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Organic solvent-induced changes in membrane geometry in human SH-SY5Y neuroblastoma cells – a common narcotic effect?

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ABSTRACT

Exposure to organic solvents may cause narcotic effects. At the cellular level, these narcotic effects have been associated with a reduction in neuronal excitability caused by changes in membrane structure and function. In order to critically test whether changes in membrane geometry contribute to these narcotic effects, cultured human SH-SY5Y neuroblastoma cells have been exposed to selected organic solvents. The solvent-induced changes in cell membrane capacitance were investigated using the whole-cell patch clamp technique for real-time capacitance measurements. Exposure of SH-SY5Y cells to the cyclic hydrocarbons *m*-xylene, toluene, and cyclohexane caused a rapid and reversible increase of membrane capacitance. The aliphatic, nonpolar *n*-hexane did not cause a detectable change of whole-cell membrane capacitance, whereas the amphiphiles *n*-hexanol and *n*-hexylamine caused an increase of membrane capacitance and a concomitant reduction in membrane resistance. Despite a large difference in dielectric properties, the chlorinated hydrocarbons *1,1,2,2*-tetrachoroethane and tetrachloroethylene caused a similar magnitude increase in membrane capacitance. The theory on membrane capacitance has been applied to deduce changes in membrane geometry caused by solvent partitioning. Although classical observations have shown that solvents increase the membrane capacitance per unit area of membrane, *i.e.*, increase membrane thickness, the present results demonstrate that solvent partitioning predominantly leads to an increase in membrane surface area and to a lesser degree to an increase in membrane thickness. Moreover, the present results indicate that the physicochemical properties of each solvent are important determinants for its specific effects on membrane geometry. This implies that the hypothesis that solvent partitioning is associated with a common perturbation of membrane structure needs to be revisited and cannot account for the commonly observed narcotic effects of different organic solvents.

1. Introduction

Organic solvents are fluids used to dissolve substances that do not dissolve in water. In general, organic solvents are relatively small molecules, often volatile with lipophilic or amphiphilic properties. Since they have multiple uses in modern society, exposure to solvents in the gas or liquid phase may occur through inhalation, swallowing, and dermal uptake. Depending on chemical properties and on the route and degree of exposure, solvents can affect multiple organs (US EPA, 2012).

Many organic solvents readily cross the blood-brain-barrier to enter the central nervous system. Long-term exposure to organic solvents is associated with solvent-induced or chronic toxic encephalopathy, and the clinical symptoms range from persisting sensorimotor polyneuropathies to neurobehavioral deficits, and comprise reduction in attention, changes in mood and reduced cognitive skills (Xiao and Levin, 2000; van Valen et al., 2009; van der Laan and Sainio, 2012). The mechanisms underlying these effects of chronic solvent exposure are not straightforward and may be distinct from the mechanisms associated with the effects of short-term, high-level solvent exposure. The latter causes symptoms of narcotic effects with prominent states of fatigue and dizziness that slow reaction time and attenuate rational judgement (*e.g.*, Dick, 1988; Viaene, 2002). It was initially presumed that these narcotic effects are caused by partitioning of organic solvents into the phospholipid membrane of neuronal cells. This has become known as the classic lipid hypothesis of anesthetic action (reviewed by *e.g.*, Seeman, 1972; Urban, 2008).

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The classic lipid hypothesis of anesthetic action, that suggests common nonspecific membrane effects, has been abandoned in favor of the protein theory, *i.e.*, the idea that volatile organic compounds selectively interact with specific protein target molecules (Franks and Lieb, 1994, 2004). By now, *in vitro* studies with cultured neuronal cells have shown that organic solvents affect the functionality of voltage-gated ion channels (*e.g.*, Shafer et al., 2005) and various natively expressed neurotransmitter receptors: organic solvents inhibit N-methyl-D-aspartate receptors (Bale et al., 2005); inhibit nicotinic acetylcholine receptors (Bale et al., 2002; Meulenberg and Vijverberg, 2003) and γ-amino-butyric acid type A ($GABA_A$) receptors (Meulenberg and Vijverberg, 2003; Bale et al., 2005). However, enhancement of $GABA_A$ receptor-mediated inhibitory postsynaptic potentials has also been observed (Beckstead et al., 2000) and receptor expression systems display a more complex pattern of organic solvent effects on receptor functionality (*e.g.*, Bushnell et al., 2005). Such selective effects on membrane proteins do not distract from the fact that organic solvents will cause changes in the structure and geometry (surface area and thickness) of the phospholipid bilayer and that they may share some common effect or mechanism on biological membranes.

Cell membrane geometry is directly reflected in membrane capacitance, as the phospholipid bilayer behaves like a parallel plate capacitor (Lindau and Neher, 1988). Using this relationship, various classes of hydrocarbons have been shown to cause a decrease of the membrane capacitance per unit area in squid giant axons (reviewed by Elliott and Urban, 1995) and lipophilic anions have been shown to cause an increase in whole-cell membrane capacitance (Oberhauser and Fernandez, 1995). In order to study the common effects of organic solvents on the geometry of the phospholipid membrane, we have measured real-time changes of whole-cell membrane capacitance of SH-SY5Y cells following exposure to selected organic solvents.

2. Materials and methods

2.1. Cell culture

Human SH-SY5Y neuroblastoma cells were cultured for up to 20 passages in a 1:1 mixture of Ham's F12 medium (Gibco, Gaithersburg, USA) and Dulbecco's modified Eagle medium (DMEM; Flow Laboratories, USA) supplemented with 15% fetal calf serum (FCS; ICN Biomedicals, USA), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.5% non-essential amino acids (Biedler et al., 1978). Cells were plated in 75 cm^2 plastic tissue culture flasks (2.5×10^6 cells/flask; Nunc, Denmark) and grown at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was refreshed every 2–3 days. After 7 days the cells were harvested and subcultured in 35 mm diameter dishes (10^5 cells/dish; Nunc) in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids. The cells were used for experiments starting on day 3 of subculture. Culture medium was replaced by external saline shortly before the start of the experiment.

