however, in P7 DRG neurons, the lidocaine-evoked cytosolic calcium increase was only statistically significantly different at 40 mM lidocaine, the highest concentration applied (fig. 2B; $P < 0.05$; one-way ANOVA). A two-way ANOVA test indicates an age difference in response to lidocaine treatment ($P < 0.05$), but no significant lidocaine concentration-dependent effect within each age group. Lidocaine inhibited KCl-evoked increases in cytosolic calcium for both P7 and adult DRG neurons (fig. 2C). This inhibition was statistically significant ($P < 0.05$; one-way ANOVA) for all concentrations of lidocaine tested in both age groups except for 10 mM lidocaine in adult DRG. Within an age group, however, the differences between groups treated with different lidocaine concentrations (except the zero-lidocaine group) were not statistically significant. A two-way ANOVA test indicates also an age-related difference between P7 and adult groups for KCl-evoked calcium responses in the presence of lidocaine ($P < 0.001$).

As DRG neurons comprise a heterogeneous population, frequency distribution histograms describing the percentage of neurons with a given calcium response to KCl alone (fig. 3A) and KCl in the presence of lidocaine (fig. 3B) were compiled for each age group. The frequency distribution profiles indicate that for both P7 and adult DRG neuron populations, the presence of lidocaine made the frequency distribution for the KCl-evoked calcium responses narrower, representing more homogeneous response magnitudes, and diminished the calcium response to KCl in a large number of the cells, especially in adult neurons (fig. 3B). In addition, whether treated by KCl alone or lidocaine with KCl, P7 DRG neurons always had a wider distribution for the KCl-evoked calcium responses than the adult group, suggesting an age-related difference.

Fluorescence Imaging of Cell Viability

Cell viability after lidocaine exposure was determined using a fluorescent assay with calcein AM and ethidium

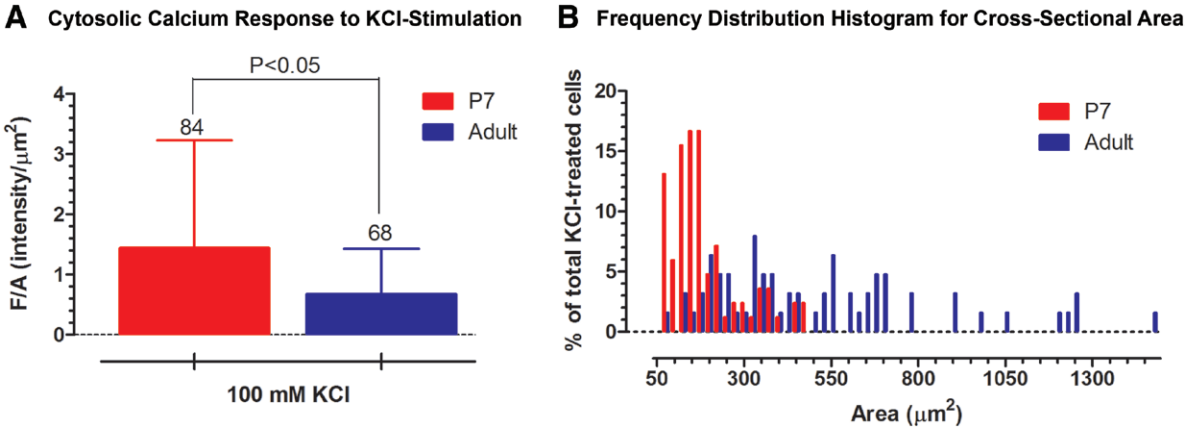

Fig. 1. (*A*) KCl-evoked cytosolic calcium transient in postnatal day 7 (P7) and adult rat dorsal root ganglia neurons. The peak height of the change in cytosolic calcium (*i.e.*, difference between the peak level upon KCl stimulation and the baseline level) was compared. The change in intracellular calcium was expressed as change in peak fluorescence intensity relative to the cross-sectional area for each cell (F/A). Two-tailed unpaired *t* test was used. Sample size (number of neurons) is indicated above each bar (three cultures per age group). (*B*) Frequency distribution histogram for cross-sectional areas of P7 and adult dorsal root ganglia neurons treated with 100 mM KCl, represented as percentage of total examined P7 or adult dorsal root ganglia neurons in this experiment.

