Toluene Effect on the Olivocochlear Reflex

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Animal studies have shown that toluene can cause hearing loss and can exacerbate the effects of noise by inhibiting the middle ear acoustic reflex. In this investigation, carried out in Long-Evans rats, the tensor tympani tendon was cut-off and the stapedius muscle was electrocoagulated in one or both middle ears. Rat hearing was evaluated by measuring cubic distortion otoacoustic emissions (2f1-f2; f1 = 8000 Hz; f2 = 9600 Hz; f1/f2 = 1.2) prior to, during, and after activation of the olivocochlear (OC) reflex. A band noise centered at 4 kHz was used as suppressor noise. It was delivered contralaterally to decrease 2f1-f2 amplitude. The strength of the inner ear acoustic reflex was tested by increasing contralateral noise intensity, and toluene injected into the carotid artery was used to study physiological efficacies. Results showed that the protective effect of the OC reflex is intensity dependent. In addition, the OC reflex was found to be less sensitive to toluene than the middle ear acoustic reflex. This may be because the efferent neurons involved in inner ear and middle ear reflexes are located differently. In conclusion, the synergistic effects on hearing of co-exposure to noise and aromatic solvents are because of solvents depressing the central nuclei, which mainly drive the middle ear acoustic reflex.

Key Words: DPOAE; toluene; noise; co-exposure; olivocochlear reflex.

Although noise is clearly the predominant occupational hazard to hearing, research on hearing conservation proves that noise is often present in occupational settings where chemical exposure also occurs (EU-OSHA, 2009; Johnson and Morata, 2010). For instance, aromatic solvents have been demonstrated to be ototoxicants and can even worsen the effects of noise.(2) Animal studies have shown that toluene can either increase or decrease efficiency of the middle ear reflex depending on the toluene concentration and the ear receiving the elicitor triggering middle ear muscle contraction (Venet et al., 2011). Thus, toluene may depress the auditory nervous system involved in ear-protective reflexes, in particular the middle ear reflex.

How toluene affects the inner ear acoustic reflex remained unknown and could not be deduced from our previous experiments. Indeed, the protective effects induced by the inner ear reflex were too weak compared with those induced by the middle ear reflex (2 vs. 16 dB) to be accurately observed.

In spite of this difficulty, we knew from the literature that the inner ear reflex, also called olivocochlear (OC) reflex, can protect the ear from noise-induced temporary threshold shift (Patuzzi and Thompson, 1991; Reiter and Liberman, 1995; Zheng et al., 1997). It was therefore of interest to study the toluene effect on the OC reflex.

The OC reflex mainly constitutes of efferent neurons originating bilaterally from brain stem regions in and around the superior olivary complex (Warr et al., 1986). In the brain stem, the latter is divided into two main subsystems: the so-called medial and lateral OC pathways (White and Warr, 1983). The medial efferent innervations of the organ of Corti are made up of crossed OC bundle (COCB) and uncrossed bundles (UOCB). The COCB contains twice as many neurons as the UOCB (Liberman, 1988). The majority of neurons terminate on either the outer hair cells (OHCs) or the eight nerve ganglion cells beneath the inner ear cells (Pujol and Lenoir, 1986). OC efferent neurons generate a sound-evoked reflex pathway to the inner ear, and activity in this pathway can suppress cochlear responses (Liberman et al., 1996; Puria et al., 1996).

To study the effects of toluene on the inner ear reflex more specifically, we carried out a series of experiments in rats with severed middle ear acoustic muscles. In these conditions, we could test the toluene effect on the OC reflex alone.

Distortion product otoemissions (DPOAEs) are low-level sounds emitted by the cochlea through the middle ear system when the auditory receptor is stimulated by two primary tones: f1 and f2. Among the different types of DPOAEs,
cubic (2f1-f2) distortion product otoacoustic emissions can be recorded in anesthetized rats and in humans. They can be measured by a sensitive microphone fitted into the outer ear canal. As a result, they allow even slight modifications because of changes in the middle ear or inner ear to be measured. They are considered reliable indicators of OHC function (Lim, 1986). Associated with a contralateral suppressor noise, 2f1-f2 DPOAEs can be used to evaluate middle ear (Puria et al., 1996; Venet et al., 2011) or inner ear acoustic reflex efficiency.

In the present study, 2f1-f2 DPOAEs were recorded while the inner ear acoustic reflex was activated by a noise suppressor (NS) emitted in the rat’s contralateral ear. The aim was to characterize the inner ear reflex using 2f1-f2 distortion otoacoustic products and to analyze the impact of toluene on efferent pathways involved in the inner ear reflex.