2.2. Solutions and chemicals

External saline contained in mM: 125 NaCl, 5.5 KCl, 1.8 $CaCl_2$, 0.8 $MgCl_2$, 20 HEPES, 25 glucose, 36.5 sucrose, pH 7.3 with NaOH (osmolarity 320 mOsm). The pipette solution contained in mM: 10 Na-HEPES, 85 CsCl, 85 CsF, 2 EGTA, 2 $MgCl_2$, pH 7.2 with HCl (osmolarity 330 mOsm). Many organic solvents have a limited water solubility (Table 1). For the less hydrophilic solvents, a saturated solution in external saline was prepared by prolonged stirring of the solvent with 100–250 ml of external saline in a tightly closed glass bottle. This saturated solution contained the upper limit concentration of the solvent to be tested and, when required, was diluted with external saline to obtain a lower concentration of solvent. Cyclohexane (purity 99%), *n*-hexane (95%), and toluene (methylbenzene, 97.7%) were obtained from Baker. Tetrachloroethylene (perchloroethylene, 99.7%) and *n*-hexanol (*1*-hydroxyhexane, 98%) were obtained from Merck. *m*-Xylene (*1,3*-dimethylbenzene, 98.5%) was obtained from Fluka and *1,1,2,2*-tetrachloroethane (99%) from BDH. Changes of pH in external saline containing *n*-hexylamine (*1*-aminohexane, 99%, from Sigma) were compensated by adding HCl. Relevant physico-chemical parameters of the compounds are listed in Table 1.

2.3. Electrophysiological recording

A culture dish containing SH-SY5Y cells in external saline was placed on the stage of an inverted phase-contrast microscope. Single cells were voltage-clamped in the whole-cell configuration (Hamill et al., 1981) using 2–6 MΩ borosilicate glass electrodes (GC150, Clark Electromedical Instruments, UK) and an EPC-7 patch clamp amplifier (List Electronics, Germany). The liquid junction potential was compensated before each experiment and remained constant within 1 mV. It was also confirmed that the solvents used did not change the junction potential. After establishing a gigaohm seal and while in the cell-attached mode capacitive current transients evoked by a small voltage step were carefully cancelled using the C_{fast} compensation circuit of the patch clamp. The value of electrode and other stray capacitance compensated in this way averaged 5.0 ± 0.1 pF (mean ± SEM; $n = 19$). Subsequently, the membrane patch was ruptured by gentle suction to enter into the whole-cell mode and the cell was voltage-clamped at −80 mV. All experiments were performed at room temperature (21–23 °C).

Real and imaginary components of the complex membrane admittance were obtained with a custom-built electronic circuit containing a lock-in amplifier (AD630, Analog Devices) and a sine/cosine generator (4423, Burr-Brown) as described previously (Lindau and Neher, 1988). A sinusoidal voltage (10 mV peak, 1606 Hz; distortion <0.2%) was superimposed on the holding

Table 1
Physicochemical properties of the solvents used (data from Haynes, 2016).

solvent (synonym)	ε_r[a]	molar volume[b] (ml mol^{-1})	log K_{ow}[c]	solubility in H_2O (mM)
n-hexane	1.9	130	4.0	0.11
n-hexylamine (*1*-aminohexane)	4.1	133	2.1	>100
n-hexanol (*1*-hydroxyhexane)	13.0	126	2.0	58
cyclohexane	2.0	109	3.4	0.69
toluene (methylbenzene)	2.4	107	2.7	5.6
m-xylene (*1,3*-dimethylbenzene)	2.4	123	3.2	1.5
tetrachloroethylene (perchloroethylene)	2.3	102	2.9	1.7
1,1,2,2-tetrachloroethane	8.5	105	2.4	17

[a]relative permittivity at 20–30 °C; [b]calculated from the ratio of molecular weight and density; [c]logarithm of *n*-octanol:water partition coefficient.

potential. The resulting membrane current was band-pass filtered (two-pole Butterworth filters, −3 dB at 150 Hz and 2.5 kHz) and fed into the lock-in amplifier. The two orthogonal outputs of the lock-in amplifier (two-pole low-pass filtered, −3 dB at 5 Hz) and DC components of membrane voltage and current (four-pole low-pass filtered, −3 dB at 5 Hz) were recorded. These signals were sampled at 1 s intervals by a four channel 12 bits AD converter (1401 plus; Cambridge Electronic Devices, UK) and stored on disk. The recording circuit was calibrated before experiments using a 100 MΩ resistor (±2%) connected between the input of the patch clamp headstage and ground. Stray capacitance of the resistor and the headstage input (~1.3 pF) was cancelled with the C_{fast} compensation of the patch clamp amplifier. For the purely resistive load remaining, the signal corresponding to the imaginary component of the admittance was adjusted to zero using an adjustable phase shifter during sine wave stimulation to compensate for phase shifts in the recording circuitry. Finally, the gain of the circuit was adjusted to obtain 500 ± 0.5 mV/100 MΩ at the lock-in amplifier output. Calibrated settings of the admittance measuring circuit and of the EPC-7 amplifier, operated at a gain of 1 mV/pA and a bandwidth of 10 kHz, were preserved during the experiment. The proper calibration and operation of the equipment were extensively tested on a realistic cell membrane equivalent circuit (see Supplemental Material). Confounding effects of changes in junction potential, stray capacitance, and osmotic pressure on the passive electrical membrane parameters have been excluded in control experiments on SH-SY5Y cells.

2.4. Single cell superfusion and solvent exposure

Immediately after obtaining a gigaohm seal a continuous, gravity-fed flow of external saline (~1 ml h^{-1}) was applied to the cell through one barrel of a two-barrel theta borosilicate glass pipette with a tip diameter of approximately 100 μm (TGC150-10, Clark Electromedical Instruments, UK). The superfusion pipette was mounted on a step motor-driven micromanipulator and positioned within a distance of 100 μm from the cell. The second barrel of the theta pipette was fed with solvent-containing external saline. Switching the superfusion from one to the other barrel of the pipette allowed for rapid application of a known concentration of solvent to the cell as well as for removal of solvent with external saline. Switching barrels of the superfusion pipette did not cause any detectable effect by itself (see Fig. 1). The level of the external saline in the culture dish (1.0–1.5 ml) was kept constant by a vacuum overflow outlet. To minimize adsorption, glass reservoirs and inert Teflon®, Kel-F® (polychlorotrifluoroethylene), and PEEK (polyetheretherketone) tubing were used. Superfusion reservoirs were covered and the reservoir headspace was minimized to prevent evaporation of solvents. Cells were exposed to a single concentration of one solvent only.