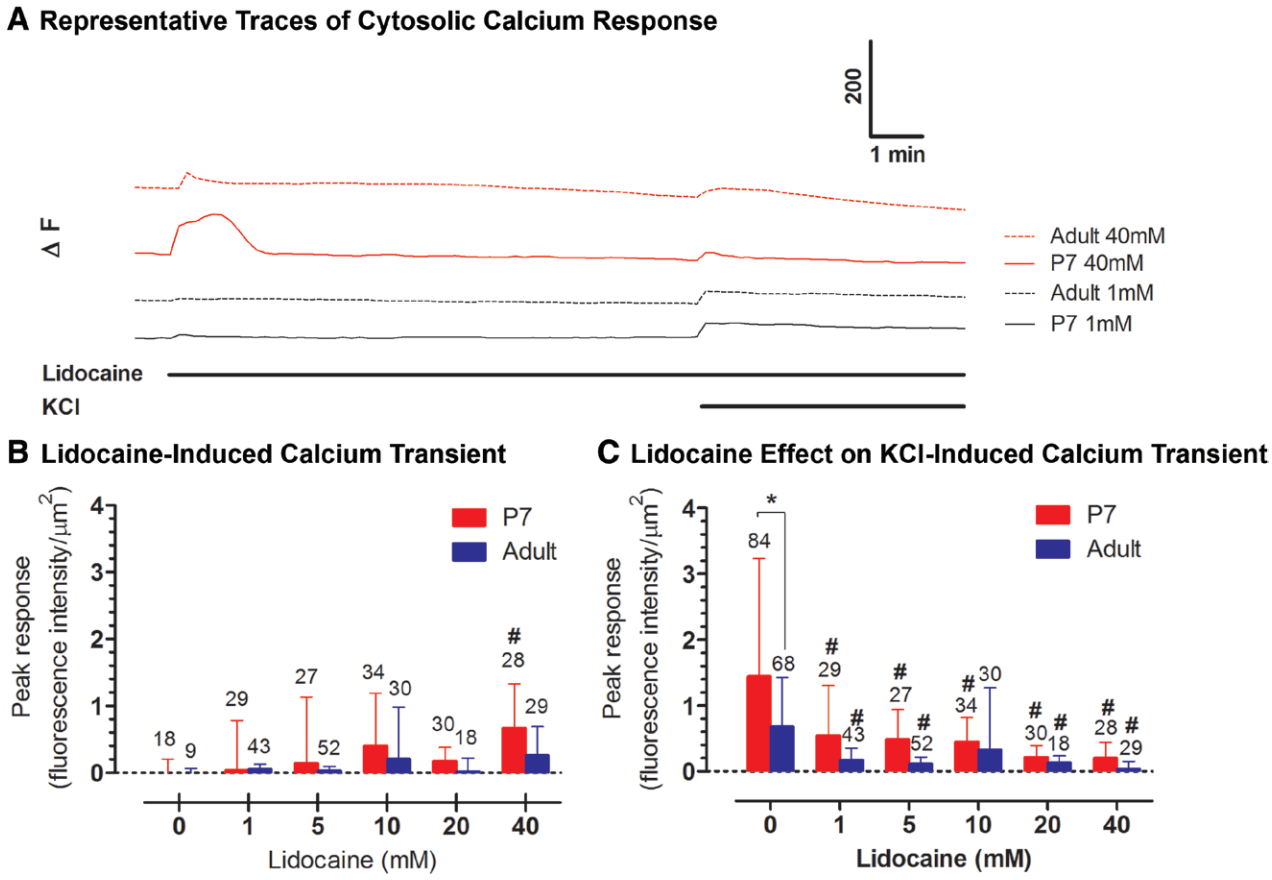

Fig. 2. Representative traces of cytosolic calcium responses in adult and postnatal day 7 (P7) individual rat dorsal root ganglia neurons in response to exposure to lidocaine for 10 min alone followed by the addition of 100 mM KCl (*A*). Effect of (*B*) lidocaine alone and (*C*) KCl stimulation in the presence of lidocaine on cytosolic calcium in P7 and adult dorsal root ganglia neurons; the peak height of the change in cytosolic calcium (*i.e.*, difference between the peak level upon lidocaine and/or KCl addition and the baseline level) was compared. The change in intracellular calcium was expressed as change in peak fluorescence intensity relative to the cross-sectional area for each cell (F/A). Statistical differences between P7 and adult and within each age group at different lidocaine concentrations were determined using one-way ANOVA. *$P < 0.05$ for comparison between P7 and adult dorsal root ganglia neurons; #$P < 0.05$ for comparison of lidocaine-treated to control cells (0 mM lidocaine) within each age group. Sample size is indicated above each bar. A two-way ANOVA test indicated significant differences between P7 and adult groups in lidocaine-induced calcium transients (*B*; $P < 0.05$), as well as in KCl-evoked transients in the presence of lidocaine (*C*; $P < 0.001$). No interaction was found between age and lidocaine concentrations.

homodimer-1 staining. DRG neurons in culture were identified by their typical round shape and light refringency. Figure 4 shows representative images of cell viability of P7 DRG neurons after 10-min treatment with varying concentrations of lidocaine. Green indicates live cells due to calcein staining, and red indicates dead cells due to ethidium bromide staining. Some cells show both green and red fluorescence, indicating impaired integrity of the plasma and nuclear membranes (compromised cells). There was an increase in the number of red cells as the lidocaine concentration was increased. There was also decreased adherence as the lidocaine concentration was increased. Similar results were seen for adult DRG neurons. Figure 5 shows the percentage of living, dead, or compromised cells at various lidocaine concentrations for adult and P7 DRG neurons in the remaining, adherent cells. Lidocaine decreased cell viability of adult and P7 neurons in a dose-dependent manner. Comparing the percentage of neurons with only green calcein staining representing intact, living cells, lidocaine at 60 mM and higher concentrations decreased viability in adult DRG neurons (fig. 5A) and at 50 mM and higher concentrations decreased viability in P7 DRG neurons in a statistically significant manner ($P < 0.05$; fig. 5B).

