Neurotoxicology 74 (2019) 58–66

Contents lists available at ScienceDirect

Neurotoxicology

journal homepage: www.elsevier.com/locate/neuro

Full Length Article

Measuring the middle-ear reflex: A quantitative method to assess effects of industrial solvents on central auditory pathways

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ARTICLE INFO

Keywords:
Solvent
Middle-ear reflex
Occupational exposure
Screening test
In vivo model

ABSTRACT

Volatile organic solvents are frequently present in industrial atmospheres. Their lipophilic properties mean they quickly reach the brain following inhalation. Acute exposure to some solvents perturbs the middle ear reflex, which could jeopardize cochlear protection against loud noises. As the physiological mechanisms involved in this protective reflex are highly complex, *in vivo* rodent models are required to allow rapid and reliable identification of any adverse effects of solvents on the middle ear reflex (MER).

In this study, MER amplitude was measured in anesthetized Brown-Norway rats by monitoring the decrease in distortion product otoacoustic emissions (DPOAEs) caused by a contralateral stimulation. Our screening test consisted in measuring the impact of inhalation of solvent vapors at 3000 ppm for 15 min on the MER amplitude. We had previously studied a selection of aromatic solvents with this model; here, we extended the analysis to volatile compounds from other chemical families. The results obtained shed light on the mechanisms involved in the interactions between solvents and their neuronal targets. Thus, benzene and chlorobenzene had the greatest effect on MER ($\geq$ + 1.8 dB), followed by a group composed of toluene, styrene, p-xylene, m-xylene, tetrachloroethylene and cyclohexane, which had a moderate effect on the MER (between + 0.3 and + 0.7 dB). Finally, trichloroethylene, n-hexane, methyl-ethyl-ketone, acetone, o-xylene, and ethylbenzene had no effect on the MER.

Thus, the effect of solvents on the MER is not simply linked to their lipophilicity, rather it depends on specific interactions with neuronal targets. These interactions appear to be governed by the compound's chemical structure, *e.g.* the presence of an aromatic ring and its steric hindrance. In addition, perturbation of the MER by a solvent is independent of its toxic effects on cochlear cells. As the MER plays a protective role against exposure to high-intensity noises, these findings could have a significant impact in terms of prevention for subjects exposed to both noise and solvents.

1. Introduction

Volatile organic solvents are present in many industrial atmospheres (Morata et al., 1993). They are used in almost all manufacturing processes and in a wide variety of applications: pharmaceuticals, paints and coatings, printing inks, cosmetics, cleaning and degreasing, automotive, dyes and pigments, adhesives, chemical manufacture, *etc.* (Dick, 2006). The term "solvent" includes several chemical families, such as aromatic and alicyclic hydrocarbons, alkenes, and ketones. These compounds differ from one another based on their structure, their chemical formula, and through their effects on the nervous system (Evans and Balster, 1991). However, as most industrial solvents are fat-soluble and volatile, they reach the bloodstream rapidly after inhalation and subsequently accumulate in lipid-rich tissues such as the brain.

Some solvents containing a benzene ring, known as aromatic solvents, have been shown to have toxic effects on the sensory systems during chronic exposure; this is the case for styrene for example, which causes hearing impairment both in animals and humans (Loquet et al., 1999; Sliwinska-Kowalska et al., 2005) and disrupts color vision (Choi et al., 2017), or for ethylbenzene, which is known to be a powerful ototoxicant by itself while also potentiating the traumatic effects of noise (Cappaert et al., 1999, 2001). The histopathological signature of chronic exposure to aromatic solvents on the cochlear neuroepithelium is loss of outer hair cells, decreasing from the third to the first row

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https://doi.org/10.1016/j.neuro.2019.05.007
Received 21 January 2019; Received in revised form 15 May 2019; Accepted 15 May 2019
Available online 20 May 2019
0161-813X/

(Lataye et al., 2001). In rats, this cochlear damage results in permanent hearing loss over a frequency range depending on the associated noise exposure (Venet et al., 2015). In previous work, we showed that in addition to this slow and insidious poisoning process, aromatic solvents could also target the central nervous pathways of the auditory system (Campo et al., 2007; Maguin et al., 2008). Thus, short-term exposure to solvents could specifically disrupt the middle-ear reflex (MER) (Wathier et al., 2016) and even if molecular mechanisms remained unclear, MER disturbances increase the risk of noise trauma. Indeed, the muscle contraction triggered by the MER plays a role in protecting the inner ear by reducing the acoustic energy carried by continuous high-intensity noise (Borg et al., 1983). The nerve pathway for this reflex starts in the cochlea and travels to the brainstem through the ventral cochlear nucleus, the olivo-cochlear complex and the nucleus of nerve VII. Thanks to cross-projections of the reflex loop, a bilateral MER can be activated in response to high-intensity ipsi and/or contralateral stimulation (Borg, 1973). Triggering of the MER causes contraction of the middle-ear muscles and adjusts the impedance of the ossicular chain (Freeman et al., 1988). Because the MER protects the auditory system against exposure to high-intensity noises, any perturbation, for example by volatile chemicals, could have a significant impact in terms of occupational prevention for subjects exposed to noise (Prasher et al., 2005). Thus, in noisy and polluted workplaces, MER perturbation could favor auditory fatigue and cochlear trauma. As solvent effects on the MER are rapid, even the shortest exposures encountered in industrial settings can weaken cochlear protection against loud noises (Campo et al., 2014).

Unfortunately, the effect of a solvent on the MER is not related to its toxic effects on the cochlea, and cannot be predicted from toxicological data available in the literature. Indeed, there is a clear discrepancy between the rapid and transient neuropharmacological actions of solvents and their delayed and irreversible cochleotoxic effects resulting from chronic exposure (Vyskocil et al., 2012; Wathier et al., 2016).

Although many *in vitro* models exist to test the action of solvents on receptors or even on organs, only a few *in vivo* tests can measure the acute effects of solvents on hearing. It is therefore essential to develop new models capable of quickly and reliably determining the possible harmful effects of solvents on MER performance, and therefore on hearing.