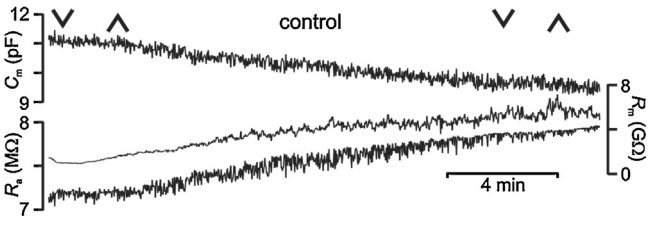

Fig. 1. A continuous 20 min recording of a SH-SY5Y neuroblastoma cell, showing slow trends of whole-cell passive electrical membrane parameters over time under control conditions The membrane holding potential was −80 mV with a 10 mV peak sinusoidal stimulus superimposed. The traces represent real-time calculated values of membrane capacitance (C_m in pF, top trace), membrane resistance (R_m in GΩ, middle trace) and access resistance (R_a in MΩ, bottom trace). The cell was continuously superfused with external saline for the entire recording period. Arrowheads indicate switching forth and back from one to the other barrel of the superfusion pipette, both containing external saline in the control experiment.

2.5. Data analysis

The values of membrane capacitance (C_m), membrane resistance (R_m), and access resistance (R_a) were calculated according to Lindau and Neher (1988) and were monitored real-time using LabVIEW graphical software (National Instruments, Austin, TX). In the analysis, the current reversal potential was assumed to be 0 mV. Note that errors or changes in reversal potential do not cause appreciable error in the estimated values of C_m and R_a when $R_m \gg R_a$ (see Supplemental Material and Fig. S2A). In all experiments $R_m > 100\ R_a$ and in many cases $R_m > 1000\ R_a$. To compare effects between cells, solvent-induced changes in passive electrical membrane parameters were normalized to values shortly prior to solvent application. Results are presented as mean ± standard error of the mean (SEM) of n independent experiments. Each cell was measured in a different culture dish and the different types of experiments were performed over periods generally including multiple culture passages. Statistical significance was assessed by a two-tailed Student's t-test, and by one-way ANOVA when determining concentration-dependence of the observed effects.

3. Results

3.1. Passive electrical membrane parameters of SH-SY5Y cells

Under control conditions, *i.e.*, in voltage-clamped SH-SY5Y cells superfused with external saline, slow trends in the passive electrical membrane parameters were observed over a prolonged period of whole-cell recording. A decrease of membrane capacitance (C_m) and an increase of membrane resistance (R_m) were generally observed (Fig. 1). Switching forth and back to the alternate barrel of the superfusion pipette, both delivering external saline in the control experiments, did not induce changes in any of the passive electrical membrane parameters (see Fig. 1). After 10–30 min of whole-cell recording R_m destabilized and dropped to a low value (not shown) and the recording was stopped.

In 19 cells superfused with external saline only, these typical slow trends in the passive electrical membrane parameters were observed. The value of C_m at the start of the experiments, *i.e.*, immediately after establishing the whole-cell configuration, starting the superfusion, and switching on the equipment, averaged 6.8 ± 0.5 pF (Table 2). After 10 min of superfusion with external saline the average reduction of C_m was less than 4% of its initial value. In the same cells, R_m showed a large increase of 230 ± 54% during the first 10 min of recording (Table 2). The increase in R_m is most likely caused by the pipette solution entering and stabilizing the cell. It is commonly observed in patch clamp experiments that internal solutions containing fluoride ions improve seal formation and stabilize the seal at high GΩ values during the initial period of whole-cell recording. When cells were maintained in the whole-cell configuration for a prolonged period, the increase in R_m gradually leveled off (see Fig. 1). The value of R_a of the control cells ranged between 5.0 and 12.5 MΩ. Approximately one third of R_a (37 ± 2%) was accounted for by the pipette resistance (2–5 MΩ). Although R_a showed a tendency to increase during prolonged whole-cell recording, marked changes were not observed within the first 10 min of whole-cell recording (Table 2).

3.2. Effects of membrane partitioning of nonpolar hydrocarbon solvents

Various nonpolar aliphatic and cyclic hydrocarbon solvents have a relative permittivity (Table 1) close to that of the cell

Table 2
Initial values and trends of the passive electrical parameters of 19 SH-SY5Y cells during control whole-cell recordings from cells superfused with external saline. Trends were expressed as a percentage of the initial value in each cell and the average change was calculated from these values.

	initial values		values after 10 min	
membrane parameter	range	mean ± SEM	range	% change rel. initial
C_m	3.8–11.8 pF	6.8 ± 0.5 pF	3.7–11.5 pF	−3.7 ± 1.3%
R_m	1.8–32.0 GΩ	7.3 ± 1.5 GΩ	3.7–48.9 GΩ	230 ± 54%
R_a	5.0–12.5 MΩ	7.7 ± 0.5 MΩ	4.7–14.3 MΩ	0.1 ± 3.4%

membrane, which is estimated to be 2.14 (Haydon et al., 1977). Because of their nonpolar nature these compounds will readily partition into the cell membrane and cause little change in the dielectric properties of the membrane. However, partitioning of the solvent into the membrane will change the bilayer volume. These compounds will cause a change in C_m only if the relative change in membrane area differs from the relative change in membrane thickness (see Supplemental material).

