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Chapter 3

Apoptosis induction by different local anesthetics in a neuroblastoma cell line

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Abstract

Background: Local anaesthetics are known to induce apoptosis in clinically relevant concentrations. Hitherto it is unknown what determines the apoptotic potency of local anaesthetics. Therefore, we compared apoptosis induction by local anaesthetics related to their physicochemical properties in human neuronal cells.

Methods: Neuroblastoma cells (SHEP) were incubated with eight local anaesthetics, two of the ester- and six of the amide-type. At least five concentrations of each local anaesthetic were evaluated. After incubation for 24 h, rates of cells in early apoptotic stages and overall cell death were evaluated by annexin V and 7-amino-actinomycin D double staining flowcytometrically. The concentrations that led to half-maximal neurotoxic effects (LD_{50}) were calculated and compared for all local anaesthetics.

Results: All local anaesthetics were neurotoxic in a concentration-dependent manner. All drugs induced similar rates of early apoptotic cell formation at low concentrations, whereas at high concentrations late apoptotic or necrotic cell death predominated. Comparison of LD_{50}-values of the different local anaesthetics resulted in the following order of apoptotic potency from high to low toxicity: Tetracaine > bupivacaine > prilocaine = mepivacaine = ropivacaine > lidocaine > procaine = articaine. The toxicity correlated with octanol/buffer coefficients as well as with experimental potency of the local anaesthetic, but was unrelated to the structure (ester- or amide-type).

Conclusions: All commonly used local anaesthetics induce neuronal apoptosis in clinically used concentrations. The neurotoxicity correlates with lipid solubility and thus with the conduction blocking potency of the local anaesthetic, but is independent of the chemical class (ester/amide).

Local neurotoxicity after neuraxial application of local anaesthetics is rare. Nevertheless, it is a severe problem when it occurs. Clinical profiles of neurotoxicity have been based on the reported incidence of cauda equina syndrome or transient neurologic syndrome (TNS) after spinal anaesthesia.[1-3] Clinically, lidocaine is the local anaesthetic which has most often been linked to local neurotoxicity, although incidental neuronal damage has been described for other local anaesthetics as well. In animal studies neurotoxicity of many local anaesthetics has been demonstrated.[5-9] Experimental evidence suggests that the mechanism of local anaesthetics-induced neurotoxicity is unrelated to the blockade of the voltage-gated sodium channel or electrical inactivation of a nerve.[8 10] Comparison of the neurotoxic potency (concentration-dependency) for different local anaesthetics is difficult in animals for biometric reasons.
Local anaesthetics induce elevations of intracellular calcium concentration through external influx or release from intracellular stores.[11-13] Furthermore, local anaesthetics activate certain kinases and inhibit the energy production in the mitochondria.[14-19] In this process apoptosis has been shown to be one mechanism of neurotoxicity *in-vitro*, especially in marginally toxic concentrations.[20-23] Recent studies have delineated the subcellular mechanism of apoptosis induction by local anaesthetics in neuronal cell cultures.[23 24] Hence, this model seems suitable for the investigation of the neurotoxic and neuroapoptotic potential of different local anaesthetics.
The investigation of the toxicity of different local anaesthetics may help to evaluate which properties of local anaesthetics are responsible for their toxic effects. Does the chemical structure, i.e. ester- or amide-type, influence their toxicity? Ester-type local anaesthetics have been considered to be somewhat more neurotoxic in comparison to amides.[11 25] Are there certain physicochemical properties that determine the toxic potential of a local anaesthetic like lipophilicity, pka-value, protein binding or molecular weight?

Therefore, we compared the concentration-dependent neuroapoptotic and neurotoxic potencies of the amide-type local anaesthetics bupivacaine, lidocaine, mepivacaine, prilocaine and ropivacaine as well as the ester type local anaesthetics procaine and tetracaine in our human neuronal cell culture model.

