NeuroToxicology 57 (2016) 13–21

Contents lists available at ScienceDirect

NeuroToxicology

Full Length Article

Membrane fluidity does not explain how solvents act on the middle-ear reflex

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ARTICLE INFO

Article history:
Received 4 January 2016
Received in revised form 26 July 2016
Accepted 3 August 2016
Available online 24 August 2016

Keywords:
Solvent
Anesthetics
Stereospecificity
Stapedial reflex
Membrane fluidity
NMR

ABSTRACT

Some volatile aromatic solvents have similar or opposite effects to anesthetics in the central nervous system. Like for anesthetics, the mechanisms of action involved are currently the subject of debate. This paper presents an *in vivo* study to determine whether direct binding or effects on membrane fluidity best explain how solvents counterbalance anesthesia's depression of the middle-ear reflex (MER). Rats were anesthetized with a mixture of ketamine and xylazine while also exposed to solvent vapors (toluene, ethylbenzene, or one of the three xylene isomers) and the amplitude of their MER was monitored. The depth of anesthesia was standardized based on the magnitude of the contraction of the muscles involved in the MER, determined by measuring cubic distortion product oto-acoustic emissions (DPOAEs) while triggering the bilateral reflex with contralateral acoustic stimulation. The effects of the aromatic solvents were quantified based on variations in the amplitude of the DPOAEs. The amplitude of the alteration to the MER measured in anesthetized rats did not correlate with solvent lipophilicity (as indicated by logKow values). Results obtained with the three xylene isomers indicated that the positions of two methyl groups around the benzene ring played a determinant role in solvent/neuronal cell interaction. Additionally, Solid-state Nuclear Magnetic Resonance (NMR) spectra for brain microsomes confirmed that brain lipid fluidity was unaffected by solvent exposure, even after three days (6 h/day) at an extremely high concentration (3000 ppm). Therefore, aromatic solvents appear to act directly on the neuroreceptors involved in the acoustic reflex circuit, rather than on membrane fluidity. The affinity of this interaction is determined by stereospecific parameters rather than lipophilicity.

1. Introduction

Despite efforts to eliminate hazardous exposure to noise in many factories, noise-induced hearing loss remains one of the most common occupational diseases in France, in the United States, and in numerous industrialized nations. One potential risk factor contributing to the prevalence of hearing loss is the influence of other agents in combination with noise exposure. Aromatic solvents are used in the manufacturing of 'adhesives, paints, varnishes, printing inks, degreasers, fuel additives, glues, thinners and plastics', ranking them among the most frequently encountered environmental hazards for workers (Dick, 2006). The main mode of occupational exposure is by inhalation, although absorption through the skin should also be taken into consideration. Exposure should be closely monitored as evidence from cases of solvent abuse (Greenberg, 1997; Lazar et al., 1983; Yamanouchi et al., 1995) indicates that chronic exposure to alkylbenzenes 'toluene, ethylbenzene, xylenes' can have effects on the central nervous system (CNS). Inhalation of toluene-based products (solvent sniffers) can induce neurologic abnormalities varying from severe dementia to elemental neurologic signs such as cerebellar ataxia, oculomotor abnormalities, tremor and deafness (Hormes et al., 1986). These effects can be exacerbated by the fact that solvents are often used in areas where noise levels are high.

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http://dx.doi.org/10.1016/j.neuro.2016.08.001
0161-813X/

The widely accepted Meyer-Overton theory (Meyer, 1899; Overton, 1901), or 'Lipid theory' (Halsey, 1992), held that the general effects of a wide variety of volatile anesthetics were related to their action on the lipid constituents of brain cells and their ability to perturb the fluidity of the neuronal plasma membrane (Engelke et al., 1992). Other volatile chemicals such as abuse inhalants were also initially thought to produce 'non-specific' effects similar to those of non-specific narcotics (Balster, 1998). However, clear evidence has emerged that inhaled solvents and volatile anesthetics act specifically on several types of ion channels expressed in neurons (Franks and Lieb, 1994; Yamakura et al., 2000), with over 25 different potential target types (Campagna et al., 2003). Like anesthetics and other CNS depressants, frequently abused inhalants such as toluene can enhance inhibitory synaptic responses (MacIver, 2009). These effects are probably linked to shared binding sites or similar mechanisms of action. For instance, N-methyl-D-aspartic acid (Cruz et al., 1998), δ-aminobutyric acid and glycine (Beckstead et al., 2000), adenosyl triphosphate (Woodward et al., 2004), serotonin (Lopreato et al., 2003), and nicotinic acetylcholine receptors (nAchR) (Bale et al., 2005, 2002) are all sensitive to toluene. nAchRs have emerged as potentially specific targets of anesthetics (Tassonyi et al., 2002; Yamashita et al., 2005), and a *in vivo* investigation carried out by our laboratory showed that toluene could also mimic the effects of nAchR antagonists and disturb voltage-mediated Ca^{2+} channels (Maguin et al., 2009).