Superfusion of voltage-clamped SH-SY5Y cells with external saline containing nonpolar hydrocarbons caused rapid changes in C_m. For example, superfusion with a saturating concentration of *m*-xylene (1.5 mM, see Table 1) induced a rapid increase in C_m that was readily reversed when the solvent was removed, while systematic effects on R_m and R_a were not observed (Fig. 2A, Table 3). Additional experiments, with a lower concentration of 0.8 mM *m*-xylene, also showed an increase in C_m, and the size of this effect was smaller than that obtained with the saturating concentration of *m*-xylene ($p < 0.01$, Table 3). Toluene (5.6 mM) caused comparable effects, *i.e.*, a marked increase in C_m without systematic effects of toluene on R_m and R_a (Table 3). Additional experiments, in which cells were superfused with external saline containing 2.8 mM toluene ($n = 7$), did not show detectable changes. The cyclic hydrocarbon cyclohexane, superfused at its saturating concentration of 0.69 mM in external saline, also caused a rapid increases in C_m (Fig. 2B), and washing with external saline readily reversed this effect. While R_m showed a tendency to decrease in the presence of cyclohexane, R_a remained stable (Table 3).

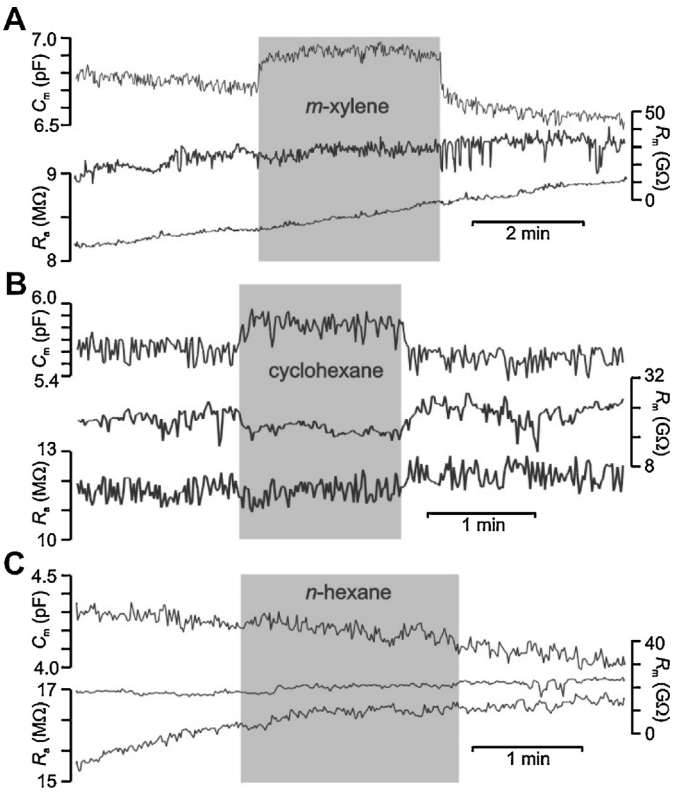

Fig. 2. Representative examples of recordings depicting changes of passive electrical membrane parameters induced by lipophilic organic solvents in whole-cell voltage-clamped SH-SY5Y neuroblastoma cells. Values of the membrane capacitance (C_m in pF, top traces), the membrane resistance (R_m in GΩ, middle traces) and the access resistance (R_a in MΩ, bottom traces) calculated from the real and imaginary parts of whole-cell membrane admittance recorded over a prolonged period at the holding potential of −80 mV. Experiments demonstrate the effect of superfusion with external saline containing (A) 1.5 mM *m*-xylene, (B) 0.69 mM cyclohexane, and (C) a saturating concentration (0.11 mM) of *n*-hexane. The periods of superfusion with solvent-containing saline are indicated by the shaded areas. The effects observed are reversed on removal of the compounds by superfusion with external saline. Note that *n*-hexane (C) did not cause a detectable change in C_m.

n-Hexane, is a hydrophobic aliphatic molecule that is supposed to partition readily into the inner region of phospholipid membranes (McIntosh et al., 1980; White et al., 1981; Pope et al., 1989; MacCallum and Tieleman, 2006).

Superfusion of voltage-clamped SH-SY5Y cells with external saline containing a saturating concentration of *n*-hexane (0.11 mM, see Table 1) for up to six minutes did not induce any detectable changes in the passive electrical parameters of voltage-clamped cells (Fig. 2C; Table 3).

3.3. Dielectric effects of membrane partitioning of chlorinated hydrocarbons

The contribution of the solvent dielectric properties to its membrane effects was investigated by testing two closely related chlorinated organic solvents with distinct relative permittivity.

Tetrachloroethylene (perchloroethylene) is lipophilic (K_{ow} ~800) and nonpolar and its relative permittivity of 2.3 is close to that of the nonpolar hydrocarbon solvents (Table 1) and to the relative permittivity of the membrane. The structurally similar *1,1,2,2*-tetrachloroethane is somewhat less lipophilic (K_{ow} ~250), but has a relative permittivity of 8.5, almost four times higher than that of the membrane. The molar volumes of the two solvents are nearly identical (Table 1). Thus, partitioning of *1,1,2,2*-tetrachloroethane will contribute a considerable change in permittivity of the membrane, while partitioning of tetrachloroethylene will not.

Superfusion of voltage-clamped SH-SY5Y cells with external saline saturated with tetrachloroethylene (1.7 mM, see Table 1) induced a reversible increase in C_m and did not cause a detectable change in R_m and R_a (Fig. 3A), similar to the nonpolar hydrocarbon solvents (see Fig. 2). The average relative increase in C_m caused by tetrachloroethylene was 2.6 ± 0.2% ($n = 3$, see Table 3). Superfusion of voltage-clamped SH-SY5Y cells with external saline containing 2 mM *1,1,2,2*-tetrachloroethane induced a comparable fast increase in C_m. The average increase amounted to 4.0 ± 0.3% ($n = 6$, see Table 3) and was somewhat larger than that observed with tetrachloroethylene, and systematic effects on R_m and R_a were not observed (Fig. 3B). In two additional experiments a lower concentration of 0.2 mM *1,1,2,2*-tetrachloroethane failed to induce detectable changes of the passive electrical membrane parameters (result not shown).