Low-amplitude variation of the MER can be measured non-invasively in (anesthetized) animals (Wathier et al., 2016) and in humans (Venet et al., 2012) by a method based on the suppression of distortion product otoacoustic emissions (DPOAEs). DPOAEs are sounds emitted by the cochlea when stimulated by a particular combination of two pure sounds, $f1$ and $f2$. DPOAEs can be measured in the external auditory canal using a microphone. The $2f1$-$f2$ cubic DPOAE is the most robust and is sensitive to changes in middle-ear impedance, making it suitable for measuring the amplitude of the MER in anesthetized animals (Avan et al., 2003; Lim, 1986). The impact of the MER on the acoustic energy penetrating the cochlea can be simply assessed: when the MER is triggered, the amplitude of the DPOAEs decreases.

DPOAE variations caused by acoustic contralateral stimulation could result from the combined effects of two protective reflexes: the MER and the medial olivocochlear reflex also named acoustic reflex. Rumeau et al. (2011) and Relkin et al. (2005) showed that the effect of the medial olivocochlear reflex is negligible in experimental conditions similar to ours. To simplify the reading, the acoustic reflex will be assimilated to the MER effect in the rest of the manuscript.

In a previous publication, we highlighted the specific neuropharmacological action of aromatic solvents on MER pathways using this model (Wathier et al., 2016). Here, we used the same model to investigate how solvents from other chemical families affect MER impedance. To perform this investigation, anesthetized rats were exposed to solvent vapors, and the amplitude of their MER was recorded in real time before, during and after exposure. Aromatic and chlorinated solvents, ketones and aliphatic hydrocarbons were tested. Data from our previous study (Wathier et al., 2016) was added to the current data for comparative purposes and to facilitate interpretation of the results.

2. Materials and methods

2.1. Animals

Adult male Brown Norway rats (n = 37), weighing 334 ± 36 g and aged between 3 and 6 months at the time of the experimentation, were purchased from Charles River breeders (Sulzfeld, 97633, Germany). The rats arrived at the animal facility one month before starting experiments. Animals were housed in individual cages (350 × 180 × 184 mm) on irradiated cellulose BCell8 bedding (ANIBED, Route de Lude, 72510 Pontvallain) on a 12 h/12 h day/night cycle. Standard laboratory diet and water were available *ad libitum*. Room temperature and relative humidity in the animal facility were maintained at 22 ± 2 °C and 55 ± 10%, respectively. The animal facilities are fully accredited (C54-547-10) and the research described in this article was conducted in line with the Guide for Care and Use of Laboratory Animals promulgated by the European parliament and council (Directive 2010/63/EU, September 22, 2010). The present study was approved by the local ethics committee (No. 00569.02 and No. APAFIS#8836).

2.2. Measuring the middle-ear reflex

The experimental steps involved in measuring the MER and hearing are detailed elsewhere (Wathier et al., 2016). Briefly, rats were anesthetized with a mixture of ketamine and xylazine (45/5 mg/kg) and tracheotomized. The intra-tracheal tube was then connected to the inhalation system. All measurements were performed inside a sound-attenuated booth. A DPOAE probe was inserted into the left outer ear canal, and an earphone was inserted into the right ear canal. Primary tones $f1$ = 8000 Hz and $f2$ = 9600 Hz ($f2/f1$ = 1.2) were delivered by two emitters (B&K type 4191) at 62 and 51 dB SPL, respectively; the two tones were generated by a frequency synthesizer (Pulse, B&K 3110). DPOAE signal at the $2f1$–$f2$ frequency (6400 Hz) was measured by the probe microphone (Knowles FG 23329-C05) and analyzed by applying a fast Fourier transform (B&K Pulse 3110). The MER was triggered by an earphone (Etymotic Research ER4B) generating a 4400-Hz tone burst at 95 dB SPL in the contralateral ear. This signal was produced by a second frequency synthesizer (Pulse, B&K 3110). Each tone burst lasted 3 s and was followed by a 27-s silent window (no noise). The MER value was calculated as follows:

$$MER = DPOAE_{(no\text{-}contra\text{-}stim)} - DPOAE_{(contra\text{-}stim)}.$$

A detailed description of the measurement protocol can be found in Venet et al. (2011). Since the MER amplitude is modulated by the dose of anesthetic, the ketamine/xylazine intra-peritoneal infusion dose was adjusted to obtain a stable MER value of approximately 1.5 dB before solvent exposure. Rats were then exposed to an atmosphere containing 3000 ppm of solvent for two 15-minute periods separated by 20 min of fresh air. Screening was performed with aromatic solvents - styrene, benzene, chlorobenzene - and non-aromatic solvents - cyclohexane, *n*-hexane, acetone, methyl-ethyl-ketone (MEK), trichloroethylene and perchloroethylene (Table 1). At the end of the second exposure period, rats were euthanized, blood was sampled, brain was removed and all samples were stored at −80 °C until solvent measurements were performed.

The solvent concentration in the air was measured using a pump (0.25 L/min) to imitate the animal's breathing, which was connected to tubes containing activated charcoal. The system used to generate the solvent-loaded atmosphere and to expose the animals generated the target concentrations of 3000 ppm with a variability of less than 15% (Table 2). The variability measured can be explained by the constraints imposed by the model: solvent vapors must be generated under a chemical hood and must then be delivered to the animal, placed in a sound-

Table 1
Solvent characteristics.

Chemical family	Solvent	CAS	Log Kow	Structure
Aromatics solvents	Benzene	71-43-2	2.13	
	Chlorobenzene	108-90-7	2.84	
	Styrene	100-42-5	3.02	
	Toluene[a]	108-88-3	2.68	
	Ethylbenzene[a]	100-41-4	3.15	
	p-xylene[a]	106-42-3	3.15	
	m-xylene[a]	108-38-3	3.2	
	o-xylene[a]	95-47-6	3.12	
Ketones	MEK	78-79-3	0.29	
	Acetone	67-64-1	−0.24	
Alkanes	Cyclohexane	110-82-7	3.44	
	n-hexane	110-54-3	3.9	
Chlorinated solvents	Trichloroethylene	79-01-6	2.42	
	Perchloroethylene	127-18-4	3.4	

[a] data published in Wathier et al. (2016).

Table 2
Differences between the target solvent concentration (3000 ppm) and the concentrations generated by the home-made exposure system, as measured on active charcoal collection tubes (3 tubes per solvent).