Figure 4F shows that few cells remained adherent after 10-min exposure to 60 mM lidocaine; therefore, we examined the cells that detached under the various conditions. The pellet recovered from the washes after lidocaine treatment contained cellular debris and structures lacking the characteristic round morphology of DRG neurons. Although reduced adherence to the coverslip could be due to several factors, few structures showed any fluorescent staining, and the ones that did were mainly stained with the red fluorescence of ethidium homodimer-1 exemplifying dead cells (data not shown). Because of this, we quantitated the number of adherent cells

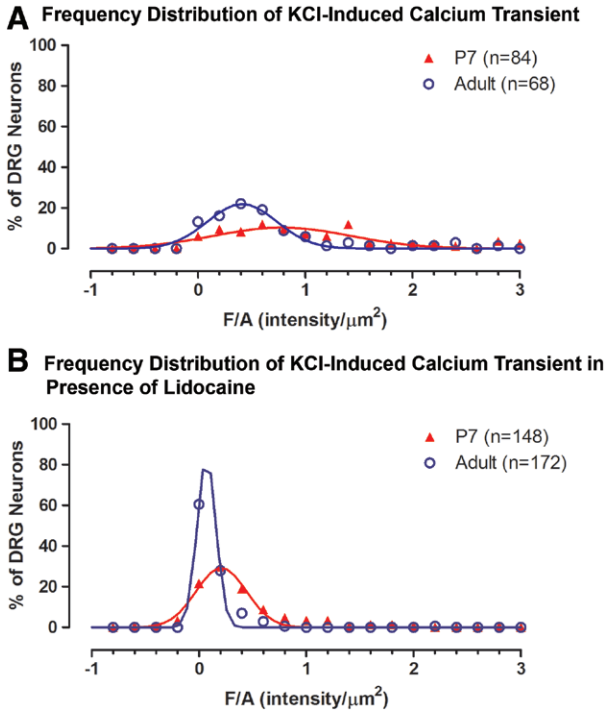

Fig. 3. Frequency distribution histograms describing the percentage of neurons with given calcium responses, measured as the peak fluorescence change relative to the cross-sectional area (F/A). (*A*) The responses to KCl stimulation alone. (*B*) The responses to KCl in the presence of lidocaine (data compiled for all lidocaine concentrations tested). The frequency distribution of the induced changes in calcium became more homogeneous in the presence of lidocaine. Sample size is indicated on each figure (three cultures per age group). DRG = dorsal root ganglia; P7 = postnatal day 7.

under the various conditions. Cell counts showed the average number of adherent neurons in four representative fields of each coverslip before the experiment were 208 ± 52 and 271 ± 47 for adult and P7 groups, respectively (n = 3 separate cultures except n = 4 for P7 dead control). Lidocaine at concentrations of 40 mM and higher caused a statistically significant decrease in the number of adherent P7 and adult neurons on coverslips in a dose-dependent manner and to the same extent (fig. 6; $P < 0.05$). Differences within each age were tested using one-way ANOVA with Dunnett posttest. The differences between adult and P7 DRG neurons were tested with two-way ANOVA with Bonferroni posttest. This is consistent with previous studies examining lidocaine toxicity in DRG neurons that showed a concentration-dependent decrease in the number of adherent DRG neurons on coverslips.[16,28] Lirk *et al.*[28] examined lidocaine toxicity in primarily cultured adult rat DRG neurons and confirmed the decrease in number of cells was due to cell death.

Based on the percentage of remaining, adherent neurons and percentage of those that were viable, dose–response curves of cytotoxicity were generated assuming detached neurons were nonviable (fig. 7). Toxicity was measured as the nonadherent percent plus the dead and compromised fraction of those that remained adherent (*i.e.*, 1 − ([percentage of adherent neurons] × [percentage of viable, adherent neurons])). The EC_{50} values (the concentration which exerts 50% of the cytotoxic effect) of lidocaine were 38 and 35 mM for adult and P7 DRG neurons, respectively; this was not a statistically significant difference ($F = 1.003$; $P = 0.37$).

Live Fluorescent Imaging of Caspase-3/7 Activation

To examine whether lidocaine triggers apoptosis and whether adult and P7 DRG neurons differ in their sensitivities to the apoptotic effects of lidocaine, caspase-3/7 activation was evaluated with live imaging. Representative images of caspase-3/7 activation in adult DRG neurons over 60 min of lidocaine exposure are shown in figure 8. Untreated control cells imaged over 60 min had no newly activated caspase-3/7 (fig. 8A). Gradual activation of caspase-3/7 occurred over time in DRG neurons treated with lidocaine (figs. 8B and C). Similar results were obtained for P7 DRG neurons.