Table 3
Overview of instant changes in passive membrane parameters induced by organic solvents, calculated as average values during solvent perfusion, and normalized to control values shortly prior to solvent application.

organic solvent	concentration (mM)	number of cells	ΔC_m	ΔR_m	ΔR_a
n-hexane	0.11	7	n.d.	n.d.	n.d.
n-hexanol	14.5	5	$4.8 \pm 0.8\%^{***}$	$-23.8 \pm 3.0\%^{****}$	$-3.5 \pm 0.8\%^{*}$
n-hexanol	2.9	3	$1.1 \pm 0.3\%^{*}$	$-3.1 \pm 1.0\%$	$-0.4 \pm 0.1\%$
n-hexylamine	16.0	5	$3.0 \pm 0.2\%^{****}$	$-20.0 \pm 3.0\%^{***}$	$-2.1 \pm 0.3\%^{***}$
cyclohexane	0.69	4	$2.5 \pm 0.4\%^{**}$	$-17.5 \pm 5.1\%^{*}$	$-2.0 \pm 0.8\%$
toluene	5.6	4	$5.7 \pm 0.9\%^{**}$	$-5.9 \pm 5.9\%$	$-0.4 \pm 0.6\%$
toluene	2.8	7	n.d.	n.d.	n.d.
m-xylene	1.5	5	$2.3 \pm 0.2\%^{***}$	$-2.4 \pm 2.4\%$	$-1.2 \pm 0.5\%$
m-xylene	0.8	3	$1.0 \pm 0.2\%^{*}$	$-1.4 \pm 1.4\%$	n.d.
1,1,2,2-tetrachloroethane	2.0	6	$4.0 \pm 0.3\%^{****}$	$11.1 \pm 7.3\%$	$-2.7 \pm 3.7\%$
1,1,2,2-tetrachloroethane	0.2	2	n.d.	n.d.	n.d.
tetrachloroethylene	1.7	3	$2.6 \pm 0.2\%^{***}$	$-8.4 \pm 8.4\%$	$-0.4 \pm 0.3\%$

Significant changes from values shortly prior to solvent application with Student's *t*-test, n.d. = no detectable change, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, $^{****}p < 0.001$.

3.4. Effects of membrane partitioning of amphiphilic molecules

In addition to the effects of nonpolar hydrocarbon solvents, like *n*-hexane and cyclohexane, membrane partitioning of the amphiphilic compounds *n*-hexanol and *n*-hexylamine was investigated.

Because of its amphiphilic nature, *n*-hexanol will partition more readily in the membrane-saline interface than into the bilayer interior (Fraser et al., 1991; Löbbecke and Cevc, 1995; Aagaard et al., 2006). Contrary to the partially negatively charged oxygen in *n*-hexanol, the nitrogen in *n*-hexylamine, with a pK_a value of 10.56 (Haynes, 2016), is protonated at physiological pH. The negative and positive charges of these amphiphilic compounds will be repulsed and attracted by the negative surface charge of the cell membrane. Although the molecular volumes of *n*-hexanol and *n*-hexylamine are in the same range as that of *n*-hexane, the relative permittivity of the amphiphiles is considerably beyond that of the phospholipid bilayer, and of the nonpolar hydrocarbon solvents (Table 1).

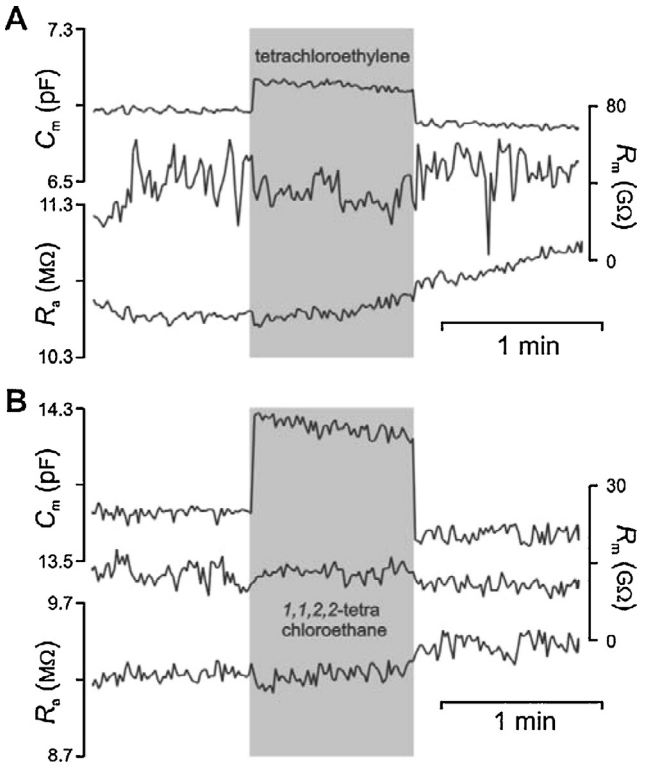

Fig. 3. Representative examples of recordings depicting changes of passive electrical membrane parameters induced by chlorinated hydrocarbons in whole-cell voltage-clamped SH-SY5Y neuroblastoma cells. Values of the membrane capacitance (C_m in pF, top traces), the membrane resistance (R_m in GΩ, middle traces) and the access resistance (R_a in MΩ, bottom traces) calculated from the real and imaginary parts of whole-cell membrane admittance recorded over a prolonged period at the holding potential of −80 mV. The traces demonstrate the effect of superfusion with external saline containing (A) a saturating concentration (1.7 mM) of tetrachloroethylene, and (B) 2 mM *1,1,2,2*-tetrachloroethane. The periods of superfusion with solvent-containing saline are indicated by the shaded areas. The effects are reversed on removal of the compounds by superfusion with external saline.

Superfusion of voltage-clamped SH-SY5Y cells with external saline containing 14.5 mM *n*-hexanol caused a rapid increase in C_m. Fig. 4A illustrates the effect of *n*-hexanol on C_m and shows a simultaneous decrease in the calculated values of R_m and R_a. These effects were all readily reversed upon removal of *n*-hexanol by superfusion with external saline. Since the solubility limit of *n*-hexanol in external saline is approximately 60 mM, the compound is suited to investigate the concentration dependence of its effects. The mean values of the effects from 3–6 experiments on C_m, R_m, and R_a, induced by various concentrations of *n*-hexanol ranging from 1.45 mM to 58 mM *n*-hexanol are shown in Fig. 4B. One-way ANOVA showed a statistically significant concentration-dependence of the effects of *n*-hexanol on C_m ($F_{4,18} = 22.7$; $p < 0.001$), on R_m ($F_{4,18} = 30.5$; $p < 0.001$), and on R_a ($F_{4,18} = 12.0$; $p < 0.001$). A significant increase in C_m was observed for 2.9 mM and higher concentrations of *n*-hexanol. Effects on R_m and R_a appeared to be significant for concentrations ≥7.25 mM *n*-hexanol. In the cells investigated the ratio R_m/R_a always remained large. Even after the largest reduction of R_m in the presence of 58 mM *n*-hexanol, $R_m > 1000\ R_a$. Control experiments for osmotic effects with sucrose showed that R_a and C_m may have actually been slightly underestimated at the highest concentration of *n*-hexanol only (see Supplemental Material).

