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Effects of volatile solvents on recombinant *N*-methyl-D-aspartate receptors expressed in *Xenopus* oocytes

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1 We have previously shown that toluene dose-dependently inhibits recombinant *N*-methyl-D-aspartate (NMDA) receptors at micromolar concentrations. This inhibition was rapid, almost complete and reversible. The NR1/2B combination was the most sensitive receptor subtype tested with an IC_{50} value for toluene of 0.17 mM.

2 We now report on the effects of other commonly abused solvents (benzene, m-xylene, ethylbenzene, propylbenzene, 1,1,1-trichlorethane (TCE) and those of a convulsive solvent, 2,2,2-trifluoroethyl ether (flurothyl), on NMDA-induced currents measured in *Xenopus* oocytes expressing NR1/2A or NR1/2B receptor subtypes.

3 All of the alkylbenzenes and TCE produced a reversible inhibition of NMDA-induced currents that was dose- and subunit-dependent. The NR1/2B receptor subtype was several times more sensitive to these compounds than the NR1/2A subtype.

4 The convulsant solvent flurothyl had no effect on NMDA responses in oocytes but potently inhibited ion flux through recombinant GABA receptors expressed in oocytes.

5 Overall, these results suggest that abused solvents display pharmacological selectivity and that NR1/2B NMDA receptors may be an important target for the actions of these compounds on the brain.

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Abbreviations: MS-222, tricaine methanesulphonate; OEL, occupational exposure limits; TCE, 1,1,1-trichlorethane

Introduction

Volatile solvents such as toluene and benzene are among the most widely used chemicals in industry and commerce. They are major constituents of a number of commercial products such as cleaning fluids, paints and glues. (Arlien-Søborg, 1992; Indulski *et al*., 1996). Contact with these substances comes from occupational exposure as well as from deliberate inhalation. According to recent surveys, an increasing number of young people around the world inhale volatile substances for recreational purposes (Greer, 1984; Kozel *et al*., 1995; Spiller & Krenzelok, 1997). There are several reported cases of death or prolonged unconsciousness after accidental exposures to high concentrations of solvents (Morley *et al*., 1970; Caplan *et al*., 1976; Takeichi *et al*., 1986) and acute mortality due to inhalant abuse is also well documented (Bass, 1970; Gresham & Treip, 1983; Bowen *et al*., 1999).

According to different authors, solvent abusers can inhale concentrations several hundreds of times the occupational exposure limits (OEL) by 'huffing' from clothes or 'sniffing' from a bag to concentrate the vapours (see Marjot & McLeod, 1989). There is considerable investigation about the effects of prolonged exposure to levels pertinent to occupational exposure (Angerer & Lehnert, 1979; Snyder, 1987; Anger, 1990) but little is known about the effects of acute exposure to high concentrations of inhalants as well as the underlying cellular mechanisms of action of these substances (Balster, 1998).

Using a variety of experimental paradigms, Evans & Balster (1991) have demonstrated that abused solvents share a pharmacological profile with other abused depressant drugs including ethanol, barbiturates and benzodiazepines. This led to the idea that inhalants may share a common mechanism of action with other abused depressant drugs such as ethanol. One of the best-studied targets of depressant drugs is the glutamate family of ionotropic receptors (Lovinger *et al*., 1989; Grant & Lovinger, 1995). Using the oocyte expression system, we have previously reported that toluene dose-dependently inhibited NMDA-mediated currents at micromolar concentrations. The inhibition of NMDA receptor currents was almost complete, rapid and reversible. The effects of toluene on NMDA receptor function were clearly subunit-dependent with NR1/2B receptors being approximately eight times more sensitive to toluene than the NR1/2A combination and 12 times more than the least sensitive combination, the NR1/2C receptor subtype (Cruz *et al*., 1998).

The purpose of the present investigation was to evaluate the effects of several abused solvents including a series of alkylbenzenes (benzene, m-xylene, ethylbenzene and propylbenzene), one halogenated hydrocarbon, TCE, and the convulsant agent flurothyl on NR1/2A and NR1/2B recombinant NMDA receptor subtypes expressed in *Xenopus* oocytes.