Lidocaine treatment caused a time- and dose-dependent increase in caspase-3/7 activation in both adult and P7 DRG neurons (fig. 9). Treatment with 40 and 60 mM lidocaine caused statistically significant greater caspase-3/7 activation compared with untreated adult DRG neurons at 50 and 60 min ($P < 0.05$; fig. 9A). For P7 DRG neurons, 20 mM lidocaine caused a statistically significant increase in caspase-3/7 activation compared with untreated neurons at 60 min ($P < 0.05$; fig. 9B). Lidocaine at the concentration of 40 mM caused statistically significant greater caspase-3/7 activation at 30 min and beyond, and 60 mM lidocaine caused statistically significant greater caspase-3/7 activation at 20 min and beyond ($P < 0.05$). The preceding statistical differences were determined using one-way ANOVA with Dunnett posttest. There were no significant differences in caspase-3/7 activation over time between adult and P7 DRG neurons at each concentration of lidocaine tested using two-way ANOVA with Bonferroni posttest.

Discussion

The current study shows lidocaine is cytotoxic, measured as a decrease in cell viability and an increase in caspase-3/7 activation, in both adult and P7 rat DRG neurons in a concentration-dependent manner. We found that for most of the concentrations tested, there were no statistically significant differences in lidocaine cytotoxicity between adult and P7 DRG neurons.

It was expected lidocaine would be cytotoxic to DRG neurons in primary culture. Our group previously demonstrated that six local anesthetics including lidocaine decreased cell viability in a concentration-dependent manner in human SH-SY5Y neuroblastoma cells.[17] One study found 15-min exposure to doses greater than 10 mM lidocaine significantly decreased viability in primary cultures of adult rat DRG neurons.[16] The DRG neurons in our study were more resistant to the effects of 10 mM lidocaine; however, the previous study tested viability in cells isolated with