Chemical family	Solvent	Difference (%)
Aromatics solvents	Benzene	−9.5
	Chlorobenzene	−14.3
	Styrene	0.5
	Toluene	−4.3
	Ethylbenzene	3.4
	p-xylene	−3.7
	m-xylene	−12.0
	o-xylene	−3.1
Ketones	MEK	−6.5
	Acetone	−5.0
Alkanes	Cyclohexane	−3.1
	n-hexane	0.0
Chlorinated solvents	Trichloroethylene	1.4
	Tetrachloroethylene	−0.8

attenuated booth for 2*f*1- *f*2 DPOAE measurement.

The “3R” rules were applied when performing animal experiments. Thus, the number of rats used to test each solvent was adjusted depending on the effect observed with the solvent: if no change in MER value was recorded in three successive animals, the absence of effect was considered relevant and the group was considered complete. In all other cases, five animals were exposed to each test solvent. The results obtained with the solvents listed in Table 1 completed those previously obtained with toluene, ethylbenzene and xylene isomers. For comparative purposes, data from these previous experiments are also presented here. These previously published data are always clearly indicated in the legends to the figures and tables.

2.3. Blood sampling, brain collection and solvent assays

Blood and brain samples were extracted with 500 µL dichloromethane (DCM), containing propylbenzene as an internal standard (1.7 g/L). Blood samples (~800 µL) were gently mixed by shaking for 10 min, while half-brains (left cerebral hemisphere, ½ cerebellum and ½ midbrain) were ground in a FastPrep instrument (MP Biomedicals) for 20 s at 4 m/s, using 2 mL lysing matrix tubes containing 1.4-mm ceramic spheres. Lysates were centrifuged for 10 min at –4 °C at either 3000 *g* (acceleration) for blood samples, or 4000 *g* for brain lysates.

Solvent contained in the DCM layer was quantified by Gas Chromatography–Mass Spectrometry (GC–MS) analysis on a QP2010 Ultra (Shimadzu) equipped with a split/splitless injector and an AOC20i auto-sampler. The system was controlled using GC–MS SU1 software, version 4.11. Samples were separated on an Rtx-1701 capillary column (30 m × 0.25 mm, film thickness 1 µm with a 4-m integra-guard column) (Restek, France) using helium as the carrier gas at a linear velocity of 40 cm/s. Samples (1 µL) were injected in split mode at a 1/20 ratio with carrier gas. The oven temperature was maintained at 40 °C for 4 min, and then increased at a rate of 15 °C/min to 235 °C; this temperature was maintained for 1 min (total duration, 18 min). Injection port, transfer line and ion source were maintained at 240 °C, 250 °C and 200 °C, respectively.

MS was performed by electron ionization (70 eV) in selected ion monitoring (SIM) mode. The following ions were selected for SIM analyses: acetone, 58; benzene, 78; chlorobenzene, 112; cyclohexane, 84, 56; ethylbenzene, 106, 91; hexane, 86, 57; MEK, 72, 43; propylbenzene, 120, 91; styrene, 104, 103, 78; tetrachloroethylene, 164; toluene, 92, 91; trichloroethylene, 130; *o,m,p*-xylene, 106, 105, 91.

In the conditions applied, the extraction efficiencies for all solvents in both tissues were almost quantitative, except for acetone and chlorobenzene in brain, for which yields were 81% and 66%, respectively. The limits of quantification for all the solvents tested in both tissues was between 1.4 and 2.1 µg/g (between 12 and 29 nmol/g), depending on the solvent. Bias, within-day and between-day precision was less than 10, 15 and 10%, respectively.

2.4. Ketamine and xylazine concentrations

Brain ketamine and xylazine concentrations were assayed using a procedure developed by Bonfanti et al. (2016). Briefly, one-quarter of a frozen brain (¼ cortical hemisphere, ¼ cerebellum and ¼ midbrain) was homogenized in a 2-mL lysing matrix tube containing 1.4-mm ceramic spheres and 500 µL PBsS solution (pH 7.4) and 100-µL Xylazine-d6 as an internal standard (50 mg/L in water). After protein precipitation with 10-µL trichloroacetic acid (20%) and centrifugation at 8000 *g*, 4 °C for 10 min, the supernatant was transferred to 2-mL Eppendorf tubes, then neutralized by adding 40 µL of NaOH (1 M), and extracted with 3 × 1 mL chloroform. Organic layers were combined and evaporated under nitrogen using a dry block heater set to 60 °C. The residue was reconstituted in 200-µL chloroform for GC/MS analysis. Samples were separated on an SLB-IL61 capillary column (30 m × 0.25 mm, film thickness 0.20 mm) (Supelco, France) with helium as the carrier gas at a constant linear velocity of 45 cm/s. The sample (1 µL) was injected in split mode at a split ratio of 1/30 with carrier gas. The oven temperature was maintained at 250 °C for 8 min, then increased to 280 °C at a rate of 10 °C/min and maintained at 280 °C for 3 min (total time, 14 min). The temperatures for the injection port, transfer line and ion source were set to 250 °C, 280 °C and 200 °C, respectively.

The MS was operated by electron ionization (70 eV) in SIM mode. The following ions were chosen for SIM analyses (ions used for quantification are underlined): ketamine: 209, 180; xylazine: 220, 205; xylazine-d6: 226, 208.

In these conditions, the limits of quantitation were 378 and 87 ng (approximately 0.76 and 0.17 µg/g of brain) for ketamine and xylazine, respectively. Bias, within-day and between-day precision was < 7% (and < 3.0%), < 11.1% (and < 3.0%) and < 20.9% (and < 10.5%), respectively, for ketamine (and xylazine).

2.5. Cochlear histology

Cochleae were perfused with a fixative solution (glutaraldehyde 2.5% in cacodylate buffer 0.1 M) and stored in the same solution for 2 weeks at 4 °C. Then, they were post-fixed for 1 h in OsO_4 1% before dehydration in ethanol 70%. After milling to remove bone, cochleae were decalcified for 48 h in a dilute ethylene-diamine-tetra-acetic acid (EDTA) solution and progressively dehydrated in graded ethanol solutions up to 100%. Finally, they were embedded in an epoxy resin (EPON812® 25%, dodecenylsuccinic anhydride (DDSA) 55%, araldite 15%, dibutyl phthalate 2% and 2, 4, 6-tris (dimethylaminomethyl) phenol (DMP30) 1.6%). Samples were finally sectioned, stained with cresyl violet dye and examined under light microscopy.