Separate voltage-clamp experiments were performed to investigate the nature of the changes in R_m induced by *n*-hexanol. Because of the very high values of R_m it was often possible to record single channel activity in the whole-cell configuration, albeit at a moderate signal-to-noise ratio. Under control conditions, occasional single channel openings were observed in SH-SY5Y cells at the holding potential of −80 mV. Superfusion of the cell with external saline containing 14.5 mM *n*-hexanol induced frequent channel openings and this enhanced single channel activity was reversed when *n*-hexanol was removed by washing with external saline (Fig. 4C and D). The single channel current recorded in the presence of *n*-hexanol shows multiple conductance levels (Fig. 4D, middle trace). These results illustrate that ion channel opening induced by high concentrations of *n*-hexanol may underlie the observed decrease of R_m.

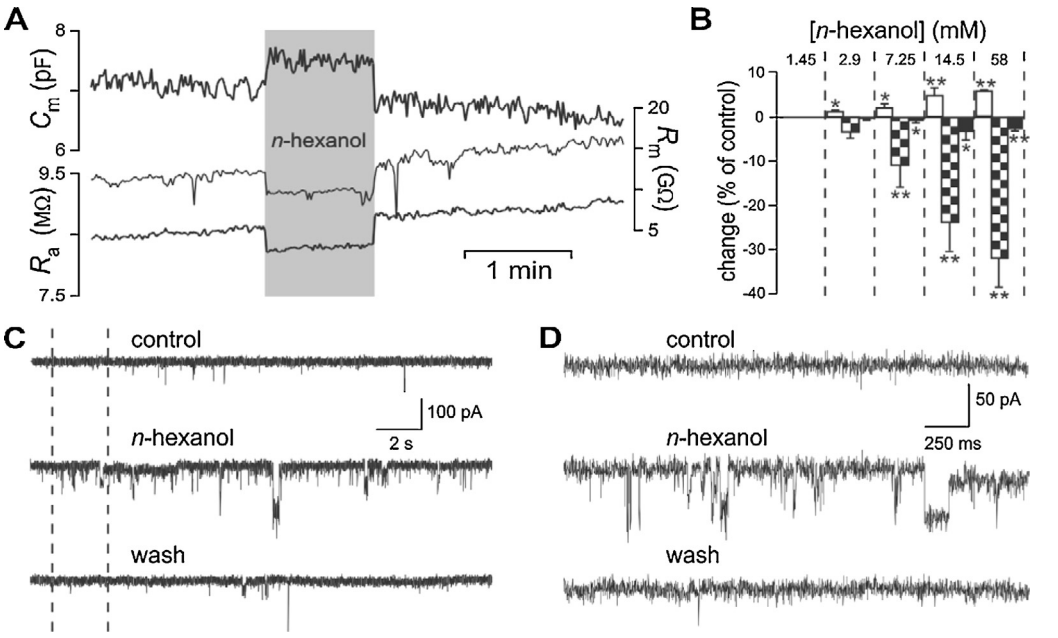

Fig. 4. Effects of *n*-hexanol on the membrane of voltage-clamped human SH-SY5Y cells. (A) Effects of superfusion of external solution with 14.5 mM *n*-hexanol (shaded area) on passive electrical membrane parameters and reversal of these effects upon removal of the compound by superfusion with external saline (C_m in pF, top trace; R_m in GΩ, middle trace; and R_a in MΩ, bottom trace). (B) Concentration dependence of the changes in passive electrical membrane parameters induced by *n*-hexanol. Calculated values of C_m (white bars); R_m (blocked bars); and R_a (black bars) were normalized to values shortly prior to *n*-hexanol application and the mean values ± SEM bars of 3–6 cells are shown in the bar diagram. At 1.45 mM, *n*-hexanol did not cause detectable effects. Asterisks denote significant changes from basal values (*$p < 0.05$; **$p < 0.005$). (C) and (*D*) whole-cell membrane current in a SH-SY5Y cell, recorded during superfusion with external saline (control, upper traces), in the presence of 14.5 mM *n*-hexanol (middle traces), and immediately after removal of *n*-hexanol by superfusion with external saline (wash, lower traces). The holding potential was −80 mV. (D) At a higher time resolution the segments of the traces between the dashed lines in (C) illustrate that the extra noise in the presence of *n*-hexanol is due to opening and closing of discrete ion channels, which are absent during superfusion with external saline (control, wash). For details see text.

Superfusion of cells with external saline containing 16 mM *n*-hexylamine also induced a rapid increase in C_m (Fig. 5, Table 3). However, this effect appeared to be transient and during continued superfusion of *n*-hexylamine the initial increase was reversed and C_m appeared to decrease to a level close to or slightly below that expected from the basal trend. The initial effect on C_m faded with an exponential time constant in the range of 5–14 s ($n = 5$). On the removal of *n*-hexylamine by washing with external saline, C_m transiently decreased before returning to its basal level. Opposite transient changes in R_a were observed. R_m showed a sustained decrease during superfusion of 16 mM *n*-hexylamine that was reversed upon wash with external saline.