Methods

Drugs and volatile agents

NMDA, glycine, GABA, tricaine methanesulphonate (MS-222) and collagenase were purchased from Sigma (St. Louis,

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MO, U.S.A.), m-xylene, propylbenzene h.p.l.c. grade and flurothyl from Aldrich (Milwaukee, WI, U.S.A.), TCE, benzene and ethylbenzene from Fischer (Pittsburgh, PA, U.S.A.) and alkamuls EL-620 (ethoxylated castor oil) from Rhone-Poulenc (Princeton, NJ, U.S.A.). NMDA receptor cDNA clones in Bluescript vectors were kindly provided by S. Nakanishi, P. Seeburg and M. Mishina. GABA cDNA clones were the kind gift of T. Verdoorn.

Synthesis of mRNA

All cDNA clones were linearized downstream from the coding sequence with the appropriate restriction enzyme, purified by phenol/chloroform extraction, precipitated by ethanol and resuspended in RNAase-free water before being used in the *in vitro* transcription reaction (Ambion, Austin, TX, U.S.A.). Formaldehyde gels were used to confirm the quality and the size of the synthesized mRNA.

Oocyte preparation and microinjections

Adult *Xenopus laevis* female frogs were purchased from Xenopus I (Ann Arbor, MI, U.S.A.). Frogs were anaesthetized before surgery by immersion in 0.25% MS-222. Stage V and VI oocytes were dissected and collagenazed (1 mg ml^{-1}) before mRNA injection (Variable Nanoject, Drummond Scientific Co., Broomall, PA, U.S.A.). Oocytes were injected with 5–10 ng of mRNA for each subunit tested at a ratio of 1 : 1. Oocytes were then maintained at 18°C in L-15 media at pH 7.4 supplemented with 10,000 U l^{-1} penicillin G, 10 mg l^{-1} streptomycin and 15.5 mg l^{-1} gentamycin for up to 7 days before recording.

Drug solutions

Solutions were prepared as previously described (Cruz *et al*., 1998) by mixing each solvent with alkamuls at a 1 : 1 ratio (v v^{-1}). This mixture was then diluted as needed with extracellular recording solution to obtain the appropriate concentrations. The recording solution consisted of (in mM) NaCl 115, KCl 2.5, N-[2Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES, 10) and $BaCl_2$ 1.8, pH 7.2. The highest concentration of alkamuls used was 0.1%. To determine the extent of solvent evaporation over time, standard solutions of each compound were prepared in extracellular recording solution as previously described, aliquotted in open containers and sampled at various time intervals. The concentrations of alkylbenzenes in the samples were determined by absorbance spectroscopy while TCE concentrations were measured by gas chromatography/mass spectrometry.

Electrophysiological recordings

Eggs were placed in a 200-μl recording chamber and continuously perfused with barium-containing extracellular recording solution at a flow rate of 4–6 ml min^{-1}. Oocytes were impaled with two low-resistance microelectrodes (0.1–0.8 MΩ) filled with a solution containing 3 M KCl and 0.8% agarose. The resting membrane potential of the oocyte was clamped at −80 mV using a GeneClamp 500 amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.). NMDA receptors were stimulated by switching the perfusion solution to one containing NMDA (100 μM) plus glycine (10 μM) in the absence or in the presence of different concentrations of solvent. For $GABA_A$ receptor stimulation, the perfusion solution contained 1 μM GABA. In all cases, non-drug-treated responses were obtained before and after each solvent concentration and were averaged to give the control response. Data were acquired, digitised with an Instrutech ITC-16 interface and analysed on a Macintosh computer running the Pulse Control module (Herrington & Bookman, 1994) under the Igor Pro graphics platform (Wave-Metrics; Lake Oswego, WS, Canada). Net agonist-stimulated current responses (in nA) were expressed as percentage of the average control value to reduce variability associated with the varying levels of expression among different batches of oocytes.

Data and statistical analysis

Data from each oocyte represent a single observation and oocytes from at least two different frogs were tested for each experimental condition. Concentration-response curves were analysed using the ALLFIT program.