2.6. Statistical analyses

All statistical analyses were performed using GraphPad Prism V7.00. A one-way ANOVA followed by a post-hoc Sidak test was used for pairwise comparison of treatment effects. A one-sample *t*-test was used to determine whether the variations in the MER amplitude changes were different from a theoretical value of zero. The statistical significance of the relationship between the MER amplitude changes and other variables (ketamine, xylazine and solvent concentrations in the brain) was computed using a Spearman correlation test, as some data were non-normally distributed. Spearman correlation coefficients (r) and p values are provided in the text. The 95% confidence interval was used as an indicator of statistical significance. Data are presented as mean ± standard deviation (SD).

3. Results

3.1. Effects of solvents on the MER amplitude

The effect of solvent inhalation on the MER amplitude was calculated by subtracting the MER amplitude during the last five minutes of exposure from the baseline MER amplitude (for the 10 min prior to the start of exposure). Fig. 1 shows two representative examples of MER amplitude changes (gray lines) induced by 3000 ppm benzene (A), and 3000 ppm MEK (B) and their respective DPOAE levels in the absence of contralateral stimulation (continuous line).

Benzene inhalation caused a rapid increase in the MER amplitude (within 2 min), which increased steadily for 10 min, reaching a plateau at about 4.8 dB. After interrupting benzene exposure, the MER amplitude immediately decreased and returned to baseline values within approximately 10 min. In contrast, MEK inhalation had no obvious effect on MER amplitude.

The one-way ANOVA reveals that the MER amplitude was significantly influenced by the treatments [$F(13, 48) = 26.79$; $p < 0.0001$]. The transient effect of a solvent was considered significant when the average MER amplitude change was statistically different from zero (*t*-test). Among the 14 solvents tested, eight significantly increased the MER amplitude (Fig. 2): six aromatic hydrocarbons (benzene, chlorobenzene, *p*-xylene, toluene, styrene and *m*-xylene), one chlorinated hydrocarbon (perchloroethylene) and one cycloalkane (cyclohexane). None of the compounds tested significantly decreased the MER amplitude. Benzene and chlorobenzene exerted the most significant effects, with increases of 2.7 ± 0.8 dB and 1.8 ± 0.4 dB, respectively (mean ± SD). Moderate effects on MER amplitude were observed with a group comprising *p*-xylene (0.7 ± 0.3 dB), styrene (0.5 ± 0.3 dB), toluene (0.5 ± 0.2 dB), perchloroethylene (0.5 ± 0.2 dB) and cyclohexane (0.5 ± 0.3 dB). Finally, *m*-xylene induced a very small, but nonetheless significant, increase in MER amplitude (0.2 ± 0.1 dB). No significant effect on the MER amplitude was observed with *o*-xylene (-0.04 ± 0.18 dB), ethylbenzene (−0.1 ± 0.3 dB), *n*-hexane (−0.03 ± 0.40 dB), trichloroethylene (−0.06 ± 0.05 dB), acetone (−0.1 ± 0.1 dB) or MEK (−0.4 ± 0.2 dB). It is noteworthy that, in the absence of contralateral stimulation, the DPOAE level was never altered by solvent inhalation, whatever the solvent considered (overall average: 0.06 ± 0.2 dB).

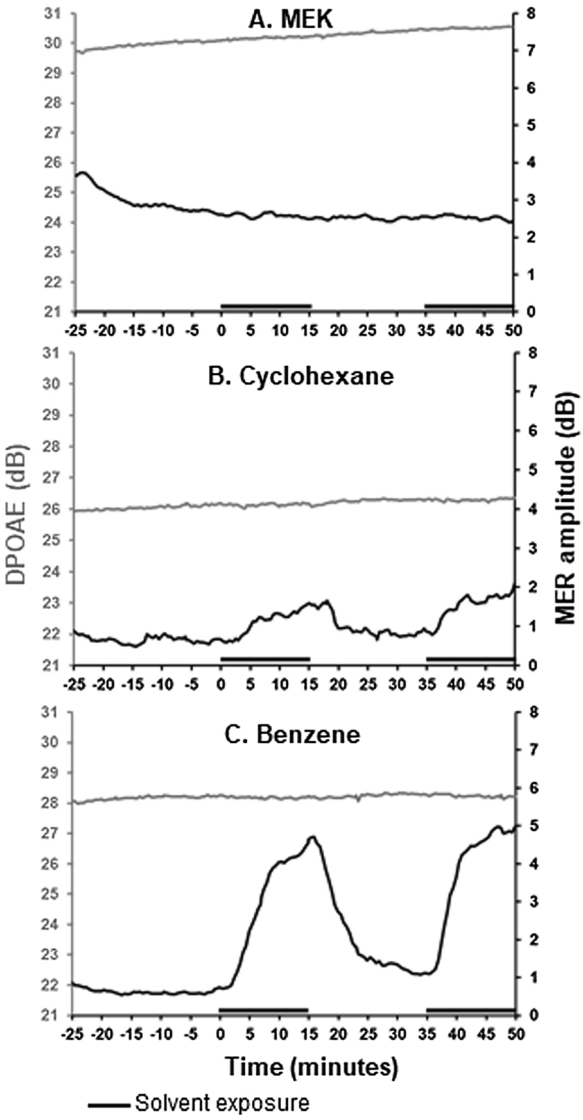

Fig. 1. Representative examples of MER amplitude modulation (black lines) by (A) 3000-ppm MEK, (B) 3000-ppm cyclohexane or (C) 3000-ppm benzene inhalation. Gray lines correspond to DPOAE levels measured in the absence of contralateral stimulation. Exposure to benzene at t = 0 min produced the most obvious effect on MER amplitude, whereas exposure to cyclohexane produced a moderate effect and MEK had no effect on MER amplitude.