Methods

Reagents

Unless stated otherwise, reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). All local anaesthetics were obtained in the highest commercially available concentration as their hydrochloride salts. Bupivacaine, lidocaine, mepivacaine and prilocaine were obtained from AstraZeneca (London, United Kingdom). Procaine was purchased from Jenapharm (Jena, Germany), while articaine and tetracaine were acquired from Sanofi Aventis (Paris, France). None of the commercially available solutions contained conservatives. Ropivacaine was kindly provided by AstraZeneca, Research and Development (Södertälje, Sweden). Phosphate-buffered saline (PBS) without calcium and magnesium was purchased from Gibco, Invitrogen (Carlsbad, CA, USA).

Cell Culture

Human neuroblastoma cells (SHEP) are a subclone derived from the human neuroblastoma cell line SK-N-SH and have been characterized before.[26-28] All cells lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with L-glutamine, supplemented with 10% heat-inactivated foetal calf serum and 50 µg ml^{-1} each of penicillin and streptomycin. All cells were cultured under equal conditions including a humidified atmosphere containing 5% carbon dioxide at 37℃.

Exposure to Local Anaesthetics and Experimental Protocol

Prior to the experiments, cells were cultured overnight in complete medium at a density of 4×10^5 cells ml^{-1} to allow logarithmic growth. Adherent cells were cultured in 3 ml samples with fresh medium alone as negative control, or one added local anaesthetic for 24 h at concentrations as indicated. Addition of local anaesthetics did not alter the pH value of the medium (7.39, range 7.35-7.43)

Apoptosis Detection Assay

The fraction of cells in an early state of apoptosis was determined by staining cells with fluorescein isothiocyanate conjugated (FITC) annexin V and counterstaining with 7-amino-actinomycin D (7-AAD). Annexin V binds to phosphatidylserine on the outer leaflet of the plasma membrane. 7-AAD is excluded by cells with intact membranes. Therefore, 7-AAD staining reveals membrane disintegration and is a marker for primary or secondary necrotic cell death. Cells staining with annexin V – FITC (below referred to as annexin V), but not with 7-AAD are defined as early-apoptotic.[29] Briefly,

for annexin V / 7-AAD staining, cell culture medium including detached cells was transferred from sample wells to analyzing tubes. Adherent cells were trypsinized for 3 min with 1.5 ml 0.05% trypsin with 1 mM EDTA per sample. Detached cells were added to corresponding analyzing tubes to pool all cells from each sample. Subsequently, cells were washed twice with cold PBS and resuspended in 97 µl annexin binding buffer (10 mM N-[2-hydroxyethyl]piperazin-N'-3[propansulfonicacid]/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of 1×10^6 cells ml^{-1}. Next, 5 µl of annexin V – FITC and 2 µl 7-AAD (50 µg ml^{-1}) were added and samples were incubated for 15 min in the dark at room temperature. Subsequently, 150 µl of annexin binding puffer were added, cells resuspended and analyzed immediately.

All FACS (fluorescence-activated cell sorting) analyses were performed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest analysis software (BD Biosciences, Franklin Lake, NJ, USA). For each determination, a minimum of 10,000 cells were analyzed.

Statistical Analysis

All experiments were performed at least in triplicate. Results are expressed as means (standard deviation). All calculations were made with the SPSS program version 15.0 (SPSS Inc. Chicago, IL, USA). Concentration-response curves of the different local anaesthetics were determined by probit regression under the guidance of our statistics department. The LD_{50} were obtained from probit analysis and compared by means of analysis of variance (ANOVA) with Tukey´s post hoc test. Correlations between LD_{50} values with lipid solubility, pKa, blocking potency, nonionized fraction, protein binding, molecular weight and experimental blocking potency were made by means of Spearman's rank correlation test. $P < 0.05$ was considered significant.

Results

Concentrations of local anaesthetics inducing cell death in less than 50% of analyzed cells resulted in a considerable fraction of cell staining positive for annexin V only. This indicates an early stage of apoptosis in at least a fraction of the analyzed cells. Nevertheless, also at these concentrations cells staining positive for annexin V staining and 7-AAD were present (Fig. 1) indicating late apoptosis or necrosis.