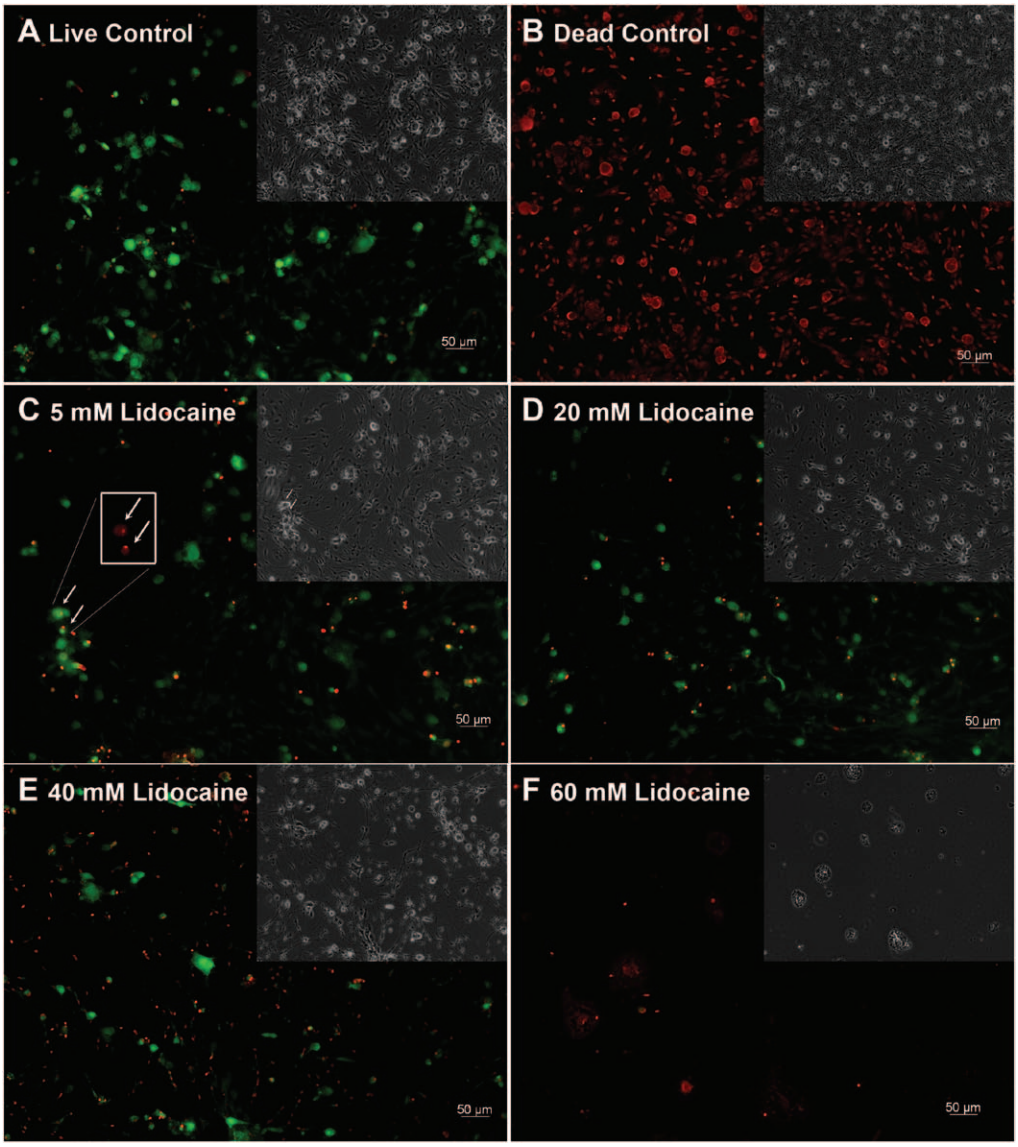

Fig. 4. Representative images showing viability of postnatal day 7 (P7) dorsal root ganglia neurons after 10-min lidocaine exposure using the LIVE/DEAD assay. The inset shows the Nomarski image of the same region. (*A*) Live control cells (without lidocaine treatment) are labeled mainly by calcein producing green fluorescence. (*B*) Dead control cells killed with 70% isopropanol are labeled with ethidium homodimer-1 producing red fluorescence. (*C–F*) P7 dorsal root ganglia neurons after 10-min exposure to increasing concentrations of lidocaine. (*C*) Shows some cells have both green and red fluorescence (compromised cells), seen in the *inset* which shows ethidium labeling alone without superposition of green calcein staining. The included *scale bar* represents 50 μm.

a longer enzymatic digestion and after less than 1 day in culture. It has been reported by Sutachan et al. that the KCl-evoked cytosolic calcium transient changes over time after dissociation, suggesting alterations of cytosolic calcium regulation soon after dissociation.[29] We expect both enzymatic treatment and mechanical trituration contribute at least in part to alterations of cytoplasmic calcium regulation after dissociation. Hence, we used a milder dissociation protocol and 2- to 3-day-old cultures to decrease the influence of enzymatic and mechanical dissociation; this is likely the reason the DRG neurons in our study were more resistant to the effects of 10 mM lidocaine than those from Gold *et al.*[16]

The precise mechanisms of local anesthetic neurotoxicity have not been clarified. Several studies have shown that local anesthetics cause an increase in cytosolic calcium that may be involved in cytotoxicity.[16,18,30] In the ND7 cell line, formed from fusion of rat DRG and mouse neuroblastoma, 60-min exposure to lidocaine at a concentration less than 1%, approximately 36.9 mM, caused transient increases in cytosolic calcium.[18] Treatment with lidocaine at 2.5 and 5%, approximately 92.32 and 184.64 mM, respectively, caused sustained increases in cytosolic calcium associated with plasma membrane lysis in some neurons. In primary cultures of adult rat DRG neurons, Gold *et al.*[16] found that 30-s exposure to

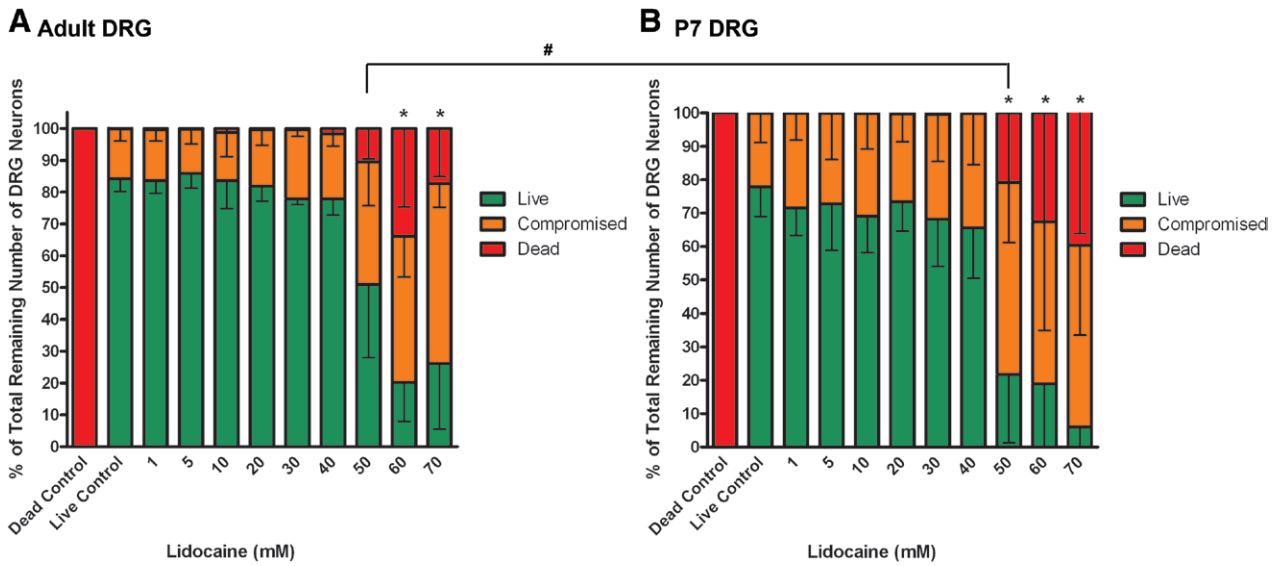

Fig. 5. Percentage of remaining, adherent neurons after a 10-min incubation with lidocaine labeled as live (*green segment*), compromised (*orange segment*), or dead (*red segment*) for (*A*) adult and (*B*) postnatal day 7 (P7) dorsal root ganglia (DRG) neurons. **P* < 0.05 for comparison of percentage of live cells in live control *versus* lidocaine-treated cells within each age; statistical differences were determined using one-way ANOVA followed by Dunnett posttest. #*P* < 0.05 for comparison of percentage of live cells in adult *versus* P7 at each lidocaine concentration; statistical differences were determined using two-way ANOVA with Bonferroni posttest. n = 3 separate cultures except n = 4 for P7 dead control.