3.2. Blood and brain solvent concentrations

Blood and brain concentrations (Fig. 3A) were measured in samples taken at the end of the second exposure period. Brain concentrations of solvent were comprised between 0.19 ± 0.04 µmol/g for *n*-hexane and 0.73 ± 0.07 µmol/g for perchloroethylene, whereas blood concentrations ranged from 0.03 ± 0.01 µmol/g for *n*-hexane to 0.44 ± 0.07 µmol/g for acetone. As expected, the blood/brain concentration ratios (Fig. 3B) indicated that the most lipophilic solvents - perchloroethylene, *n*-hexane and cyclohexane - preferentially accumulated in the brain.

Fig. 4A shows the relationship between the MER amplitude changes obtained in each animal and the amount of solvent measured in their respective brains. No significant association was observed (Spearman

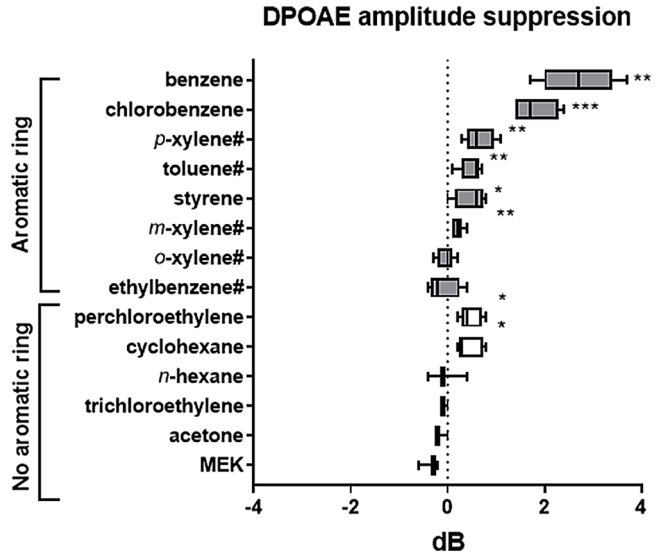

Fig. 2. MER amplitude changes induced by inhalation of air containing 3000 ppm of 14 different solvents. Solvents are divided into two groups: aromatic solvents (gray box-plots) and non-aromatic solvents (white box-plots). Within each group, solvents were ranked based on the average MER amplitude change. For comparative purposes, data from Wathier et al. (2016) are included in the graph, they are indicated by #. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$: probability that the MER amplitude change is different from zero. Box and whisker graphs represent median, 25th and 75th percentile and min / max values. n = 5 for solvents having a significant effect on the MER and n = 3 for solvents having no effect.

r = 0.0674; $p = 0.61$), suggesting that the MER amplitude change measured in our experiments was not influenced by the solvent burden in the brain. For comparative purposes, the MER amplitude changes obtained in all animals were normalized relative to the amount of solvent detected in the brain. Thus, the MER perturbing "potency" was expressed as dB/μmol/g of brain (Fig. 4B). Apart from minor statistical *p*-value changes, this normalization did not change the results presented in Fig. 2. This result suggests that the screening method used effectively reflects the specific impact of the solvent on the reflex rather than the effect of solvent burden on neuronal activity.

3.3. Ketamine/xylazine concentrations

The animals were anesthetized throughout the experiment and therefore during solvent exposure. Statistical tests indicated that ketamine/xylazine concentrations did not influence the effects of solvents on the MER amplitude (Fig. 5). Indeed, no significant relationship was found between the individual MER amplitude and the brain ketamine (Spearman r = -0.1093; $p = 0.40$) or xylazine (Spearman r = −0.0979; $p = 0.45$) concentrations in animals exposed to the different solvents.

3.4. Histology

The organ of Corti, the stria vascularis and the median spiral ganglion were analyzed by histological methods in cochleae from exposed and control rats (Fig. 6). Microscopic examination of the sections revealed no cochlear distress in exposed rats, whatever the solvent tested. In addition, no morphological differences were observed in tissues harvested from exposed animals or control rats.

4. Discussion

The main objective of the work presented here was to validate the method of the screening test elaborated in order to determine the impact of different classes of solvents on the MER. The effect of solvents on the MER is important because it can compromise cochlear protection against high-intensity noise, and therefore potentiate cochlear trauma when co-exposure with noise occurs (Borg et al., 1983). Data from our previous work suggested that MER changes caused by aromatic solvents were due to a specific pharmacological modulation of the neurons involved in the reflex, rather than a nonspecific action on cellular membranes (Maguin et al., 2008; Wathier et al., 2016). After excluding experimental parameters that could influence the MER disturbances, the current study further explored the chemical parameters involved in the effects of solvents on the MER by extending the test to other chemical families.

The amplitude of the 2*f*1-*f*2 acoustic distortion product (DPOAE) was recorded throughout the experiment. In the absence of contralateral stimulation, the DPOAE was not significantly altered (0.06 ± 0.2 dB) by solvent exposure, suggesting that solvent inhalation at 3000 ppm for 15 min did not fundamentally impair outer hair cell function. In addition, the cells in the cochlear neurosensory epithelium of exposed rats were morphologically similar to those from control rats

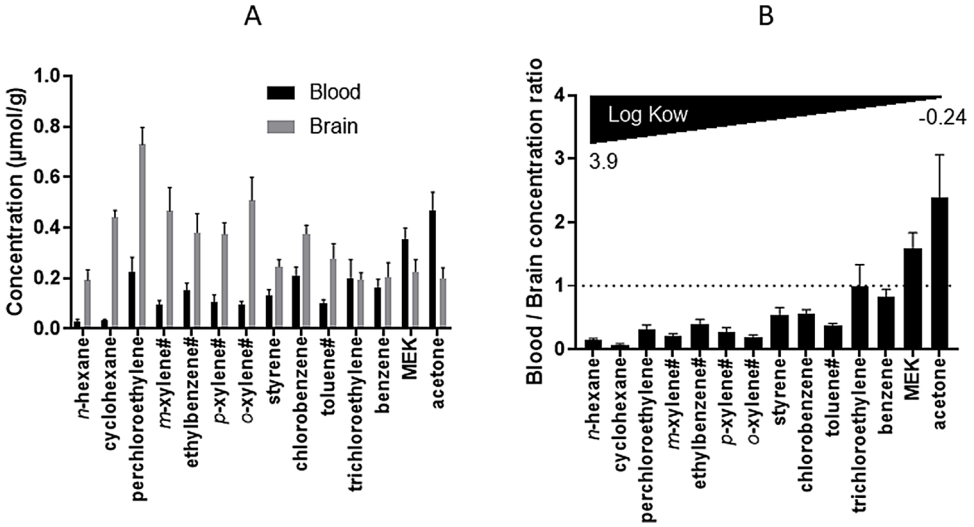

Fig. 3. (A) Blood and brain concentrations (μmol/g) analyzed from samples collected at the end of the second 15-minute solvent exposure. (B) Blood/brain concentration ratios. Solvents are ranked according to their lipophilicity (Log Kow). Data correspond to mean ± SD.

