The Neurotoxic Effects of Amitriptyline Are Mediated by Apoptosis and are Effectively Blocked by Inhibition of Caspase Activity

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Oral tricyclic antidepressants, widely used as adjuncts in the treatment of chronic pain, block sodium channels *in vitro* and nerve conduction *in vivo*. However, toxicity of amitriptyline has been observed after neural application. We therefore investigated the mechanism and possible prevention of amitriptyline neurotoxicity. To assess dose-dependent neurotoxicity of amitriptyline, we incubated neuron cultures from adult rat dorsal root ganglia with amitriptyline and quantified neuronal survival. Additionally, we investigated accepted markers of apoptosis (mitochondrial membrane potential, cytosolic cytochrome c, and activated caspase-3) and co-incubated amitriptyline with an inhibitor of caspase activity, z-vad-fmk, to assess the effect on cell survival. We found a dose-dependent neurotoxic effect of amitriptyline. Neurons incubated with amitriptyline exhibited loss of mitochondrial membrane potential, release of cytochrome c into the cytoplasm, and activation of caspase-3. Co-incubation with z-vad-fmk substantially improved neuronal survival in culture. In conclusion, amitriptyline-induced neurotoxicity is mediated by apoptosis and is attenuated by inhibition of caspase activity, suggesting that inhibition of apoptotic pathways may be efficient at alleviating local anesthetic–induced neurotoxicity. *In vivo* studies will have to corroborate whether the co-injection of anti-apoptotic drugs with local anesthetics decreases neurotoxic side effects.

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One major problem with the clinical use of local anesthetics is their neurotoxic potential when they are applied directly to a nerve (1). Prevention of local anesthetic–induced neurotoxicity would be a significant advancement not only for currently used local anesthetics but also for investigational drugs potentially suitable for conduction block. To attain this aim, however, we need to gain a better understanding of the subcellular pathways by which local anesthetics elicit neurotoxicity.

Systemic therapy with antidepressants has long been known to benefit patients suffering from neuropathic (2) and inflammatory (3) pain. Recently, a role for tricyclic antidepressants (TCA) as novel local anesthetics has been suggested because of their ability to block Na^+ channels (4), similar to "conventional" local anesthetics. Indeed, preclinical studies suggest that the prototype TCA, amitriptyline (and derivatives thereof), may provide a more potent and long-lasting conduction block than bupivacaine (5,6). Moreover, locally applied amitriptyline attenuates hyperalgesia following experimental spinal nerve ligation (7). However, TCA have been shown to cause neurotoxic effects when applied directly to a peripheral nerve *in vivo* (8,9).

Amitriptyline has synergistic activity with local anesthetics such as bupivacaine, thereby significantly decreasing the dose of amitriptyline needed to achieve conduction block and potentially decreasing side effects at the same time. Although one would expect

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that a significant decrease of dose would result in a corresponding reduction in neurotoxicity, the issue of amitriptyline neurotoxicity remains clinically significant. We therefore sought to characterize the mechanism of TCA-induced neuronal toxicity and the possible prevention thereof.

We hypothesized that amitriptyline exerts neurotoxicity in primary sensory neuron cultures in a dose-dependent manner, that this effect is mediated by apoptosis (indicated by typical hallmarks such as loss of mitochondrial membrane potential, release of cytochrome c, and activation of caspase-3), and that pharmacologic inhibition of apoptotic pathways alleviates neurotoxicity.

Methods

Experimental Design

To test dose-dependency and apoptosis as a possible mechanism of neurotoxicity directly in adult peripheral neurons, we used the model of dissociated rat dorsal root ganglion (DRG) culture, repeatedly described as a sensitive and well-established *in vitro* paradigm for neurotoxicity testing (10,11). We incubated primary sensory neuron cultures with amitriptyline, or novel derivatives thereof, with or without the addition of a caspase inhibitor, and assessed cell survival.

Experimental Drugs

Unless stated otherwise, drugs were purchased from Sigma Aldrich (Sigma, Vienna, Austria). Stock solutions were prepared by dissolving substances in dimethyl sulfoxide (DMSO, $< 0.1\%$). Test solutions were prepared freshly, and pH of the final amitriptyline solution was 7.38.