Figure 1:

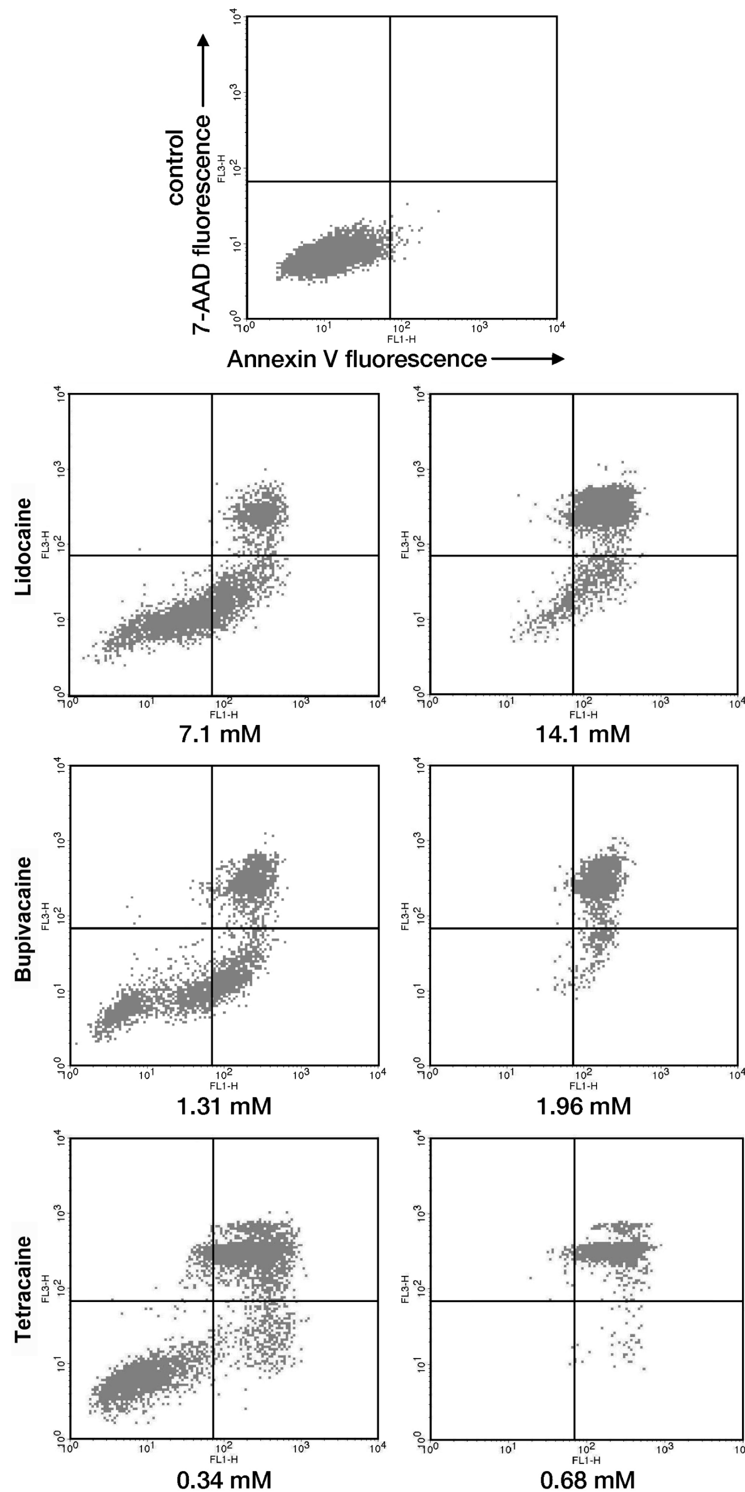

Fig 1 Flowcytometric analysis of neuroblastoma cells incubated for 24 h with investigated local anaesthetics. Dot plots represent typical results after incubation with medium alone (control), lidocaine, bupivacaine or tetracaine. Intensity of red fluorescence by 7-AAD stained cells is indicated on the ordinate, while intensity of green fluorescence emerging from cell-bound annexin V - FITC is indicated on the abscissa. Unstained and therefore vital cells lead to a population of dots in the lower left quadrants as in controls. Cells in the lower right quadrants are stained positive for annexin V – FITC only and are therefore in an early apoptotic stage, while cells in the upper right quadrants were stained positive for annexin V - FITC and 7-AAD indicating late apoptosis or primary necrosis. Note that vital cells and early apoptotic cells can be found at lower concentrations of all local anaesthetics, while at high concentrations almost all cells show signs of lost cell membrane integrity.