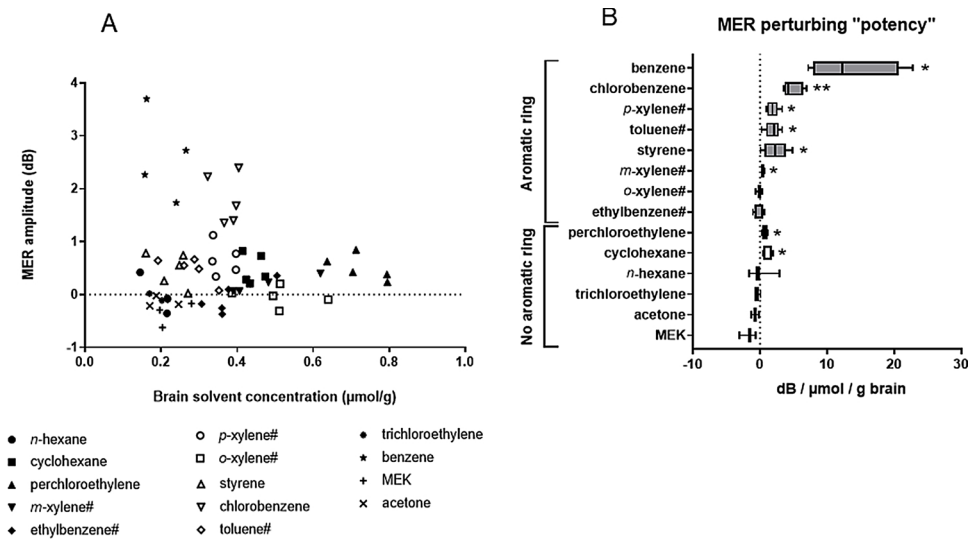

Fig. 4. (A) Relation between the MER amplitude and solvent concentrations in brain (µmol/g). (B) MER perturbing "potency" as represented by the values of MER amplitude change (dB) normalized relative to the amount of solvent detected in the brain (µmol/g). Solvent ranking was similar to that in Fig. 2. Box and whisker graphs represent median, 25th and 75th percentiles, and min / max values. n = 5 for solvents having a significant effect on the MER and n = 3 for solvents having no effect.

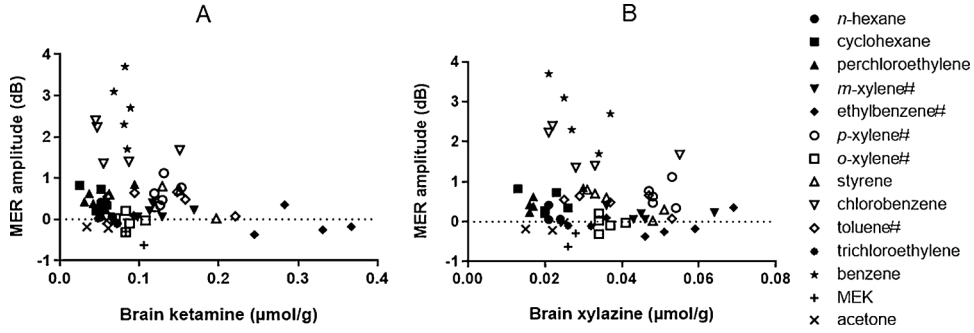

Fig. 5. Relation between the MER amplitude and (A) ketamine or (B) xylazine brain concentrations (µmol/g). Ketamine and xylazine concentrations were obtained from the brain of the animals anesthetized for approximately 2 h and exposed 2 x 15 min to different solvents.

(Fig. 6). Therefore, the effects observed are most likely caused by neuronal alteration of the reflex arc rather than by dysfunctions of the inner ear.

This *in vivo* model revealed statistically significant differences between the effects on the MER of fourteen test-solvents (Fig. 4B). The greatest impact on MER (≥1.8 dB) was measured with benzene and chlorobenzene. Then, there was a group of moderate-activity solvents (between 0.3 and 0.7 dB): toluene, styrene, perchloroethylene, cyclohexane, *m*-xylene and *p*-xylene. Finally, a group of solvents (trichloroethylene, *o*-xylene, *n*-hexane, acetone, ethylbenzene and MEK) had no significant impact on the MER (≤0 dB).

To safely interpret these differences, we verified that the experimental variability due to the use of an *in vivo* model, with differences in anesthesia and solvent uptake, did not significantly influence the results.

Although the solvents were all inhaled at 3000 ppm (Table 2), the resulting brain concentrations were (not surprisingly) heterogeneous, ranging from 0.19 ± 0.04 µmol/g for *n*-hexane to 0.73 ± 0.07 µmol/g for perchloroethylene (Fig. 3A). However, when MER amplitude variations were normalized against the solvent burden in the brain (expressed as dB /µmol/g of brain tissue), which represents the potency of the solvent, the values obtained were quite similar to the raw values expressed in dB. Thus, MER amplitude variations were not directly determined by solvent absorption (Fig. 4A). In other words, the solvent concentrations in the brain alone, and by extension differences in solvent absorption profile, cannot explain the variations in MER amplitude observed.