Neuron Culture

Adult neurons were obtained from rat dorsal root ganglia, desheathed, and incubated in collagenase, 5,000 U/mL, for 90 min at 37°C followed by 15 min of treatment in 0.25% trypsin/EDTA. Adult rats were aged between 6–8 weeks, and weight was 200–250 g. Each culture contained approximately 400–500 neurons. After dissociation of the neurons in Roswell Park Memorial Institute (RPMI) medium containing 10% horse/5% fetal bovine serum, they were plated in RPMI medium supplemented with N_2 additives and antibiotics. Neurons were allowed to adhere for 24 h to the glass floor of dishes coated with poly-D-lysine/laminin. Subsequently, medium was changed and cultures were subjected to different experimental protocols, while maintaining culture conditions as described above.

Dose-dependency of Neurotoxicity

To confirm whether neurotoxicity of amitriptyline is dose-dependent, we incubated DRG cultures with amitriptyline HCl at final concentrations between 0.1 and 500 μM for 24 h. In this and all subsequent experiments, control cultures were incubated with vehicle DMSO corresponding to the highest concentration of amitriptyline. Subsequently, cultures were fixed with 4% paraformaldehyde for 30 min at 4°C. Neurons were visualized fluoroscopically, by adding monoclonal antibodies to neurofilament (N52m, 1:800) for 2 h at 37°C or overnight at 4°C after neurons were permeabilized with 0.5% Tween20 for 10 min at room temperature. Neuron cultures then were treated with anti-mouse IgG (Alexa mouse red, 1:2000, Molecular Probes, Eugene, OR) for 1 h at room temperature in darkness and subsequently washed twice with PBS.

To determine whether the neurotoxicity profile was different for novel derivatives of amitriptyline, we incubated DRG neuron cultures for 24 h with amitriptyline, N-propyl amitriptyline, and N-phenylethyl amitriptyline, respectively, at a concentration of 100 μM.

For morphologic and morphometric analysis of neurons, a Zeiss Axiovert 100M microscope (Zeiss, Vienna, Austria) equipped for inverted fluorescence was used. Images of neurons were taken at 20× magnification with a digital camera (Spot RT, Visitron, Munich, Germany) connected to a PC and analyzed with Metamorph software (version 4.5r5, Visitron Systems, Munich, Germany). Axonal outgrowth was determined by measuring the longest vector from the cell body to one of the growth cones (maximal axonal distance). This parameter correlates with the total axonal length. The proportion of cells exhibiting outgrowth of axons longer than their cellular diameter was determined as previously described (12).

Detection of Apoptotic Markers

Loss of Mitochondrial Membrane Potential. We used a fluorescent marker (JC-1, Calbiochem, USA) of mitochondrial membrane potential ($\Delta\Psi_m$), a cationic lipophilic fluorescence dye that is selectively incorporated into the mitochondrial membrane. After excitation at a wavelength of 490 nm, intact mitochondria feature red fluorescence (emission at 590 nm) whereas a loss of $\Delta\Psi_m$ is indicated by green fluorescence (emission at 527 nm). After treatment with 100 μM amitriptyline (or vehicle) for 6 h, neurons were incubated with 15 μM JC-1 for 45 min at 37°C, followed by two washes with RPMI medium supplemented with N_2 additives and antibiotics. Subsequently, we determined the ratio of green/red fluorescence of each neuron, which can be used as an indicator of mitochondrial membrane potential.

Release of Cytochrome C. To detect liberation of cytochrome C into the cytoplasm, we stained cultures

fixed using 4% paraformaldehyde after 4 hours incubation with amitriptyline with a selective rabbit polyclonal antibody to cytochrome c (Cyto-C H-104, 1:400, Santa Cruz Biotechnology, CA) for 2 h at room temperature. Subsequently, cultures were treated with anti-rabbit IgG (Alexa rabbit green, Molecular Probes, Eugene, OR) for 1 h at room temperature.

Activation of Cytochrome C. We aimed to detect the active subunit p17 of caspase-3, a typical effector caspase. Cultures were fixed and permeabilized as described above and incubated with a rabbit polyclonal antibody to the cleaved (active) p17 fragment of caspase-3 (1:400, Chemicon, Temecula, CA) for 2 h at room temperature followed by secondary staining with anti-rabbit IgG (Alexa rabbit green, Molecular Probes, Eugene, OR). Cultures were compared with controls undergoing the same procedure of staining, including omission of primary or secondary antibodies on parallel dishes.

For both caspase-3 and cytochrome assays, the excitation wavelength was 488 nm, the absorption wavelength 496 nm and the emission wavelength 519 nm.