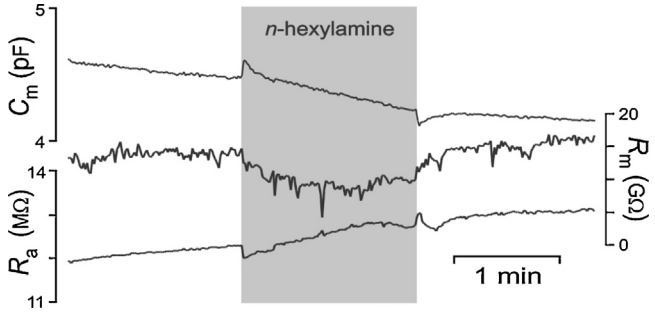

Fig. 5. Representative example of recordings depicting changes of passive electrical membrane parameters induced by *n*-hexylamine in whole-cell voltage-clamped SH-SY5Y neuroblastoma cell. Effects of superfusion of external solution with 16 mM *n*-hexylamine (shaded area) on passive electrical membrane parameters showing transient changes during continuous superfusion with *n*-hexylamine (C_m in pF, top trace; R_m in GΩ, middle trace; and R_a in MΩ, bottom trace).

4. Discussion

4.1. Fidelity of membrane capacitance recordings

The present study shows control values of R_m and R_a that are representative for tight seal, whole-cell recording with low access resistance and low membrane leakage, *i.e.*, optimal conditions for uncompensated whole-cell membrane capacitance measurement (Lindau and Neher, 1988; Zorec et al., 1991; Gillis, 1995). From the initial values of C_m of 3.8–11.8 pF ($n = 19$) the specific membrane capacitance of SH-SY5Y cells can be estimated. Assuming a smooth cell surface and spherical geometry of the 10–20 μm diameter cells, calculated values are in the range of 0.94–1.21 μF cm^{-2}, which is close to the biological membrane capacitance reported in other studies (Cole, 1968; Fenwick et al., 1982; Zupančič et al., 1994; Gentet et al., 2000). Since control experiments show that the trends in the passive electrical membrane parameters are gradual and slow, SH-SY5Y cells are a suitable model to measure the rapid changes in C_m caused by the partitioning of solvents into the membrane. Thus, the methods used are appropriate to gain new insight on the membrane partitioning behavior of organic solvents.

4.2. Organic solvents induce lateral membrane expansion

Nonpolar solvents with relative permittivity close to that of the cell membrane, cause a reproducible, significant increase in C_m amounting up to 6% of the total membrane capacitance. This effect is rapid, depends on the solvent concentration, and persists as long as the solvent is present. In addition, the increase in C_m is rapidly and completely reversed when the solvent is removed by superfusion of the cell with external saline (Fig. 2A and B). A net increase in C_m indicates that the relative increase in surface area exceeds the relative increase in membrane thickness, *i.e.*, that the predominant effect of solvent partitioning consists of lateral

membrane expansion. These effects are opposite to an expected decrease of C_m to occur with an increase in membrane thickness, which is part of the classic lipid hypothesis. In the squid giant axon it was shown that various cyclic hydrocarbons and aliphatic hydrocarbons, including *n*-hexane, cause a decrease of the membrane capacitance per unit area (Haydon et al., 1980; Haydon and Urban, 1983; Hendry et al., 1985): for example 2.7 mM benzene reduced the capacitance per unit area of membrane with 9% (Hendry et al., 1985). The experiments on squid giant axons provide estimates of the membrane capacitance per unit area only and, as a consequence, any lateral membrane expansion due to solvent partitioning will have gone unnoticed in these experiments. The present results provide estimates of the net, opposing effects of membrane thickening and lateral expansion and show that the latter is, by proportion, the larger effect. If it is assumed that membrane thickening occurs in the SH-SY5Y cell membrane to the same extent as in squid giant axons, the present results demonstrate that lateral expansion may well exceed 10% of the cell membrane area.

Despite the low concentration of *n*-hexane in the superfusate, the concentration of *n*-hexane in the membrane is expected to be in the same order of magnitude or higher than that of the other nonpolar cyclic hydrocarbons, since the K_{ow} value of *n*-hexane is 1–2 orders of magnitude higher than the K_{ow} values of the other nonpolar solvents. Thus it appears that hexane in the membrane does not affect the passive electrical parameters, whereas cyclohexane and several aromatic hydrocarbons induce a rapid and reversible increase in C_m consistent with a predominant increase in membrane surface area. The absence of an effect of the aliphatic hydrocarbon *n*-hexane on C_m (Fig. 2C) is remarkable. X-ray diffraction has shown that *n*-hexane causes an increase in the thickness of the hydrocarbon region of phospholipid vesicles (McIntosh et al., 1980), but neutron diffraction has shown that *n*-hexane partitions into the core of phospholipid bilayers without an apparent increase in the volume of the hydrocarbon region (White et al., 1981; King et al., 1985; MacCallum and Tieleman, 2006). These observations indicate that, as expected for a small lipophilic and flexible molecule, *n*-hexane partitions into the lipid region of membrane phospholipids, but the resulting effect on membrane geometry remains unclear. As it appears that all hydrocarbons, including cyclohexane and the amphiphilic *n*-hexanol and *n*-hexylamine cause some degree of lateral membrane expansion, it is concluded that *n*-hexane causes the membrane area and thickness to change to comparable extents, with no net changes in membrane geometry. Whether these effects are large or small is not revealed by the present whole-cell recording of membrane capacitance.

It is also remarkable that tetrachloroethylene and *1,1,2,2*-tetrachloroethane cause a comparable increase of membrane capacitance, *i.e.*, 2.6% and 4%, respectively. The K_{ow} value of tetrachloroethylene is 3-fold that of tetrachloroethane. As the applied concentrations of tetrachloroethylene and *1,1,2,2*-tetrachloroethane are comparable, the final membrane concentration of tetrachloroethylene must be about a 3-fold higher. Based on the comparable size, shape and molar volume, the nature of the membrane perturbing effects of these chlorinated hydrocarbons are expected to be comparable. For tetrachloroethylene, with its permittivity close to that of the membrane, the small effect may reflect a net change in membrane geometry. However, the relative permittivity of *1,1,2,2*-tetrachloroethane is nearly 4 times higher than that of the membrane. According to Eq. (5) (Supplemental material), this would result in a 4-fold larger contribution of lateral expansion (left hand term of Eq. (5)), and a 4-fold smaller contribution in thickness (right hand term of Eq. (5)) to the effect on C_m. Hence, the observed 4% increase of C_m induced by *1,1,2,2*-tetrachloroethane is caused almost entirely by lateral membrane expansion. Furthermore, when this effect is compensated for the 4-fold higher permittivity of *1,1,2,2*-tetrachloroethane as compared to the permittivity of the membrane, the actual increase of membrane area would be in the order of 1% only. If both solvents would cause a large change in membrane thickness, the net positive effect observed for C_m by tetrachloroethylene should be much smaller than the net effect of *1,1,2,2*-tetrachloroethane. Since the effect on C_m is the resultant of changes in area and in thickness, the results demonstrate that for chlorinated hydrocarbons, the relative increase of the membrane area exceeds the relative increase in membrane thickness by far.