The MER assessment had to be carried out under anesthesia, but anesthesia was also a factor that could cause skewing of the data. For example, ketamine, as an NMDA receptor antagonist (Orser et al., 1997) and an acetylcholine nicotinic receptor antagonist (Flood and Krasowski, 2000), can reduce MER amplitude (Campo et al., 2013) by disrupting both the afferent (glutamate) and the efferent (acetylcholine) pathways of the MER control arc (Reuss et al., 2009). Indeed, solvents are known to act on the same receptors as ketamine (Bale et al., 2005; Cruz et al., 2000, 1998; Raines, 2004), among others (Cruz et al., 2000, 1998; Raines, 2004). Therefore, in our experimental conditions, it was impossible to separate the effect of solvents from those of ketamine on the amplitude of the MER. To reduce the confounding influence of anesthesia on MER measurements, we stabilized the MER at around 1.5 dB before starting solvent inhalation. This required the injection of variable volumes of anesthetics determined by the individual susceptibility of the animals. Nevertheless, no relation was found between the effects of solvents on the MER and the brain ketamine or xylazine load (Fig. 5), which suggests that MER amplitude changes following solvent administration result from a specific action of the solvent and are not a by-product of the anesthetic burden.

Based on all these results, we concluded that this *in vivo* model is appropriate to classify the potency of solvents based on their effect on the reflex loop of the MER, regardless of the concentration of anesthetics used (Fig. 4B).

Another factor that had to be taken into account in the interpretation of the MER changes was the variable lipophilicity of the different solvents tested. Our results show that there was no relationship between the effects of solvents and their log Kow. We therefore assume that the MER changes were due to specific action of solvents governed by their steric hindrance rather than by a nonspecific action linked to their affinity for fatty tissues. Nevertheless, this specific action of the

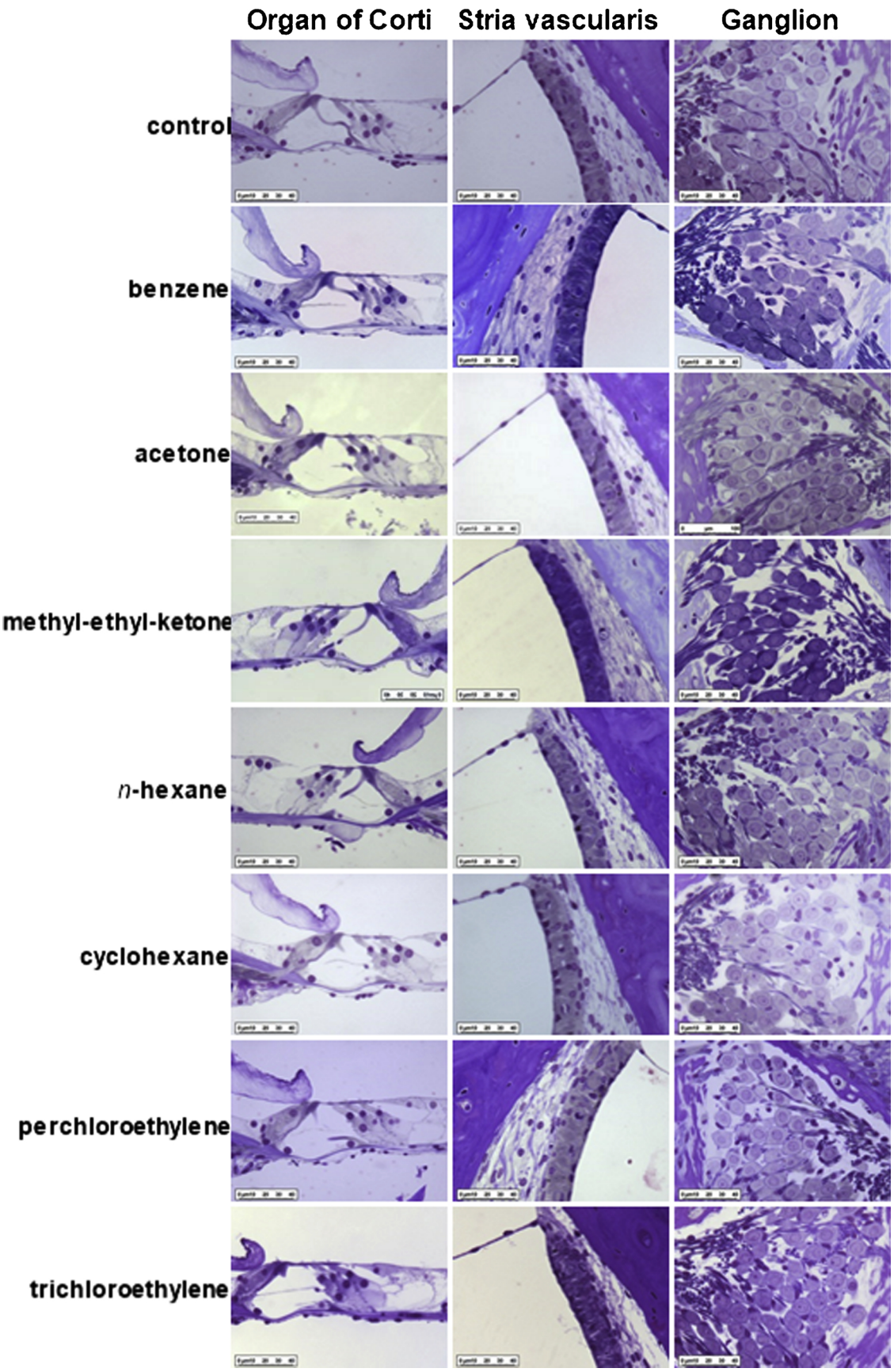

Fig. 6. Photomicrographs of histological sections of the medial turn in the cochlea. Organ of Corti, stria vascularis and ganglion were harvested after 2 x 15 min exposure to the different solvents (3000 ppm).

Table 3
How blood concentrations (μg/L) measured in rats at the end of the exposure compare to the biological exposure index set for exposed workers.

Chemical family	Solvent	Solvent blood concentration (μg/L)	Human BEI[a] (μg/L)[b]	Ratio ([rat]/[human])
Aromatic solvents	Benzene	11186	28	400
	Chlorobenzene	26407	210	125
	Styrene	12180	550	22
Ketones	Methyl-ethyl-ketone	20503	4000	5
	Acetone	20125	8300	2
Alcanes	Cyclohexane	2157	65	33
	n-hexane	1585	150	10
Chlorinated solvents	Trichloroethylene	37784	4000	9
	Tetrachloroethylene	60361	500	120

[a] Biological Exposure Index.
[b] Biotox database (http://www.inrs.fr/publications/bdd/biotox.html).

solvents was deducted from the results obtained and further investigations are required to identify the neuronal targets involved in this phenomenon.