Pharmacologic Inhibition of Caspase Activity

To assess whether inhibiting caspase activity would attenuate neurotoxicity, we incubated neurons with 100 μM amitriptyline either with or without 20 μM fluoromethyl ketone peptide inhibitor of caspase (z-vad-fmk, Promega, Germany). After 24 h, cultures were fixed with paraformaldehyde and stained with N52 anti-neurofilament antibodies as described above and the survival of adherent neurons was evaluated.

Statistics

Summarized results are given as mean ± SD (SD). Analysis of variance with *post hoc* Bonferroni correction was used to compare effects of treatment between groups. Unpaired *t*-tests were used to compare the fluorescence of control cultures with that of cultures treated with amitriptyline.

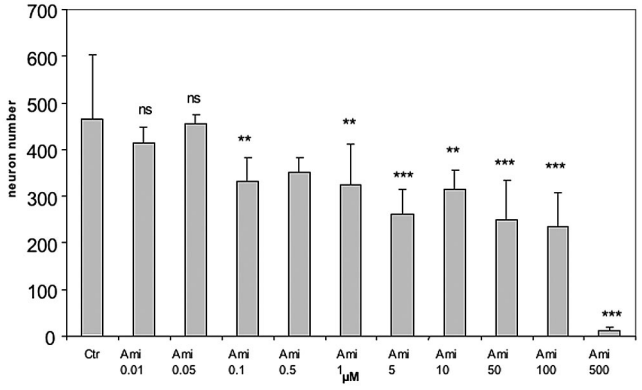

Figure 1. Dose-dependent neurotoxicity of amitriptyline. ** $P < 0.01$; *** $P < 0.001$ as compared to controls.

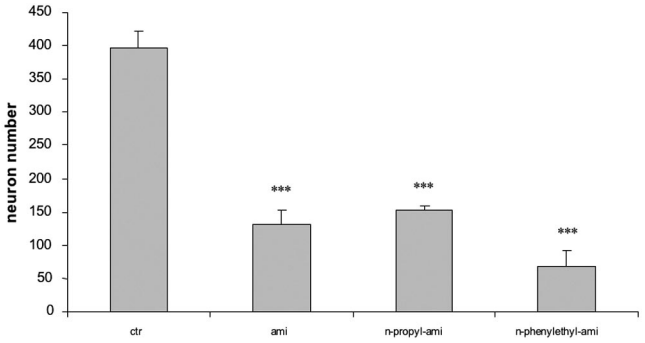

Figure 2. Comparative neurotoxicity of amitriptyline, N-propyl amitritptyline, and N-phenylethyl amitriptyline. Abbreviations: *ami*, amitriptyline, *Ctr*, controls. *** $P < 0.001$ as compared to controls.

Results

Dose-dependent Amitriptyline Neurotoxicity

We found a dose-dependent toxic effect in neurons exposed to amitriptyline (Fig. 1). The average number of neurons was not significantly decreased in cultures incubated with amitriptyline at concentrations of 0.01 μM (415 ± 34, $n = 3$) or 0.05 μM (455 ± 18, $n = 3$) as compared with the number in control cultures (465 ± 140, $n = 13$) In contrast, significantly fewer neurons were observed in cultures incubated with amitriptyline at concentrations of 0.1 μM (332 ± 49, $n = 9$, $P < 0.01$), 0.5 μM (350 ± 32, $n = 8$, $P = 0.07$), 1 μM (322 ± 89, $n = 8$, $P < 0.01$), 5 μM (260 ± 54, $n = 6$, $P < 0.001$), 10 μM (315 ± 39, $n = 8$, $P < 0.01$), 50 μM (250 ± 83, $n = 9$, $P < 0.001$), 100 μM (223 ± 72, $n = 12$, $P < 0.001$), and 500 μM (9 ± 10, $n = 12$, $P < 0.001$). In cultures incubated with 100 μM amitriptyline, the number of neurons present after 24 h of incubation was reduced by about 50%. Therefore, this concentration was chosen for further experiments. With 500 μM amitriptyline, the number of surviving cells declined steeply.