lidocaine caused transient increases in cytosolic calcium in a dose-dependent manner. In their model, Gold *et al.* found 15-min exposure to 30 mM lidocaine caused a significant decrease in cell viability compared with control, which was correlated with increases in cytosolic calcium. Consistent with this idea, when DRG neurons were preloaded with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid,, lidocaine toxicity was decreased. We found lidocaine caused increases in cytosolic calcium in P7 and adult DRG neurons. However, both the lidocaine- and KCl-evoked cytosolic calcium responses were different in P7 and adult DRG neurons, suggesting that the similarity in lidocaine cytotoxicity in P7 and adult DRG neurons probably involves other actions in addition to lidocaine-evoked increases in cytosolic calcium.

Several studies have shown that local anesthetics induce apoptosis, usually in a dose-dependent manner, with low concentrations of local anesthetic causing apoptosis and high

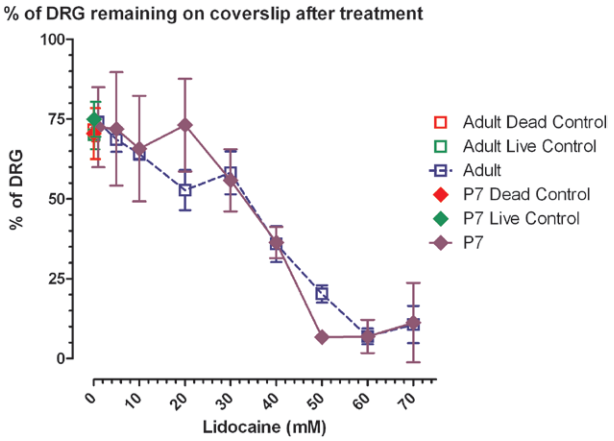

Fig. 6. The percentage of cells remaining adherent to coverslips after lidocaine treatment decreased in a dose-dependent manner for both adult and postnatal day 7 (P7) dorsal root ganglia (DRG) neurons. Images of four representative fields were taken before and after 10-min lidocaine incubation. Neurons were counted, and the percentage of cells remaining after treatment was determined. n = 3 separate cultures except n = 4 for P7 dead control.

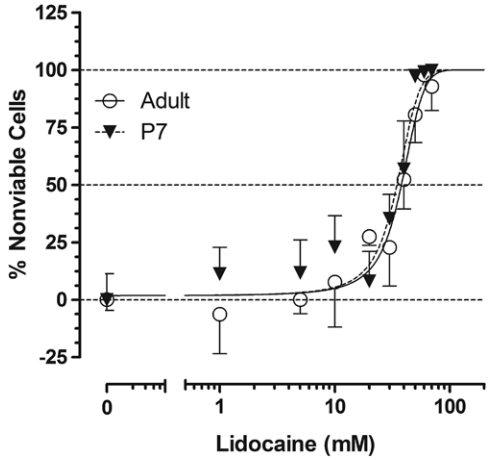

Fig. 7. Percentage of nonviable cells after 10-min lidocaine incubation. Data points were calculated as 1 – ([percentage of adherent neurons] × [percentage of viable, adherent neurons]), assuming detached neurons were nonviable. Dose–response curves were generated by nonlinear fitting using the following equation: Y = 100/(1+10^((LogEC$_{50}$ – X) × Hill Slope)) (data analyzed using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA)). P7 = postnatal day 7.