Results

As previously described, the vehicle alkamuls at concentrations up to 0.1% had no effect on oocyte membrane leak currents or NMDA-induced responses (Cruz *et al*., 1998). Similarly, exposure of oocytes to alkylbenzenes up to concentrations of approximately 10 mM did not induce measurable changes in membrane leak currents. In contrast, all alkylbenzene solvents tested in this study inhibited NMDA-induced currents in oocytes expressing NR1/2A or NR1/2B receptors. An example of this inhibition is shown in Figure 1 that shows the effects of 11 mM benzene on NR1/2A and NR1/2B receptors. As mentioned, benzene alone did not alter the membrane leak current of uninjected oocytes voltage-clamped at −80 mV (Figure 1A). However, when co-applied with agonist, benzene produced a clear inhibition of NMDA-induced currents in oocytes expressing either NR1/2A and NR1/2B receptors (Figure 1B and C, respectively). As shown previously for the related solvent toluene (Cruz *et al*., 1998), the effect of benzene and the other solvents was biphasic with a more pronounced inhibition occurring within a few seconds of drug administration. The slow onset of the solvent inhibition is primarily due to the large size of the oocyte and the relative slowness with which solutions can be applied without compromising the integrity of the voltage-clamp. All measurements of solvent inhibition reported in this study were made at the end of the 20 s agonist/solvent application when the effects of the solvent had reached equilibrium. As shown in the third trace of Figure 1B and C, the inhibition of NMDA-induced currents by benzene was readily reversed upon removal of the test solvent.

Figure 2 shows that the concentration-response relationships for the inhibition of NR1/2A and NR1/2B receptors for the alkylbenzene solvents tested as well as for TCE. For all solvents tested, the receptors composed of NR1/2B subunits were more sensitive to inhibition than those composed of NR1/2A subunits. Interestingly, this differential sensitivity of NR1/2B receptors was slightly more pronounced for ethylbenzene. Comparison of the solvent concentration-response curves also suggested that there were differences in the relative potency and maximum inhibitory efficacy of the various alkylbenzenes tested. In addition, these results showed that TCE was less potent as an inhibitor of NMDA responses than the alkylbenzenes. However, measurements of solvent evaporation revealed that there were differential rates

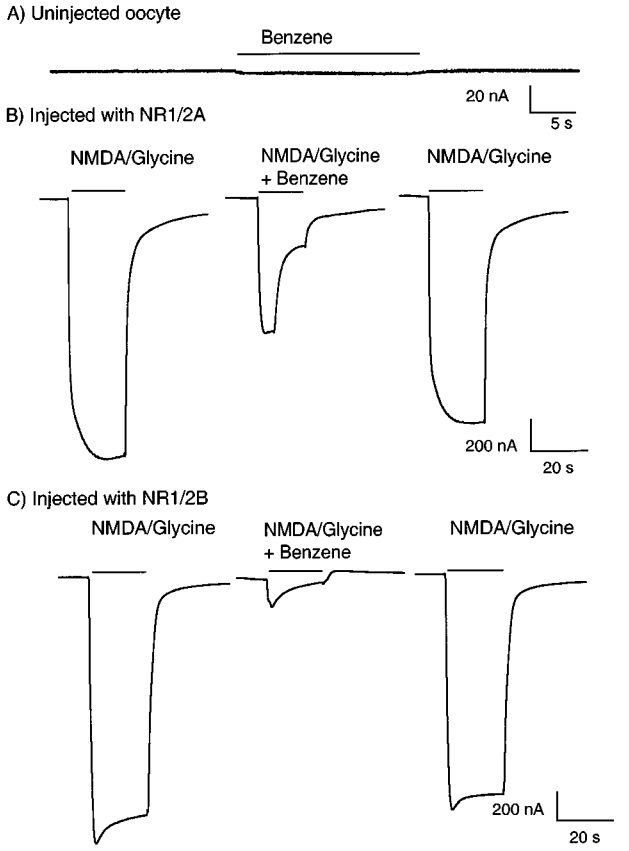

Figure 1 Effects of 11 mM benzene on membrane currents in uninjected (A) and NMDA-induced currents in NR1/2A and NR1/2B injected eggs (B and C, respectively). Oocytes held at −80 mV in extracellular recording medium were exposed for 20 s to solutions containing either benzene alone (A) or 100 μM NMDA plus 10 μM glycine (B and C) in the absence (first and third traces) and presence (middle trace) of benzene. Horizontal bars above current recordings indicate the time of drug application.

of solvent loss for each compound tested that affected the apparent potency determinations.