Comparative Neurotoxicity of Amitriptyline and Novel Derivatives

As compared with control cultures (396 ± 25, $n = 3$), cultures incubated with 100 μM amitriptyline contained significantly fewer neurons (130 ± 23, $n = 3$, $P < 0.001$). The same concentration of N-propyl amitriptyline had toxicity similar to that of amitriptyline, whereas the same concentration of N-phenylethyl amitriptyline was significantly more toxic than amitriptyline (67 ± 25, $n = 3$, $P < 0.05$, Fig. 2).

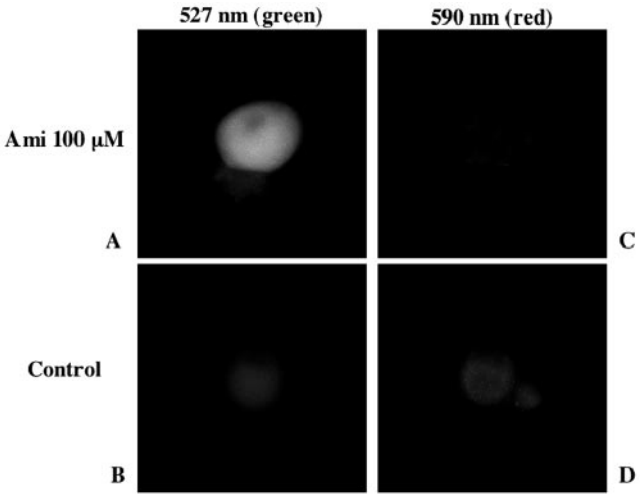

Figure 3. Representative neurons in experiments detecting mitochondrial depolarization. Left column: Green fluorescence indicating loss of $\Delta\Psi_m$ in cultures incubated with amitriptyline (A) but not in controls (B). Right column: Red fluorescence indicating that intact mitochondria are diminished in cultures incubated with amitriptyline (C) as compared with controls (D).

Amitriptyline Reduces Axonal Diameter at High Concentrations

The mean maximum axonal distance was not significantly lower in cultures incubated with amitriptyline at concentrations of 0.1 μM (269 ± 152 μm, $n = 68$), 1 μM (261 ± 109 μm, $n = 107$), 5 μM (302 ± 165 μm, $n = 129$), 10 μM (281 ± 123 μm, $n = 129$), 50 μM (322 ± 175 μm, $n = 70$), or 100 μM (281 ± 164 μm, $n = 193$) than in control cultures (286 ± 154 μm, $n = 165$). Axonal distance was significantly reduced only in cultures incubated with 500 μM amitriptyline (127 ± 57 μm, $n = 6$, $P < 0.05$).

The proportion of cells exhibiting outgrowth of axons larger than the diameter of their cell body was not significantly lower in cultures incubated with amitriptyline at concentrations of 0.1 μM (16 ± 5%, $n = 3$), 1 μM (23 ± 4%, $n = 3$), 5 μM (24 ± 4%, $n = 10$), 10 μM (23 ± 3%, $n = 3$), 50 μM (23 ± 5%, $n = 3$), or 100 μM (24 ± 9%, $n = 10$) than in control cultures (27 ± 10%, $n = 10$). In contrast, cultures incubated with 500 μM amitriptyline featured a significantly reduced share of cells exhibiting outgrowth of axons beyond 20 μm (10 ± 21%, $n = 9$, $P < 0.05$).

Detection of Apoptotic Markers

Loss of Mitochondrial Membrane Potential. Neurons incubated with 100 μM amitriptyline for 4 h exhibited loss of $\Delta\Psi_m$, highlighted by positive JC-1 staining, as shown for a representative neuron in Figure 3. The ratio of mean intensity for green ($\Delta\Psi_m$ depolarized) versus red ($\Delta\Psi_m$ intact) fluorescence was significantly different in controls (0.33 ± 0.12, $n = 115$ cells) and cultures incubated with amitriptyline (0.54 ± 0.88, $n = 82$ cells, $P < 0.01$).

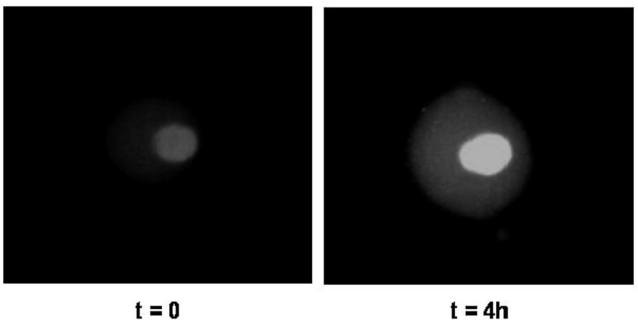

Figure 4. Representative neurons in experiments detecting release of cytochrome c into the cytoplasm, measured as an increase in cytoplasmic fluorescence intensity after 4 h.