Here, we present a conceptualized hypothesis to explain the different interactions between solvents and neuronal receptors. Three solvents – benzene, cyclohexane and *n*-hexane – with the same number of carbon atoms but different organizations exerted different effects. Benzene, which has an unsaturated ring with a flat conformation, had a stronger effect (Sidak, $p < 0.0001$) on the MER (2.7 dB) than cyclohexane (0.49 dB), which has a cycle that can take different, but more cumbersome, conformations (chair or boat). In contrast, the chain structure of *n*-hexane failed to disrupt the MER. From these results, it appears that the three-dimensional chemical structure influences the interactions.

The results obtained with the aromatics tested in this study support the assumption that the steric hindrance around the benzene ring is a major factor in modulating the reflex. Indeed, among the aromatics tested, benzene has the smallest steric bulk, since it bears no side-atom or chemical group. It was also the molecule that had the most impact on the amplitude of the MER (2.7 dB) (Fig. 3B). When a side-group is added to the benzene ring, such as a chlorine atom (chlorobenzene), the effect on the MER was significantly decreased (from 2.7 to 1.8 dB; Sidak, $p = 0.0002$). If the benzene ring bears a methyl group (toluene), which has a greater steric bulk than the chlorine atom, the effect on the MER was further reduced (from 1.8 to 0.5 dB; Sidak, $p < 0.0001$). The same pattern was found with the three isomers of xylene, for which different steric hindrances were associated with different effects on the MER.

In contrast, the length of the lateral chain does not seem to increase the potency of the molecule on the receptors. Thus, styrene, which is an aromatic carrying a 2C side chain, had an equivalent effect to toluene (Sidak, $p = 0.93$) or *p*-xylene (Sidak, $p = 0.41$), which both bear a single C chain. In addition, styrene and ethylbenzene, which both have a 2C lateral chain, had different effects on MER (Sidak, $p = 0.012$). This difference could be explained by the vinyl group carried by styrene, which confers rigidity on the molecule, whereas the ethylbenzene side chain allows the methyl group to move freely in space. Thus, the reduced effect of ethylbenzene on the MER compared to styrene may be due to its greater steric hindrance.

Among the halogenated solvents, perchloroethylene had an effect on the MER, whereas trichloroethylene did not. In this case, the metabolism of these compounds could shed light on the differences between them (Sidak, $p = 0.033$). Indeed, trichloroethylene is metabolized to 2,2,2 trichloroethanol, which binds to $GABA_A$ receptors (Peoples and Weight, 1994). The GABAergic inhibitory effect of this metabolite could indeed mask the effect of trichloroethylene on ketamine receptors.

Finally, the ketones, MEK and acetone, which carry a C=O bond on their carbon chain, did not disrupt the MER despite their well-known anti-convulsive and anesthetic properties (Hasebe et al., 2010; Yang et al., 2007). However, the concentrations of MEK and acetone measured in our study were lower than those tested by Hasebe et al. (2010) and Yang et al. (2007). It is therefore possible that the brain concentrations of these compounds were not sufficient to exert an effect on NMDA receptors.

Solvents act quickly on the MER nerve pathways, with effects on the amplitude of the MER detectable just a few minutes after the start of inhalation. Nevertheless, a possible action of their main metabolites should not be neglected, particularly for benzene and chlorobenzene which produce metabolites, such as epoxybenzene and 4-chlorobenzene-1, 2-epoxide, that are known to have toxic effects (Valentine et al., 1996; Willhite and Book, 1990). In summary, the parent molecule may not be the only molecule responsible for the effect observed on the MER. This observation does not reduce the relevance of the model, the main purpose of which is to identify solvents (and/or their metabolites) likely to alter cochlear protection upon co-exposure with noise. Readers should keep in mind that these experiments were carried out in rats, which display similar solvent metabolism to humans (Lovern et al., 1997; Nedelcheva and Gut, 1994). Therefore, the same reactive metabolites are likely to be produced in humans.

As exposure was performed with anesthetized rats, solvent concentrations had to be increased to 3000 ppm. The blood concentrations obtained in rats exposed to these atmospheric concentrations were not excessive compared to those defined by the biological exposure indices (BEI) established for exposed workers (Table 3). In fact, the ratio between the blood solvent concentrations measured in rats and the BEI was between 2, for acetone, and 125, for chlorobenzene. These numbers are less than or close to the safety factor of 100 that is generally applied in toxicology to take inter-species and inter-individual differences into account. Only benzene was excluded from this comparison, because it has a very low occupational exposure limit (1 ppm) as a result of its carcinogenic effects. The exposure conditions for our model therefore appear to be relevant to the concentrations observed in real occupational exposure scenarios.

In summary, the *in vivo* model developed here highlighted and allowed classification of the effect of solvents on the MER. As a result, it allows us to anticipate the effects of co-exposure to solvents and noise, and to prioritize chronic *in vivo* studies, which require large groups of animals. Perturbation of the MER by solvents is rapid, and can thus occur even during short-lived exposure, a scenario which is frequently observed in occupational settings. As a result, workers and employers should keep in mind that even short-term solvent exposure can modify the cochlea's natural protection against loud noises.

5. Conclusions

This study highlights a poorly known mode of action of solvents. Indeed, although solvents are known to be toxic for hair cells and cells in the organ of Corti, this study provides evidence that the acoustic reflexes can also be disrupted by solvents through an action that seems dependent on the steric hindrance of the molecule. The results obtained illustrate the distinction between the two phenomena: cochleotoxicity and neuropharmacology. For instance, benzene is not cochleotoxic, but it significantly perturbs the reflex, whereas ethylbenzene is a powerful cochleotoxic, but has no effect on the MER. In other words, volatile substances can be dangerous for hearing in two different ways: they can have a rapid and reversible pharmacological action on the nerve nuclei and / or they can display direct irreversible cochleotoxicity.

Conflicts of interest

The authors declare no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgment

The authors wish to thank Aurélie Thomas, Lise Merlen and Stéphane Boucard for performing the histological analyses.

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