Figure 3 shows the loss of each solvent in aqueous solution over time expressed as a per cent of the initial time zero control value. As expected, the rate of solvent loss was correlated with the vapour pressure for each solvent with TCE being particularly prone to evaporative loss. In the present studies, recordings with these solvents were generally completed within 15–30 min after placing solvent solutions in open containers. Thus, the solvent evaporation curves were used to adjust the dose-response data to account for the differential loss of solvents.

Table 1 shows some physico-chemical properties of the solvents used, and Table 2 shows the uncorrected and corrected IC_{50} values and Hill coefficients (n_H) for solvent effects on NR1/2B receptors. These uncorrected IC_{50} values were based on the nominal concentrations of each solvent and come from the data shown in the dose-response curves (Figure 2). IC_{50} values were corrected by adjusting the solvent concentration values at a fixed time of 30 min. Corrected IC_{50} values are all relatively similar and suggest that alkylbenzenes and TCE are roughly equipotent in inhibiting NR1/2B receptors.

The uncorrected and corrected IC_{50} values for benzene inhibition of NR1/2A receptor subtype was 2.7 ± 0.9 and 1.29 mM, respectively. IC_{50} values for inhibition of NR1/2A receptors by the other solvents were not calculated due to difficulty in obtaining full inhibition of NR1/2A responses over the concentrations tested. Attempts to use higher concentrations of solvents ($\geqslant$20 mM) caused irreversible effects on membrane leak currents in oocytes.

Finally, the effects of flurothyl on NMDA receptors were determined to examine the selectivity of the alkylbenzenes and TCE on NMDA receptors. Flurothyl is a convulsant solvent whose behavioural actions are not consistent with blockade of NMDA receptors. As expected, flurothyl (0.1–10 mM) had no significant effects on either NR1/2A or NR1/2B NMDA receptors expressed in oocytes. In contrast, flurothyl dose-dependently inhibited GABA-induced currents in oocytes expressing the $\alpha_1\beta_2\gamma_{2S}$ subunit combination under the same experimental conditions used in the present study (Figure 4).

Discussion

The main findings of this work can be summarized as follows: (a) benzene, m-xylene, ethylbenzene, propylbenzene and TCE all inhibited NR1/2B receptors over a range of concentrations that had no effects on membrane permeability. This inhibition was rapid, dose-dependent and reversible, (b) the same compounds were less potent and efficient as inhibitors of the NR1/2A receptor subtype and (c) flurothyl had no effect on NMDA receptors at the concentrations tested but potently inhibited $GABA_A$ receptors.

All substances used in this study are similar in that they are highly lipophilic and are easily distributed into lipid-rich organs, such as the brain and adipose tissue (Table 1). Flurothyl, although having similar physico-chemical properties (MW: 182.07; density: 1.41 g ml^{-1}; bp: 63.9°C) to the alkylbenzenes and TCE is a potent convulsant agent (Adler, 1975) that has recently been shown to act as a non-competitive antagonist at $GABA_A$ receptors (Krasowski, 2000). Several authors have proposed that volatile solvents, owing to their lipophilic nature, produce their effects *via* interaction with the lipids of cell membranes (Gustafson & Tagesson, 1985; Engelke *et al.*, 1992). In this study, concentrations of solvents that dramatically affected the function of ligand-gated ion channels (<10 mM) had no effect on membrane leak currents. Although membrane integrity could be compromised by higher ($\geqslant$20 mM) concentrations of solvents, these data strongly support the effects of abused volatile solvents on these channels are not due to non-specific disruption of lipid membranes.

Humans come in contact with volatile solvents due to: (a) occupational exposure, (b) voluntary, or (c) accidental inhalation of concentrated vapours. Therefore, relevant concentrations of these compounds range from hundreds to several thousands p.p.m. in the air. Since relatively few reports exist on the concentrations achieved in blood and/or other tissues when exposed to controlled atmospheres it is difficult to establish cause-effect relationships. In a previous study we found that concentrations of toluene (0.1–10 mM) that inhibit NMDA receptors *in vitro*, appeared to be related to those associated with behavioural effects of this volatile solvent in humans and animals (Cruz *et al.*, 1998). Regarding other solvents, Angerer & Lehnert (1979) reported that workers in a histology laboratory that were exposed to concentrations of mixed xylene from 34 to 68 p.p.m. had 1.65–2.47 mg l^{-1} of xylene in blood (approximately 0.02 mM). Morley and colleagues (1970) reported that an accidental exposure to 10,000-p.p.m. xylene resulted in the death of a worker and prolonged unconsciousness of other two. However, in that study, blood concentrations of xylene