Release of Cytochrome C. Incubation with 100 μM amitriptyline for 4 h resulted in release of cytochrome c into the cytoplasm, measured as an increase in fluorescence intensity from 1423 ± 370 ($n = 204$ cells) to 1644 ± 421 ($n = 179$ cells, $P < 0.001$). Figure 4 depicts a representative neuron releasing cytochrome c.

Activation of Caspase-3. Last, incubation with 100 μM amitriptyline for 4 h caused a significant increase in staining for active caspase-3, measured as an increase in fluorescence intensity from 879 ± 202 ($n = 287$ cells) to 972 ± 313 ($n = 291$ cells, $P < 0.001$). Figure 5 depicts a representative neuron demonstrating caspase-3 activation.

Inhibition of Caspase Activity Is Neuroprotective

To assess whether inhibiting caspase activity would attenuate neurotoxicity, we incubated neurons with 100 μM amitriptyline either with or without an inhibitor of caspase activity. Incubation of neurons with amitriptyline alone significantly reduced neuronal survival (from 739 ± 9 ($n = 6$ cultures) to 259 ± 15 ($n = 3$ cultures), $P < 0.001$, see Figure 6). In contrast, co-incubation with 20 μM z-vad-fmk alleviated the decrease in neuron number (average number 676 ± 85, $n = 3$ cultures). The latter survival rate was significantly higher than in cultures incubated with amitriptyline 100 μM alone ($P = 0.007$), and not significantly different from controls ($P > 0.05$).

Discussion

The main findings of the present study are that amitriptyline exerts a dose-dependent toxic effect on primary sensory neurons that is most likely mediated by apoptosis and attenuated by inhibition of caspase activity.

Neurotoxicity of TCA has been cited as a major factor impeding widespread use of this substance

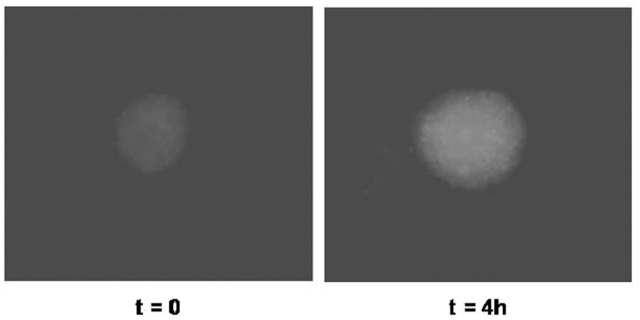

Figure 5. Representative neurons in experiments detecting activation of caspase-3, measured as an increase in cytoplasmic fluorescence intensity after 4 h.

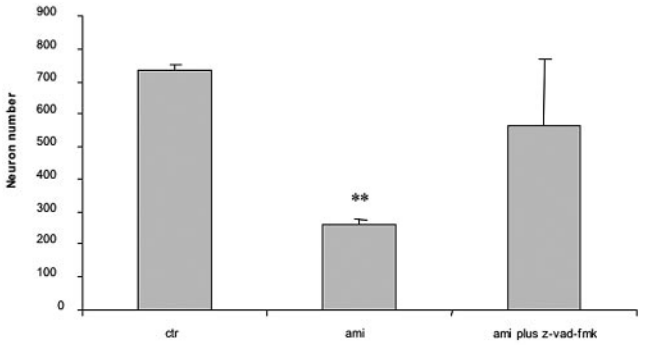

Figure 6. The caspase inhibitor z-vad-fmk attenuates neurotoxicity elicited by amitriptyline. Abbreviations: *Ctr*, controls, *ami*, amitriptyline 100 μM. *** $P < 0.001$ as compared to controls.

class for regional anesthesia (8). Furthermore, neurological sequelae of using of amitriptyline for conduction block in healthy volunteers have been reported, although not more frequently than reported for conventional local anesthetics (8).

Our findings of dose-dependent neurotoxicity *in vitro* correlate with previous *in vivo* data. Specifically, Estebe and Myers (9) observed toxic effects of amitriptyline on peripheral neuronal and glial cells *in vivo* following topical application of relatively high doses (20–80 mM) as compared with those used in the present study. This adverse effect was already discernible at doses well below those reported to elicit conduction block in previous experiments (8,9). Similarly, intrathecal amitriptyline at 25 mM was found to elicit neuronal and glial degeneration in the rodent model (13).