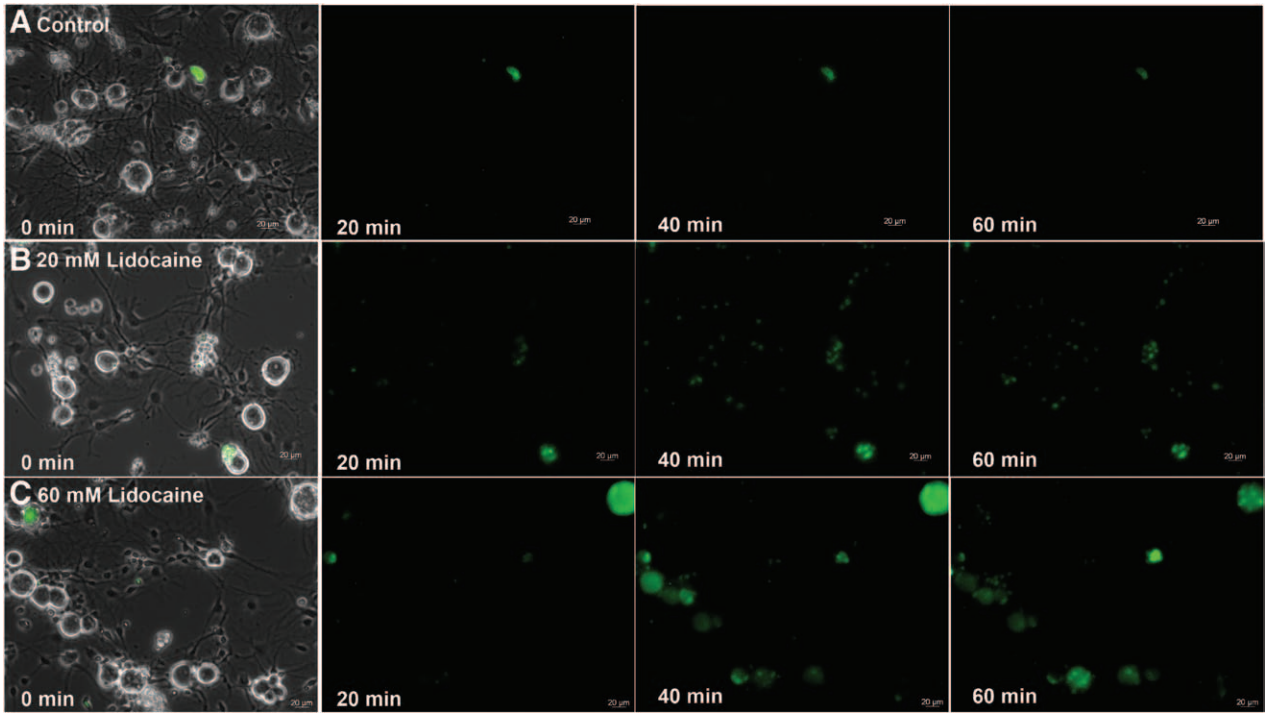

Fig. 8. Representative images showing caspase-3/7 activation in adult dorsal root ganglia neurons over 60 min of lidocaine exposure. (*A*) Control cells (without lidocaine treatment) show no new caspase activation over time. (*B*) 20 mM and (*C*) 60 mM lidocaine treatment increases caspase-3/7 activation over 60 min. The included *scale bar* represents 20 μm.

concentrations causing necrosis.[18,31,32] In this study in adult and P7 DRG neurons, 10-min exposure to lidocaine caused a concentration-dependent increase in cell death. At 10 min, there was no caspase activation at any lidocaine concentration for adult DRG neurons and less than 20% of P7 DRG neurons showed caspase-3/7 activation at 60 mM lidocaine (fig. 9). It is possible then that with a 10-min exposure to lidocaine, most cell death was necrotic as apoptosis may not have had a chance to manifest itself. Caspase-3 and -7 are effector caspases that recognize specific tetrapeptide sequences containing aspartic acid, hydrolyzing bonds at aspartic acid residues, which eventually leads to cell disassembly. Several other studies have shown that local anesthetics cause caspase activation *in vitro*.[17,19,33] Other steps in the apoptotic pathway have been studied. Local anesthetics have been shown to cause mitochondrial cytochrome c release.[19,33] In the ND7 cell line, 37 mm lidocaine caused release of mitochondrial cytochrome c associated with decreased mitochondrial membrane potential.[19] In a study on human Jurkat T-lymphoma cells, lidocaine decreased mitochondrial membrane potential in a dose-dependent manner, tested at 3, 6, and 10 mM concentrations.[33] Cytochrome c release was seen after treatment with 3 and 6 mM lidocaine but was less evident with 10 mM treatment. Jurkat cells overexpressing B-cell lymphoma-2

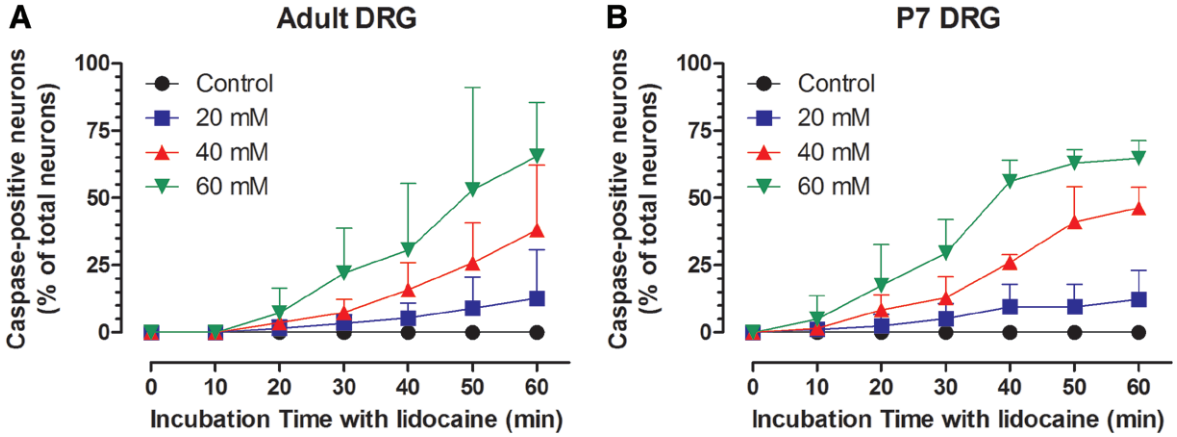

Fig. 9. Activation of caspase-3/7 upon lidocaine exposure in (*A*) adult and (*B*) postnatal day 7 (P7) dorsal root ganglia (DRG) neurons. n = 3 separate cultures.