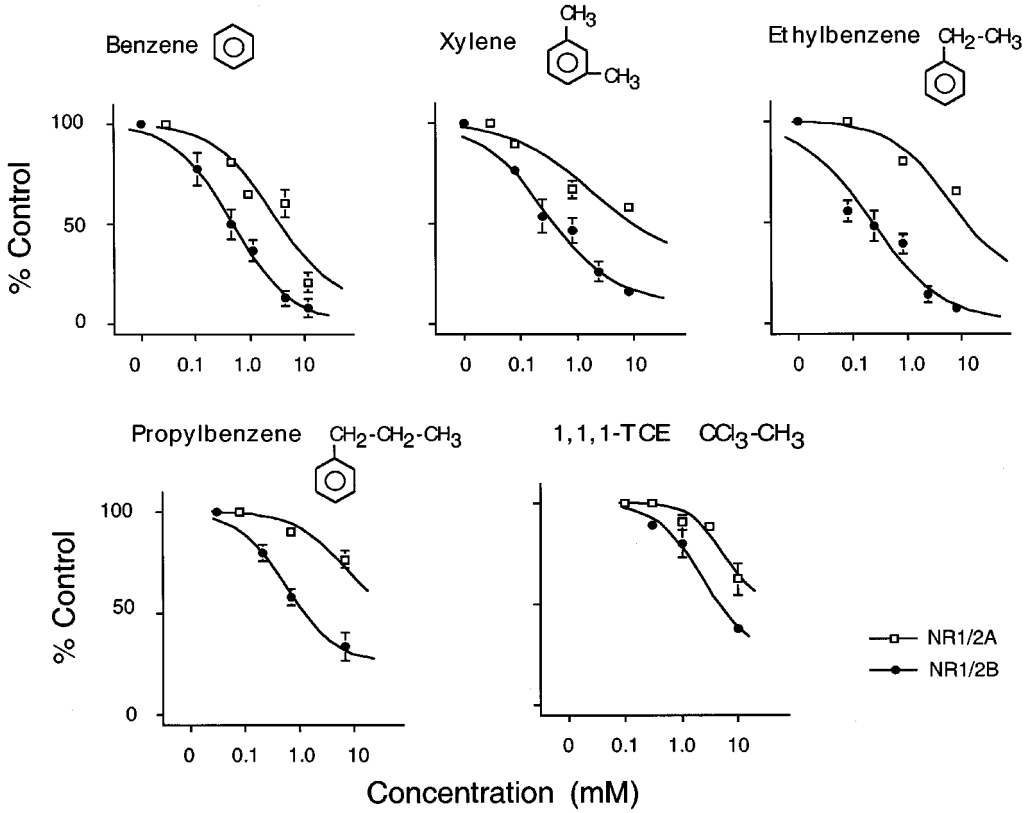

Figure 2 Concentration-response curves for inhibition of NMDA-induced currents by several alkylbenzenes and TCE in oocytes expressing NR1/2A or NR1/2B receptors. Each point is expressed as a per cent of the non-solvent control response and is the mean ± s.e.mean of at least five oocytes from two different frogs.