Higher concentrations (leading to cell death in more than 50% of analyzed cells) of all investigated local anaesthetics led to increased fractions of cell staining positive both for annexin V and 7-AAD. This indicates a late stage of apoptosis or primary necrosis. Thus, all investigated local anaesthetics induced cell death in a concentration-dependent manner (Fig. 2). The toxic concentrations inducing early stages of apoptosis and late stages of cell death varied over a wide range (almost hundredfold) for the different local anaesthetics.

Figure 2:

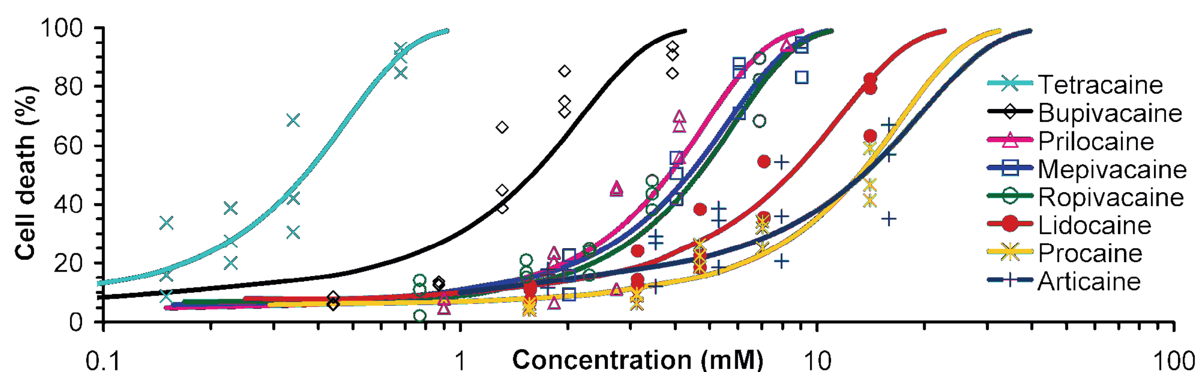

Fig 2 Concentration-dependent in vitro neurotoxicity of local anaesthetics. Sigmoid shaped curves for concentration-response relationship were calculated by probit regression analysis using the results from triplicate experiments with five different concentrations of each local anaesthetic. Single symbols represent results from single experiments.

In order to compare the toxicity of all eight local anaesthetics, the concentration-toxicity functions derived from regression analysis were used to calculate the concentrations that induced approximately 50% overall cell death (LD_{50}). Overall cell death was defined as the sum of cells staining positive for annexin V only and cells staining positive for annexin V and 7-AAD. The different LD_{50} -values are displayed in figure 3. Overall ANOVA of LD_{50}-values revealed significant differences between the eight local anaesthetics ($P < 0.001$). Tukey´s post-hoc-test comparison yielded the following order of toxicity: Tetracaine > bupivacaine > prilocaine = mepivacaine = ropivacaine > lidocaine > procaine = articaine ($P < 0.05$).

In order to identify factors affecting toxicity, the LD_{50}-values were correlated to a number of known physicochemical properties as displayed in table 1. LD_{50}-values correlated well with octanol/buffer distribution coefficients. Spearman's rank correlation coefficient was - 0.88 ($P < 0.01$), i.e. the higher the LA solubility in oil, the

lower was the observed toxic concentration. In contrast LD_{50}-values did not correlate with pka-values, fraction of nonionized local anaesthetic or protein binding. Finally, LD_{50}-values correlated positively with experimental effective anaesthetic concentrations (correlation coefficient: 0.81, P < 0.05).[30-33]