MATERIALS AND METHODS

Animals. Adult Long-Evans rats weighing over 350 g were used (n = 22). Animals were purchased from Charles River breeding laboratories (Saint Aubin-les-Elbeuf, France) 2 weeks before the start of experiments. They were housed in individual cages (350 x 180 x 184 mm) with irradiated pine wood bedding (supplier: Special Diets Services, France, ref: Gold cob 891180). Food and tap water were available ad libitum. A normal day/night cycle was maintained: lighting was on 12 h/day. Room temperature and relative humidity were controlled in the animal facility at 22 ± 2°C and 55 ± 10%, respectively. The animal facilities have full accreditation, and experiments described in this article complied with the recommendations in the Guide for Care and Use of Laboratory Animals as promulgated by the French Conseil d’Etat through decree no. 87 848, published in the French Journal Officiel on 20 October 1987.

Anesthesia. General anesthesia was required to record DPOAEs in rats equipped with an intracartid catheter. To minimize stress in animals, levorepromazine (12.5 mg/kg) was administered ip 15 min prior to general anesthesia. Deep anesthesia was induced by injection of a mixture of ketamine (50 mg/kg) and xylazine (6 mg/kg). Supplemental doses of anesthesia, one-fifth of the original bolus, were administered approximately once per hour or when the rat responded to a toe pinch. A heating pad system was used to maintain rectal body temperature around 36°C. Before recording DPOAEs, otoscopy examination was used to verify that the tympanic membranes did not display any obstruction, infection, or other abnormalities.

Surgery. The tympanic bullae were ventrally exposed and opened with the extremity of a surgical blade. The tendon of the tensor tympani muscles was cut with a pair of microscissors at the point where the tendon is attached to the malleus. The stapled muscle was destroyed by electrocoagulation using an insulated platinum wire denuded at the tip.

To eliminate the possibility of a noise artifact in our measurements, the contralateral cochlea were destroyed in three additional rats (n = 3).

Reflex Measurement. DPOAE recording. All measurements were performed inside a sound-attenuated booth. The custom-designed DPOAE probe consisted of two transducers generating the primary tones: f1 = 8000 Hz and f2 = 9600 Hz and a microphone measuring the acoustic pressure within the outer ear canal. Primary tones f1 and f2 were delivered at 65 and 60 dB sound pressure level (SPL), respectively, to the ipsilateral (left) ear. The highest level of sound was limited to 65 dB SPL. The ratio of f1 to f2 was 1.2, which is suitable both for rats (Henley et al., 1989) and humans (Faskill and Brown, 1990). The primary tone signals were produced by frequency synthesizers (Pulse, B&K 3110) and emitted by two miniature speakers (Microphone, B&K type 4191). The elicitor sounds were considered as the ipsilateral (ipsi) acoustic stimulation in our protocol, as illustrated in Figure 1.

The calibration procedure ensured that the primary tones were always emitted at the target intensities. Calibration was performed with a 1/8 inch microphone (B&K type 4138) placed in a specifically designed cavity of equivalent volume to the rat’s outer ear canal. DPOAEs were recorded with a microphone (Knowles FG 23329-C05) fitted into the probe. The three transducers were enclosed in the probe. The probe tip was pressed gently against the opening of the ear canal.

The response was measured by a Fast Fourier Transform (FFT) analyzer (B&K PULSE 3110). DPOAE amplitude was determined from a linear averaged (N = 148) spectrum of 500 ms, given that each FFT epoch lasted 10 ms (overlap 66.7%). Spectrum averaging was recorded every 500 ms to trace the DPOAE amplitude evolution as a function of inner ear acoustic reflex activity.

Contralateral acoustic stimulations. The contralateral noise (NS) was an 800 Hz band noise centered at 4 kHz (BN 4 kHz). Each tone burst lasted 2.5 s and was followed by a 9.5-s silent window before the next tone burst, as illustrated in Figure 1. NS intensity for contralateral experiments varied between 90 and 120 dB SPL. The signal was synthesized by a B&K Pulse 3110 and emitted by an Etymonic Research ER4 B earphone. DPOAEs were measured prior to and during delivery of the NS.