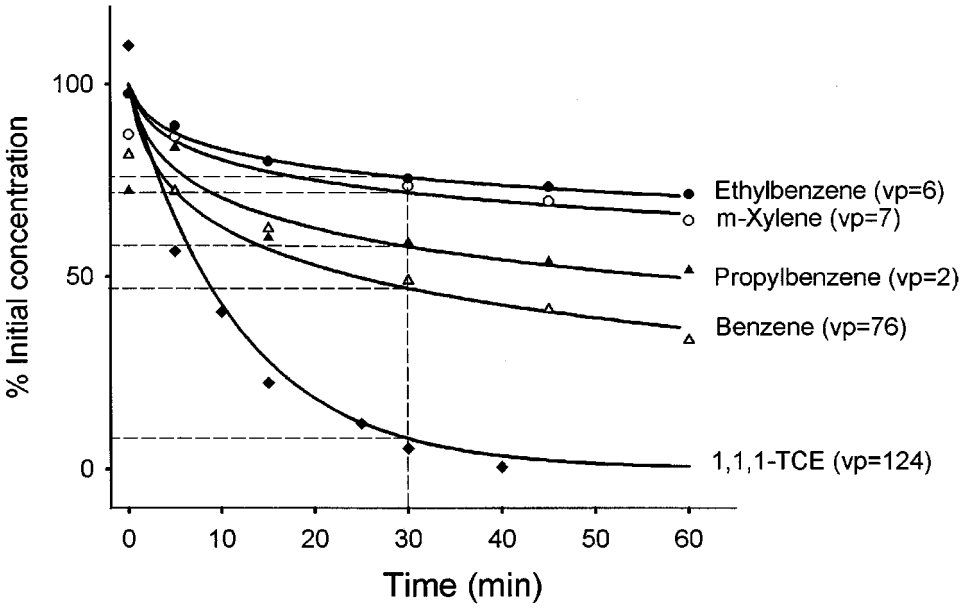

Figure 3 Estimated loss of each solvent in solution over time. The concentrations of alkylbenzenes were measured by absorbance spectroscopy while TCE concentrations were determined by gas chromatography/mass spectrometry. Each point is the mean of triplicate determinations. Experimental data were adjusted with the following equations: Ethylbenzene $y = -0.125 \log(x) + 0.930$, ($r^2 = 0.992$); m-xylene $y = -0.521 \log(x) + 2.765$, ($r^2 = 0.975$); propylbenzene $y = -0.994 \log(x) + 3.516$, ($r^2 = 0.910$); benzene $y = -1.134 \log(x) + 3.188$, ($r^2 = 0.958$); 1,1,1-TCE $y = 3.529\, e^{-0.0848x}$, ($r^2 = 0.986$).

were not measured. There is also some information on the effects of different concentrations of TCE in humans. According to Stewart & Dodd (1964) immersion of a hand in pure solvent for 30 min resulted in an alveolar concentration of 22 p.p.m. (0.16 mM), which is equivalent to inhalation of 50–250 p.p.m. for a period of similar duration. Post-mortem samples from one boy who died after exposure to high concentrations of TCE revealed a blood concentration of 27 mg 100 ml^{-1}, which corresponds to approximately 2.7 mM. Exposure to ethylbenzene could occur by exposure to technical xylene, a commercial mixture of the three isomers of xylene (orto-, meta- and para-) that contains

Table 1 Physico-chemical properties of volatile solvents

Solvent	*Chemical formula*	*MW*	*Density* (g ml^{-1})	*log Po/w*	*Blood : gas*	*Vapour pressure* (mmHg)	*bp* (°C)	*LD_{50}* (mg kg^{-1})
Benzene	C_6H_6	78.1	0.88	2.05	7.5	76	80.1	3.8
m-Xylene	1,3-$(CH_3)_2C_6H_4$	106.1	0.86	2.98	29	7	139.1	7.7
Ethylbenzene	$C_6H_5C_2H_5$	106.1	0.87	2.91	28.4	6	136.2	6.3
Propylbenzene	$C_6H_5C_3H_7$	120.2	0.86	3.31	47	2	159.2	7
1,1,1-TCE	CCI_3CH_3	133.4	1.34	2.40	3.3	124	74.1	NA

Log $P_{o/w}$: logarythmic value of the oil/water partition coefficient. Taken from Hansch & Leo, 1979. Blood: gas, blood/gas partition coefficient. Taken from Sato & Nakajima, 1979. TCE values from U.S. Department of Health and Human Services, 1995. LD_{50} values, orally in rats. General references: Budavari, 1996; Lide, 1990. NA: not available.