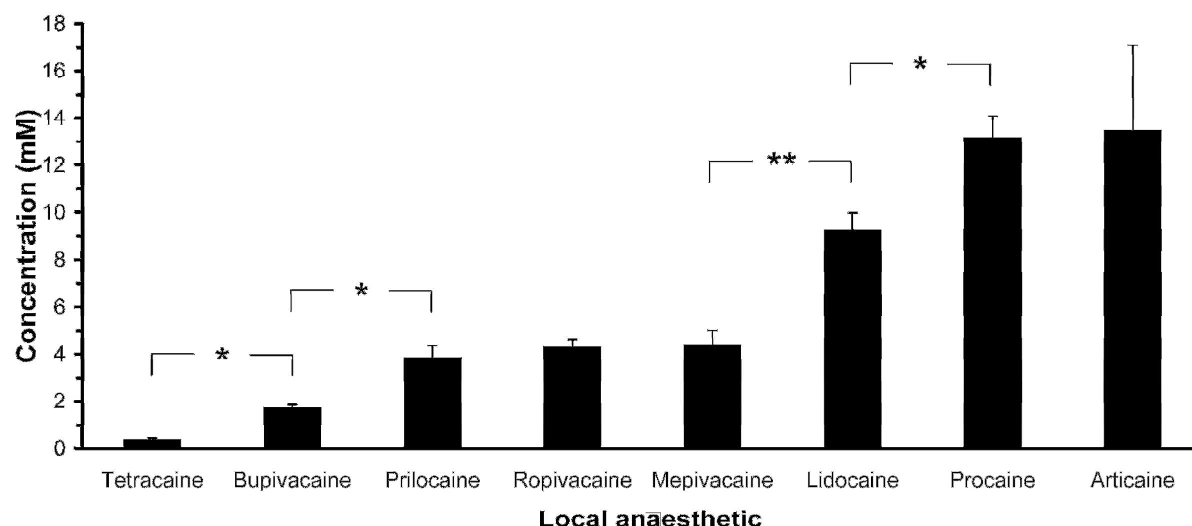

Fig 3 Half-maximal neurotoxic concentrations (LD_{50}) of investigated local anaesthetics. LD_{50}-values were calculated from probit analysis and are mean (SD). Note that not only the LD_{50}-values of two local anaesthetics adjacent to a significance sign were different, but also LD_{50}-values of all local anaesthetics on different sides of a significance sign. E.g. the LD_{50}-value of lidocaine was significantly higher than the values for mepivacaine, ropivacaine, prilocaine, bupivacaine and tetracaine. Furthermore, this value was significantly lower than that of procaine or articaine. ANOVA with Tukey´s post-hoc test. * P < 0.05; ** P < 0.01

Table 1 Correlation between physicochemical properties and toxicity of local anaesthetics. Concentrations of local anaesthetics (mM and % solution of hydrochloride salts) leading to half-maximal neurotoxic effects **(LD_{50})** after 24 h of incubation were calculated by probit regression analysis of concentration-response relationship experiments in SHEP neuroblastoma cells. Values are mean (SD).Partition coefficients **(O/B PQ)** with h-octanol/buffer at pH 7.4 and 25℃,[31-33] ionisation constants (**pKa**),[30, 32-33] unionised fractions at pH 7.4 **(LA_b pH 7.4)**,[30, 32-33] protein binding **(PB)**,[30, 32-33] molecular weight **(MW)** of the local anaesthetics' base in Dalton units[31-33] and relative experimentally effective anaesthetic concentrations **(EAC)** from rat sciatic nerve blocking procedures[31-33] were used to calculate Spearman's rank correlation coefficients **(Spearman's ρ)**.