The acoustic reflex in the rat can be considered as the sum of the effects of the inner ear reflex (medial olivocochlear reflex) and those of the middle ear reflex (MER). Nevertheless, Rumeau et al. (2011) and Relkin et al. (2005) showed that the main effect are due to the MER in a ratio 2/3. To simplify the reading, the acoustic reflex will be assimilated to the MER effect in the rest of the publication.

The MER is sensitive to both anesthetics (Borg and Moller, 1975) and certain aromatic solvents (Campo et al., 2007). However, to our knowledge, only one study so far has compared the effects of anesthetics and inhaled toluene on acoustic reflex performances in the same *in vivo* experimental protocol (Campo et al., 2013). In this study, the authors showed that inhaled toluene counterbalances the effects of anesthetics in a dose-dependent manner. In other terms, toluene can increase the amplitude of the MER in anesthetized rats, whatever the nature of the anesthetic used (pentobarbital, isoflurane, ketamine/xylazine). In humans and rats, the magnitude of the MER triggered by noise depends on the depth of anesthesia (Bissinger et al., 2000), but certain aromatic solvents, including toluene, can alter this magnitude. Based on the results presented by Campo et al. (2013), it was impossible to determine whether the alterations to the MER resulted from modifications to membrane fluidity or from direct competition between anesthetics and solvents for binding to specific targets. Thus, the interactions of anesthetics and solvents with their targets could be either non-specific or specific (Urban et al., 2006).

From a biophysical point of view, membrane fluidity can be quantified based on the amplitude and rotational speed of the lipid molecular motion. Lipid bilayers are known to adopt different physical phases (crystal (L_C), gel ($L_{\beta'}$), or liquid-crystalline phase (L_α)) depending on temperature, nature and content (Huang and Li, 1999). By altering the packing and cooperativity of the lipid chains in a cellular membrane, solvents could modify overall membrane fluidity. Such an effect would be revealed by changes to the phase transition temperatures which depend on interactions between the components making up the bilayers. Since Nuclear Magnetic Resonance (NMR) properties are sensitive to molecular motion, hydrogen-1 (^1H) NMR measurements on the fatty acid "tails" can give access to these transition temperatures. Additionally, phosphorus-31 (^{31}P) NMR properties of the lipid "head" group are sensitive to rotational diffusion of the lipids (Seelig, 1978), therefore their measurement will also reflects the membrane plasticity.

This paper presents a preliminary investigation into the combined effects of exposure to a mixture of ketamine and xylazine, and inhaled solvents in anesthetized rats. Except for toluene, the laboratory reference molecule (log Kow = 2.68), the solvents were chosen to test the possible impact of the lipophilicity [octanol/water partition coefficient (Kow)] or that of the structure of the molecule on the MER amplitude. The three xylene isomers were chosen because they have log Kow which are close (*para*: 3.15, *meta*: 3.2, and *ortho*: 3.12) but different structures, with the position of the two methyl groups differing between isomers. Ethylbenzene was chosen because it has the same log Kow value as *p*-xylene (3.15), but an extra carbon on the lateral chain.

The respective effects of anesthetics and solvents on the MER amplitude were investigated using a previously-described method (Venet et al., 2011). The effect of solvent exposure (toluene) on membrane fluidity was estimated by solid-state NMR analysis of ^{31}P residual Chemical Shift Anisotropy (CSA). Additionally, the T_m gel ($L_{\beta'}$) to liquid-crystalline phase (L_α) transition temperature was evaluated by variable temperature ^1H NMR measurements, to assess how solvent molecules modify overall membrane fluidity.

2. Materials and methods

2.1. Animals

Adult male Brown Norway rats weighing 290 ± 25 g were purchased from Janvier breeders (Le Genest St Isle, St Berthevin, 53941, France). 104 rats were used to carry out the study: 5 animals per solvent were used (n_{it} = 25) for the intra-tracheal exposure, whereas 5 animals per solvent/dose were used (n_{ld} = 15 × 5 = 75) for the long-duration exposure. Finally, 2 controls and 2 toluene-exposed rats were used for the MNR experiments.

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 tap 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, 22 September 2010). The present study was approved by the local ethics committee (n° 00569.02).

2.2. Anesthesia and animal preparation

Anesthesia was induced with a single injection of ketamine and xylazine (45/5 mg/kg). An intra-peritoneal catheter was placed for subsequent dosage of anesthetic. Body temperature was maintained at 34–36 °C, based on continuous monitoring using a rectal probe connected to a temperature-regulation device. Both left (ipsilateral) and right (contralateral side) tympanic membranes were then pierced with the tip of a pulled glass electrode (1–2 μm) to avoid variations in the middle-ear pressure caused by anesthetics (Acar et al., 2010). A distortion product oto-acoustic emission (DPOAE) probe was inserted into the left outer ear canal, and an earphone was inserted into the right canal. An intra-tracheal tube was connected to the home-made inhalation system, as illustrated in Fig. 1.

Anesthesia was adjusted with a syringe pump to obtain a MER amplitude between 1.5 and 2 dB. The mixture of ketamine and xylazine (33/3.7 mg/kg) was injected intra-peritoneally at a rate of