Table 2 Dose-response curve parameters for solvent inhibition of NMDA-induced currents

	NR1/2B		
	Estimated IC_{50} (mM)	n_H	*Corrected IC_{50}* (mM)
Benzene	0.48±0.04	0.8±0.6	0.23
m-Xylene	0.29±0.07	0.7±0.1	0.21
Ethylbenzene	0.22±0.1	0.7±0.2	0.17
Propylbenzene	0.60±0.1	0.9±0.1	0.35
1,1,1-TCE	2.36±0.4	1.1±0.1	0.18

Values are expressed as the mean±s.e.mean of at least five oocytes from two different frogs. IC_{50} values given in the third column were corrected by adjusting the solvent concentration values at fixed time of 30 min for solvent evaporation (see Figure 3). n_H: Hill coefficient.

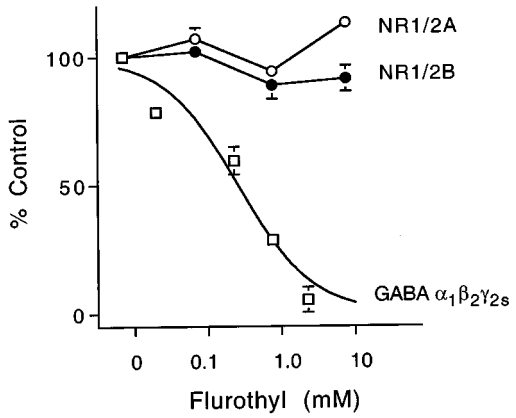

Figure 4 Concentration-response curves for the effects of flurothyl on oocytes expressing either NR1/2A, NR1/2B or $GABA_A$ $\alpha_1/\beta_1/\gamma_{2s}$ receptors. Each point is expressed as a per cent of the non-flurothyl control response and is the mean±s.e.mean of at least five oocytes from two different frogs.

about 20% ethylbenzene (Gamberale *et al*., 1978; Fishbein, 1985). This compound, as well as propylbenzene, has a low acute toxicity in animals and humans even at high concentrations (Engström *et al*., 1987). Although the range of concentration used in this study was relatively wide, it is important to note that all alkylbenzenes inhibited NR1/2B receptors at micromolar concentrations. Based on the animal and human studies discussed above, it can be concluded that the concentrations of alkylbenzenes tested in this study are relevant to those associated with human exposure.

In a previous study, we reported that toluene inhibited NR1/2B receptors with an IC_{50} value of 0.17 mM (Cruz *et al*., 1998). That study also showed that there is only a small evaporative loss of toluene after 30 min of storage in the open containers used in the oocyte recording studies. Thus, for toluene, the IC_{50} value calculated from nominal concentrations of the solvent is very close to the actual concentration present at the time of the experiment. We now report IC_{50} values for the effects of various alkylbenzenes and TCE at the same receptor subtypes. Since there was a considerable loss of some of these solvents over time, a correction based on solvent evaporation was applied to prevent under-estimating the potency values for these compounds. Data were corrected using solvent evaporation values at 30 min, as this represents an average time at which most of the recordings were completed. Analysis of these corrected IC_{50} values shows that alkylbenzenes including toluene all inhibit NR1/2B receptors in the 100–350 μM range. TCE appears to somewhat more potent than either toluene or the other alkylbenzenes. However, this conclusion should be viewed in light of the significant volatility of TCE, which may preclude an accurate measure of its potency.

The major hypothesis underlying these studies was that NMDA receptors might be particularly sensitive to inhibition by abused volatile solvents. Our results support this hypothesis and clearly show that alkylbenzenes and TCE can significantly inhibit the function of this ion channel. NMDA receptors thus appear to be inhibited by several classes of compounds that show abuse liability including phencyclidine, nitrous oxide, ethanol, and now abused volatile solvents (Lodge & Johnson, 1990; Jevtovic-Todorovic *et al*., 1998; Lovinger *et al*., 1989; Kuner *et al*., 1993; Masood *et al*., 1994, Mirshahi & Woodward, 1995). While some of these compounds may be relatively selective for NMDA receptors (phencyclidine, nitrous oxide), others like ethanol appear to be effective at a number of important ligand-gated ion channels including those activated by GABA and glycine. Interestingly, a recent study has demonstrated that toluene and TCE enhance the function of native and recombinant $GABA_A$ and glycine receptors (Beckstead *et al*., 2000). Taken together with the results of the present study, these findings suggest that alteration of ligand-gated ion channel signaling may underlie some of the neurobehavioural effects of abused volatile solvents.

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