	LD_{50} (mM)	**LD_{50} (%)**	**O/B PQ**	**pKa**	**LA_b pH 7.4 (%)**	**PB (%)**	**MW (Da)**	**EAC**
Tetracaine	0.37 (0.08)	0.011	221	8.5	7	76	264	0.25
Bupivacaine	1.73 (0.12)	0.056	346	8.1	15	95	288	0.25
Prilocaine	3.85 (0.53)	0.099	25	7.9	24	55	220	1
Ropivacaine	4.32 (0.28)	0.134	115	8.1	15	94	274	0.5
Mepivacaine	4.37 (0.65)	0.123	21	7.6	39	77	246	1
Lidocaine	9.23 (0.74)	0.249	2.4	7.9	25	64	234	1
Procaine	13.14 (0.93)	0.358	1.7	8.9	3	6	236	2
Articaine	13.43 (3.66)	0.480	17	7.8	28	70	321	1
Spearman's coefficient			- 0.88	- 0.33	0.36	- 0.47	0.02	0.81
P Values			< 0.01	0.43	0.38	0.23	0.96	< 0.05

The percentage of early apoptosis (Annexin V+, 7-AAD-) of all cells firstly increased in a concentration-dependent manner, reaching a maximum with LA concentrations well below the LD_{50}-values (Fig. 4). The highest percentage of cell death by early apoptosis varied between the different local anaesthetics between 14 and 28% after 24 h of incubation. With a further increase in concentration the percentage of early apoptotic cell formation decreased and returned to baseline for almost all local anaesthetics (Fig. 4).

Figure 4:

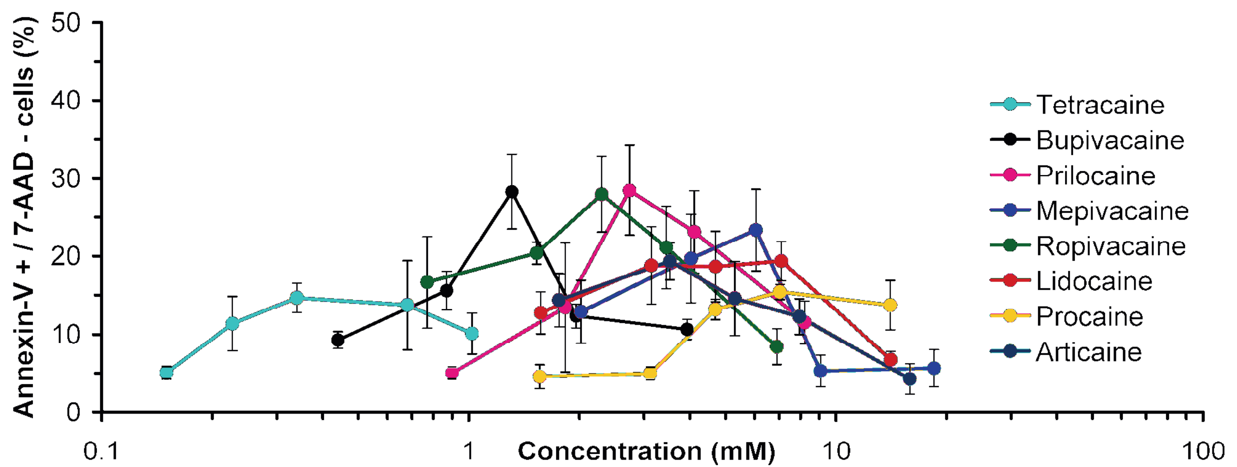

Fig 4 Concentration-dependent induction of early apoptotic cells. The fractions of cells undergoing an early stage of apoptosis were identified by positive annexin V - FITC and negative 7-AAD staining after 24 h incubation with the different investigated local anaesthetics. Note that induction of early apoptosis increases within the first three concentrations of each local anaesthetic and at higher concentrations the fraction of cells with signs of early apoptosis decrease again revealing a change in the mechanism of cell death from apoptosis to necrosis.

Discussion

All local anaesthetics investigated were neurotoxic at concentrations observed intrathecally after spinal anaesthesia. They induced in a concentration-dependent manner first apoptotic and with higher concentrations necrotic cell death. The toxicity of the local anaesthetics correlated with their octanol/buffer partition coefficient and thus their relative clinical potency.

Several publications demonstrated that lidocaine and other local anaesthetics can induce apoptosis in neuronal and non-neuronal cells.[14 15 20-22 37-41] Most of these studies investigated one drug only.[14 21 22 37-41] Therefore, toxic potency of various local anaesthetics from different models cannot be compared for methodological reasons. Boselli and co-workers compared the toxic and apoptotic potential of lidocaine and ropivacaine in human lymphocytes.[20] Unfortunately, in these studies only one concentration of lidocaine and a different - not equipotent - concentration of ropivacaine were investigated. These shortcomings are discussed by the authors themselves and they advocate studies with more concentrations and local anaesthetics in human neuronal cell lines, as presented here.