Catheter Implantation and Solvent Injections. A ventrolateral incision was made in the neck to expose the left (ipsilateral) or right (contralateral) carotid artery. The external carotid artery was ligated to drive the bolus toward the brain stem. A circular custom-made catheter (Te in polypropylene internal diameter (i.d.) = 1.6 mm extended with silicon catheters [i.d. = 0.635 mm]) filled with a solution of 0.9% NaCl and hepargin (50 U/ml) was fitted into the common carotid trunk. This type of catheter allows normal blood flow to be maintained while the vehicle containing the solvent is injected (Lataye et al., 2007). Once inserted, the circular catheter was filled with vehicle consisting of a 10% fatty emulsion of purified soya oil and essential fatty acids (intralipid Ref: 830513161, Fresenius Kabi company). All injections were performed with a syringe pump calibrated to deliver a 266-µl bolus over 80 s (Fig. 1). Toluene (Prolabo 20675294) concentration was diluted to 116.2 nm in vehicle prior to injection. It is important to bear in mind that the concentration is given as the concentrations inside the syringe, not the effective concentrations reaching the brain and/or cochlea. Each animal was tested with a single concentration.

Data Recording. The 2f1-f2 DPOAE changes measured with and without NS (noNS) are illustrated in Figure 2. The inner ear acoustic reflex metric followed the decrease in DPOAE amplitude and could be modeled as follows:

inner ear acoustic reflex = DPOAE_{noNS} - DPOAE_{NS}.
Prior to injection, at least four inner ear acoustic reflex values were triggered. The average magnitude of the inner ear acoustic reflex (avgIER) was calculated for all animals using the four values recorded prior to injection.

Statistics. Statgraphics Centurion XV software was used to perform all statistical analyses. A one-way ANOVA was run to test the effect of the intensity on the $f_1$-$f_2$ variations and to compare the different experimental conditions. The statistical result is expressed as follows: $F(\text{df}_b,\text{ df}_i) = F$-ratio; $p = p$ value, in which $\text{df}_i$ is the number of degrees of freedom between groups and $\text{df}_i$ the number of degrees of freedom within a group. $F$-ratio is the mean square value between groups divided by the mean square value within a group. Post hoc analysis was performed using Bonferroni method. Comparisons between groups used the Mann-Whitney (MW) test.

RESULTS

Measurement of the OC Reflex Amplitude

Despite the surgical interventions on the tested ear, $f_1$-$f_2$ DPOAE amplitudes of approximately 28 dB SPL were measured. In addition, we obtained a significant (MW $= 0$; $p = 0.002$) decrease in $f_1$-$f_2$ DPOAE amplitude because of the NS delivered to the contralateral side (Fig. 3A). The avgIER values obtained from four representative animals, as plotted in Figure 3, were approximately 1.8 dB.

In animals in which the contralateral cochlea was destroyed, no variations in $f_1$-$f_2$ amplitudes in the intact ipsilateral ear were measured (data not shown). Thus, inflexions of $f_1$-$f_2$ amplitudes observed in animals with intact cochlea were because of elicitation of the inner ear reflex and not artifact of transmission of the NS from one ear to the other. In other terms, sound conduction through the skull bones was not factor in generating our results.

The relationship between NS intensity (BN 4 kHz) and variations in $f_1$-$f_2$ amplitudes is shown in the Table 1. Tensor tympani and stapedius muscles were severed in the ipsilateral ear in all animals. The maximal amplitude measured with this technique was approximately 2 dB. Amplitude differences obtained with a NS delivered at 90, 100, or 120 dB were large enough to provide statistical reliability with the 95% confidence interval ($F(2,9) = 21.9$; $p < 0.05$). Bonferroni post hoc analyses showed that the 120 dB group was different from the 90 and 100 dB SPL groups.

Contralateral Stimulation, Intralipid Injection in Left or Right (ipsilateral/CONTRA) Artery, and Middle Ear Muscles Severed in Left Side (ipsilateral)

Injection of toluene at 0 mM (intralipid alone) did not elicit variations in $f_1$-$f_2$ DPOAE amplitude, regardless of injection side. The data obtained with a representative subject, out of four rats tested in this control group ($n = 4$), are shown in Figure 3A. Therefore, the vehicle (intralipid) is neutral in the phenomenon observed in this series of experiments. Only the effects of contralateral injection are illustrated in this paper (Fig. 3A).

The middle ear muscles of the tested ear were cut to allow inner ear reflex effects to be assessed using the acoustic setup described.