Amitriptyline has been the main focus of research into the use of TCA for regional anesthesia. However, similar local anesthetic properties have been described for other TCA (6) and for derivatives of amitriptyline such as N-phenylethyl amitriptyline (14). To assess whether different derivatives of amitriptyline that are potentially suitable for conduction blockade exhibit a similar side-effect profile, we compared the toxicity of amitriptyline, N-propyl amitriptyline, and N-phenylethyl amitriptyline. It is interesting that the last drug was both more potent and substantially more toxic than amitriptyline. Therefore, differences in the side-effect profiles of different TCA and their derivatives may be inferred. Our results regarding the neurotoxicity of N-phenylethyl amitriptyline confirm previous observations of its narrow therapeutic range and substantial neurotoxic potential (14).

One key marker of neuronal viability is the capability to initiate and sustain axonal outgrowth. The present results suggest that amitriptyline at doses below 500 μM does not significantly influence this vital indicator. This contrasts with a previous investigation that used early developmental chick cerebral neurons describing inhibition of neurite outgrowth by amitriptyline at concentrations of 10 μM (15,16). The difference in model systems most likely explains the discrepancy in results connected with axonal outgrowth. First, early embryological stages of neurons develop developing growth cones more easily and depend on an array of neurotrophic factors for survival. Moreover, central and peripheral neurons feature distinctly different patterns of axonal growth. Our results suggest that TCA elicit neurotoxicity primarily via effects on the cellular metabolism without first inducing axonal degeneration.

Furthermore, we demonstrate that apoptosis mediates, at least in part, the neurotoxic sequelae of amitriptyline *in vitro*. Mitochondrial depolarization, release of cytochrome c, and activation of caspase-3 are generally accepted markers of apoptosis. Our results regarding these apoptotic markers are in agreement with previous mechanistic investigations of TCA toxicity in quiescent and proliferating lymphocyte cultures (17). The local anesthetic lidocaine similarly causes mitochondrial depolarization and caspase activation in immortalized cell lines *in vitro*, eventually leading to apoptosis (18). It should be noted that neurotoxic effects are common not only among TCA and their derivatives but also among currently used local anesthetics, albeit to a lesser degree (8,18,19).

Because the process culminating in apoptosis is at least partially reversible, neuroprotective drugs that intervene in apoptotic pathways may be of considerable benefit. We found that the caspase inhibitor z-vad-fmk substantially reduced the pronounced neurotoxic effect of amitriptyline, confirming immunohistochemical results that suggest caspase activation during amitriptyline-mediated neurotoxicity. Caspase activation has also been described as a pivotal event in neurotoxicity elicited by lidocaine (18). The finding that apoptosis is responsible for TCA-induced neurotoxicity suggests that pathways leading to neuronal damage seem to overlap, at least in part, between classic local anesthetics such as lidocaine and investigational compounds such as amitriptyline. Therefore, an improved understanding of the pathogenic mechanisms involved in neurotoxicity may contribute to a

safer clinical application of both classical and novel drugs. This could be achieved, for example, by co-injecting inhibitors of apoptotic pathways.

The trigger of apoptosis ultimately responsible for local anesthetic–induced neurotoxicity is unclear. Two mechanisms responsible for initiating apoptosis following TCA application have been proposed. First, the generation of reactive oxygen species following amitriptyline application was described previously in HL-60 leukemia cells as a pivotal step in the elicitation of apoptosis by TCA, immediately preceding loss of mitochondrial membrane potential (20). Second, increased cytoplasmic levels of Ca^{2+} were reported after application of both amitriptyline (21) and the conventional local anesthetics lidocaine and bupivacaine in an immortalized neuronal cell line (22).

In conclusion, amitriptyline causes a dose-related cytotoxic effect in neurons beginning at clinically relevant concentrations. This effect is most likely mediated by apoptosis and is efficiently blocked by an inhibitor of caspase activity. Inhibition of apoptotic pathways may be efficient in alleviating neurotoxicity induced by local anesthetics. *In vivo* studies will have to corroborate whether the co-injection of anti-apoptotic drugs with local anesthetics can decrease neurotoxic side-effects.

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