More recently, Perez-Castro and co-workers compared the cytotoxic effects of short-time (10 min) exposure with procaine, mepivacaine, lidocaine, chloroprocaine, ropivacaine and bupivacaine in human SH-SY5Y neuroblastoma cells and found the same order of toxicity as seen in the data presented here.[42] In contrast to the results presented here, they found apoptosis (non-quantitative caspase activation) only after exposure to high concentrations of lidocaine and bupivacaine, respectively. Probably exposure to very high concentrations for a very short time leads to a greater predominance of necrotic cell death, whereas during long-time exposure of neurons apoptosis is one major mechanism of cell death.

Lirk and colleagues compared the neurotoxic potentials of lidocaine, bupivacaine and ropivacaine in equipotent concentrations in primary cell cultures of rat dorsal root ganglia.[15] They evaluated their blocking potential on voltage-gated sodium channels $Na_V1.1$-3 and $Na_V1.6$ in rat pituitary cells. Then they applied one equipotent concentration of each of the three local anaesthetics on primary cell cultures of rat dorsal root ganglia for 24 h. In these equipotent concentrations the percentage of cell death did not differ between the three local anaesthetics. Although they compared only one concentration of three different local anaesthetics, their

results are reconfirmed and generalized to more local anaesthetics through the results of the present study.

The concentrations of local anaesthetics that induced apoptosis in our model are within the same range as those observed intrathecally after single-shot spinal anaesthesia in primates and in sciatic nerves of rodents during nerve blockade.[31,] Therefore, the concentrations inducing neurotoxicity may be reached clinically.[36] However, after a single-shot spinal anaesthesia or peripheral nerve block these concentrations are only reached for about one hour, whereas in the presented cell culture model the concentration was kept constant for 24 h. It is well known that beyond the concentration, the time of exposure to a local anaesthetic is important for the development of neurotoxicity, therefore neurotoxicity after single application is a rare complication clinically. Thus, intraneural injection of lidocaine 2% (≈ 78 mM) for single shot sciatic nerve block did not lead to any functional nerve damage, although this concentration is more than eight times the here observed LD_{50} concentration.[43]

Although we used human tumour cells which might generally be resistant to apoptosis induction, they were actually more sensitive to apoptosis induced by local anaesthetics than primary rodent dorsal root cell cultures and hybrid immortalized dorsal root ganglia.[15 21] Obviously, the cell culture model used for the presented study has several limitations in translating data to the in vivo situation: Human SHEP neuroblastoma cells are growing and dividing during the local anaesthetic exposure rather than being mature terminally differentiated neurons. They are derived from malignant neural crest cells which would ordinarily differentiate into the sympathetic chain, adrenals or dorsal root ganglia. Nevertheless, despite those limitations our model of a human neuronal cell line seems even more sensitive in detecting minor differences between different local anaesthetics compared to in vivo studies.

Older animal studies compared also the neurotoxic, but not the apoptotic potency of different local anaesthetics.[5 6 8 48] For biometric reasons no study compared a wide range of local anaesthetics in varying concentrations. Nevertheless, a few studies compared different local anaesthetics in equipotent concentrations. Myers and colleagues investigated the effect of 2-chloro-procaine, tetracaine, lidocaine and bupivacaine in high concentrations on the sciatic nerve of rats.[5] All drugs induced swelling, but 2-chloro-procaine and tetracaine significantly more than lidocaine or bupivacaine. However, the authors compared the effects of the different local anaesthetics with only one concentration, thus equipotency could not be

ensured. The same group investigated the blocking and toxic concentrations of etidocaine, lidocaine, 2-chloroprocaine and procaine on the sciatic nerve of rats two days after a single-shot injection. They found a perfect correlation between nerve blocking concentrations and toxic concentrations.[49] Unfortunately, they instituted only two commonly used drugs. Nevertheless, their findings regarding procaine and lidocaine are in accordance with the data presented here.