In contrast to *n*-hexane, *n*-hexanol and *n*-hexylamine applied at the external cell membrane surface cause parallel changes in R_a and C_m. However, *n*-hexanol and *n*-hexylamine do not readily partition into bilayers. *n*-Hexanol, due to its amphiphilic nature and high permittivity, prefers and disturbs the membrane-saline interface (Fraser et al., 1991; Hille, 1992; Löbbecke and Cevc, 1995; Aagaard et al., 2006). It has been shown that the hydrocarbon chain of *n*-hexanol points toward the hydrocarbon core of the membrane, which causes the lipid chains to become disordered and creates energetically unfavorable void spaces (Adachi et al., 1995; Löbbecke and Cevc, 1995; Kranenburg et al., 2004). The presently observed increase in C_m in the presence of *n*-hexanol reflects a net increase in membrane area, consistent with partitioning of *n*-hexanol at the membrane-saline interface. That *n*-hexanol induces changes at the membrane-saline interface is further supported by the activation of ion channels, and the saturation of the changes in R_m and R_a at high concentration. A finite number of sites in the membrane, *i.e.*, either the phospholipids or the ion channels, can account for such saturation, similar to what is proposed for anesthetic compounds (Franks and Lieb, 1994). *n*-Hexylamine with a pKa of 10.56 (Haynes, 2016) will be protonated in saline at the physiological pH of 7.3. On partitioning from saline into the cell membrane, the positive charge of *n*-hexylamine may interact with negative charges of membrane proteins and/or phospholipid headgroups at the membrane surface and *n*-hexylamine may be deprotonated and trapped in the saline-membrane interface (Rustenbeck et al., 1998; Chávez et al., 2000). In experiments with chloroplast thylakoid membranes, it has been shown that *n*-hexylamine is able to cross the membrane (Allnutt et al., 1991). The present findings indicate that a protonated *n*-hexylamine molecule partitions into the membrane-saline interface and causes lateral expansion, like *n*-hexanol. However, after deprotonation the uncharged *n*-hexylamine molecule will move into the core of the membrane, much like *n*-hexane. This would account for the transient increase in C_m observed after exposure of cells to *n*-hexylamine (see Fig. 5). The observation that the time constant of decay of the transient increase in C_m induced by *n*-hexylamine is slow (5–14 s) further indicates that the partitioning and deprotonation of *n*-hexylamine in the membrane-saline interface are slow processes.

4.3. Applicability of the model

From the effects of various solvents on the membrane of SH-SY5Y cells it is not possible to predict how different membrane properties might influence solvent effects. Although SH-SY5Y cells are the most widely used human neuroblastoma cell line for neurotoxicity studies (Westerink, 2013), they are just one subclone of the SK-N-SH line, human metastatic neuroblastoma cells isolated from a bone marrow biopsy (Biedler et al., 1978). A single type of tumor cell or any single type of neuron can neither form a representative model for the cell membrane in general nor for the neuronal cell membrane specifically. Cell membrane composition will depend on specific properties of the cell type, and may also vary with the culture conditions *in vitro* or with the diet *in vivo*

(*e.g.*, Reynolds et al., 2001). Thus, the present results merely compare the effects of solvents with distinct properties on a model membrane. Observed changes in membrane conductance may be highly specific to SH-SY5Y cell membranes and may be due to mechanical effects, changes in membrane fluidity, or direct effects of solvents on membrane proteins. Conversely, the similarity of the basic solvent effects on the SH-SY5Y cell membrane capacitance reported here and effects of solvents and other hydrophobic molecules reported by others, *e.g.*, on the squid giant axon (Haydon et al., 1980), mouse mast cells and bovine adrenal medulla cells (Oberhauser and Fernandez, 1995), and artificial lipid bilayers (Haydon et al., 1977), indicates that the concept of membrane expansion is generally applicable. The present whole-cell membrane capacitance measurements provide information on the combined changes in membrane thickness and area, and reveal lateral expansion as a prominent feature. Whether solvent effects also depend on membrane composition becomes within experimental reach, as it is now possible to study the effects of solvents in a variety of cells and under a range of different culture conditions. Ideally, experiments should also be repeated in freshly isolated neuronal and non-neuronal cells to further validate the present results and conclusions.

4.4. Conclusions on membrane expansion and its contribution to solvent-induced narcotic effects

The present results demonstrate that solvent partitioning into the cell membrane leads to rapid and reversible changes in membrane capacitance. The different classes of organic solvents tested each cause lateral membrane expansion, which for almost all solvents is larger than the change in membrane thickness. However, the results also show specific effects on the passive electrical membrane parameters for each solvent. Therefore, the present results are contradictory to the notion that a shared membrane effect of organic solvents leads to common narcotic effects.

Even though the present findings indicate that membrane effects of solvents do not lead to a common narcotic effect, the membrane effects were measured at high concentrations of solvents, which are generally associated with 'narcotic' or even with lethal effects. In contrast to the relation between ambient air concentration and narcotic effects, only little information is available on tissue concentrations associated with acute adverse effects of solvents. Two case studies, containing compiled available data on toluene concentrations during acute intoxication, report brain concentrations between 189 μM and 755 μM. In fatal cases, postmortem brain concentrations ranged up to 5.6 mM (Yajima et al., 2005; Tanaka et al., 2016). As outlined in the introduction, specific neurotoxic effects, which may be observed on subchronic or chronic and often low-level exposure to solvents, more likely involves interactions of solvents or of their biotransformation products with specific targets in the nervous system.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neuro.2016.05.013.

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