protein or deficient in caspase-9 were protected against lidocaine-induced apoptosis at 3 and 6 mM, whereas cells deficient in the Fas-associated protein with death domain or caspase-8 showed no attenuation in apoptosis, suggesting that the intrinsic pathway was involved in lidocaine-induced apoptosis at these concentrations. At higher concentrations of lidocaine, B-cell lymphoma-2 protein overexpression or caspase-9 deficiency was not protective, however, suggesting lidocaine at higher concentrations induced necrosis. p38 mitogen-activated protein kinase (MAPK) has been shown to play a role in lidocaine-induced toxicity. In adult rat DRG neurons, p38 MAPK was activated by lidocaine exposure, and inhibition of p38 MAPK attenuated lidocaine-induced cell death.[34] p38 MAPK plays a role in a wide array of cellular functions, including the response to stress and inflammation and in apoptosis.[35] Another mechanism of local anesthetic toxicity includes inhibition of mitochondrial respiration.[19,20] It is possible that necrotic cell death is due to inhibition of mitochondrial energetics or to other changes in cell homeostasis such as alterations in calcium. The precise role, however, of increases in intracellular calcium in response to lidocaine has not been elucidated. p38 MAPK has been shown to be activated by increases in calcium.[36] Therefore, it is possible lidocaine-evoked alterations in calcium homeostasis may affect cell viability through different mechanisms, and this together with other lidocaine actions thus result in similar levels of lidocaine cytotoxicity between P7 and adult DRG neurons.

In using DRG cells in culture as a model to determine whether a difference in cytotoxicity to local anesthetics exists between P7 and adult rat DRG neurons, it is important to ask whether these cells possess an age-related phenotype after days in culture. Others have shown marked differences in calcium currents, transmitter expression, and gene expression in rat neonatal and adult DRG in culture.[24,25,37] Physiologic differences have been noted between neonatal and adult DRG neurons in culture for 3–13 days.[24] The density of low-threshold calcium currents was highest in P2 to P7 cells and declined in adult and old DRG neurons.[24] Another study examined the presence of chemical messengers, peptides, and neurotransmitters in neonatal and adult rat DRG in culture for 5–7 days. They found distinct patterns of transmitter expression and in particular noted substance P was present in 36 and 34% of P2 and P9 DRG neurons, respectively, whereas only present in 22% of adult DRG neurons.[37] Microarray analysis of adult and neonatal (P0-1) rat DRG neurons showed differences in ion channel and signaling molecule messenger RNA expression.[25] In our study, a phenotypic difference between P7 and adult DRG was manifest by the KCl-induced calcium transients, which were greater in a statistically significant manner for P7 than adult DRG neurons. The observed higher KCl-evoked responses per area in P7 neurons cannot be accounted for by differences in cell volume because the frequency distribution histogram for cell sizes (fig. 1B) showed the cross-sectional area of the P7 neurons were on average smaller than those of the adult.

The KCl-induced calcium transient represents the opening and influx of calcium through voltage-dependent calcium channels and the release of calcium from intracellular stores. P7 DRG neurons were also more heterogeneous in their calcium responses to KCl stimulation and lidocaine exposure than adult DRG neurons. Interestingly, this difference in the KCl-evoked response decreased in the presence of lidocaine; hence, in the presence of lidocaine, some aspects of cytosolic calcium regulation may be altered, becoming less different and possibly contributing to similarity in lidocaine toxicity between P7 and adult DRG neurons.

We have shown that lidocaine was cytotoxic to both adult and P7 DRG neurons at clinically relevant concentrations and that the lidocaine dose–responses showed no statistically significant differences in the cytotoxic and apoptotic effects of lidocaine between age groups. Our *in vitro* study suggests that in spite of physiological differences between P7 and adult DRG neurons, lidocaine is essentially equally cytotoxic in these age groups. Although potentially reassuring that lidocaine was not more toxic in P7 than adult DRG neurons, this study has limitations to being extrapolated to what may occur clinically or *in vivo* as the actual lidocaine concentration at the DRG neurons may be affected by differences in blood supply and other environmental differences between P7 and adult DRG. The sheath of satellite cells surrounding DRG neurons has been shown to increase with age in rats.[38] This would be expected to offer less protection to young DRG during exposure to lidocaine, and thus more neuronal damage may be expected *in vivo* in P7 DRG. Further studies need to evaluate these and other possibilities, examining, for instance, effects at the ventral nerve root and at varying ages, including younger than P7 *in vivo*. Behavioral outcomes also need to be assessed because toxicity may manifest itself through changes in development or synaptogenesis.

In summary, in spite of their physiological differences, lidocaine is similarly cytotoxic to adult and neonatal rat DRG neurons in primary culture. More extensive and comparative studies on neuraxial local anesthetics are needed to meaningfully translate the results from this study to the *in vivo* setting and to humans.

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Competing Interests

The authors declare no competing interests.

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