Sakura et al. evaluated in their model of spinal anaesthesia in rats equipotent concentrations of lidocaine and bupivacaine with an equal sensory deficit after 4 days.[8] In a subsequent study Sakura found an increased functional and morphological neurotoxicity of lidocaine in comparison to bupivacaine, probably because this study was more appropriately powered.[50] Equally, Yamashita and colleagues in a similar model found bupivacaine and ropivacaine to be significantly less toxic then lidocaine and tetracaine.[51]

Recently, Umbrain et al. demonstrated, that intrathecal administration of equipotent doses levobupivacaine and lidocaine led to a release of glutamate and prostaglandine E_2.[52] On repeated injection only lidocaine but not levobupivacaine led to an enhanced prostaglandin release.[52]

To summarize the results of in-vivo animal studies, most of them found orders of toxicity in accordance with our results. The only difference is that lidocaine seems to be more toxic in some studies. This varying toxicity of lidocaine may be related to other mechanisms not detected in all models. Thus, e.g. lidocaine may release inflammatory substances like CGRP as recently discovered.[53] When linked to the clinical situation, the release of inflammatory substances merely under certain circumstances may explain why the incidence of TNS varies within a wide range between 4 and 33%.[1]

Astonishing is the high degree of correlation between the toxicity and octanol/buffer coefficient. Since the lipophilic properties of local anaesthetics correlate with their potency, a correlation between toxicity and potency at the sodium channel is obvious. That might suggest that the toxicity is mediated via the sodium channel. Studies inducing a long-term blockade with tetrodotoxin[8 54] or functionally inactivating a nerve[10] have shown, that a conduction block *per se* does not lead to nerve fibre degeneration. Furthermore, apoptosis induction and toxicity have also been shown in cells not expressing a voltage-gated sodium channel.[23 37 39 40] This implicates, that all local anaesthetics developed on the basis of the known structures

neurotoxic depending on the lipophilic properties. Therefore, one may argue that we have to look out for local anaesthetics with a completely different structure in order to completely avoid this toxicity, e.g. tetrodotoxine which displays no local toxicity.[8] Disappointingly, also the structurally different tricyclic antidepressant amitriptyline which also inhibits the voltage-dependent sodium channel induces apoptosis and local neurotoxicity.[55] More recently, lipophilic effects of a series of amino-amide local anaesthetics on human potassium channels have been reported.[56] This interaction may also be involved in toxic actions of local anaesthetics. Thus, again the lipophilic properties seem to play an important role in determining the toxic potential.

Some studies have attributed the cytotoxicity of local anesthetics and amitriptyline to an unspecific membrane effect as a detergent.[57] [58] However, these physicochemical effects occur with concentrations approximately 10-fold higher. Furthermore, findings that cells are protected against local anesthetic induced apoptosis by Bcl-2 overexpression, caspase-9 deficiency, caspase-inhibitors and p38 mitogen-activated-kinase-inhibitors argue against a detergent-like effect of local anesthetics as the principal cause of cell death.[15-17] [23] [59] Nevertheless, it is conceivable that higher concentrations of local anesthetics, which induce necrosis, might be caused by such a more unspecific effect.

Since tetracaine is the most toxic of all local anaesthetics investigated here, one may ask whether the chemical structure (amide vs. ester type) has influence on the neurotoxicity. In agreement with Tan and colleagues we found that tetracaine and procaine, as the two ester-type local anaesthetics investigated, are at the two ends of the toxicity scale.[14] Therefore, a possible influence of the ester-amide bond on their neurotoxic effects is highly unlikely.

In conclusion, all local anaesthetics are concentration-dependent neuroapoptotic and neurotoxic. The toxicity correlates with the lipophilicity and therefore with the potency of the local anaesthetic. Ester- or amide-type local anaesthetics are equally neurotoxic. Thus, local neurotoxicity seems to be a universal phenomenon of all clinically used local anaesthetics and linked more to physicochemical properties than to their molecular structure.

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