Contralateral Stimulation, Ipsilateral Toluene Injection, and Neutralization of Middle Ear Muscles

With contralateral stimulation combined with ipsilateral toluene injection and severing of the middle ear muscles, we obtained avgIER values of approximately 1.7 dB. As previously described, the data obtained with a representative subject, out of four rats tested in this group ($n = 4$), are shown (Fig. 3B). Toluene was injected into the ipsilateral (IPS1) artery, i.e., the artery connected to the ear into which the $f_1$ and $f_2$ primaries were emitted. Injection of 116 mM toluene did not significantly change the average amplitude of the inner ear reflex (Bonferroni test before vs. during injection $[0.014 \pm 0.218]$) (Fig. 3B).
Contralateral Stimulation, Toluene Injection, and Ipsilateral Neutralization of Middle Ear Muscles

In combination with severed ipsilateral middle ear muscles, contralateral stimulation in rats and injection of 116mM toluene on the same side provoked an unexpected increase in amplitude of the inner ear reflex (Fig. 3C). The increase from 2 to 3 dB SPL ($n = 4$) was significant ($F(4,25) = 10.8, p < 0.001$, Bonferroni test: significant difference $0.586 \pm 0.218$).

Contralateral Stimulation, Toluene Injection, and Bilateral Severing of Middle Ear Muscles

In rats where both sets of middle ear muscles had been severed, contralateral stimulation with or without toluene injection on the same side nevertheless gave rise to $2f_1-f_2$ DPOAE amplitudes approximately 27 dB SPL and a decrease in amplitude of approximately 1.2 dB (Fig. 3D). Only three rats were tested in this experimental condition ($n = 3$). No significant difference between amplitudes for the inner ear reflex was found for before versus during toluene injection (Bonferroni test: $0.054 \pm 0.239$).

DISCUSSION

In a previous paper (Venet et al., 2011), we showed that toluene could either increase or decrease the efficiency of the middle ear acoustic reflex depending on the toluene concentration applied and the ear receiving NS stimulation. Only the presence of interneurons between the facial nucleus and the superior olivary complex could explain these findings. The role played by the superior olivary complex in the middle ear reflex is therefore more important than that reported by the literature (Lee et al., 2006). Because this complex also plays a key role in the inner ear reflex, a similar strong toluene effect was expected on this reflex.

OC efferent neurons form a sound-evoked reflex pathway to the inner ear. Activity in this pathway can control how sounds are processed in the auditory periphery, allowing improved detection of signals in background noise, also known as frequency discrimination. OC neuron activity can also decrease cochlear responses, protecting the peripheral receptor, the cochlea, from damage caused by overly loud sounds. This is mainly achieved through modification of cochlear tympani and stapedius muscles were severed in the IPSI side. Primary tones $f_1 = 8$ kHz and $f_2 = 9.6$ kHz were emitted at $L_1 = 65$ dB SPL and $L_2 = 60$ dB, respectively. Frequency ratio was 1.2. The NS was an 800-Hz BN 4 kHz emitted at 110 dB. The darker traces were measured on the left hand Y-axis scale and the lighter traces on the right hand Y-axis scale. (A) 0mM toluene, using intralip as vehicle ($n = 4$); (B) 116mM toluene injected in the IPSI side ($n = 4$); (C) 116mM toluene injected in the CONTRA side ($n = 4$); (D) 116mM toluene injected in the CONTRA side with severed tensor tympani and stapedius muscles in both sides ($n = 3$). Data were obtained from individual representative subjects.

FIG. 3. Variations in $2f_1-f_2$ DPOAE amplitude and inner ear acoustic reflex efficiency prior to, during, and after toluene injection. In all cases, tensor

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micromechanics. The OHCs play a crucial role in this modification (Cooper and Guinan, 2006; Lim, 1986; Pujol, 1994).

OC efferent neuron effects have been assessed in a number of ways using either auditory-evoked potentials (Puel et al., 1988; Puria et al., 1996) or evoked otoacoustic emissions (Liberman et al., 1996). In the present investigation, otoacoustic emissions and more specifically 2f1-f2 distortion product otoacoustic emissions were used as an efficient tool to assess the role played by OC system in cochlear protection. With contralateral sound suppression and neutralization of middle ear muscles, a modest but significant decrease in amplitude of 2f1-f2 distortion product otoacoustic emission was measured (up to 3 dB). We succeeded in measuring intensity-dependent responses, despite the narrow amplitude range, 0.5–2 dB (Table 1). This finding is unexpected because mechanical inhibition induced by the OC efferent is strong for moderate intensities and become progressively weaker as intensity increases (Cooper and Guinan, 2006). How can our results be explained? The analysis performed considers the magnitude of the cubic distortion otoacoustic product measured at 6.4 kHz along the cochlear partition, namely, at the geometric mean of the two primary tones, rather than at 4 kHz, which is the characteristic frequency of the suppressor noise. By increasing NS intensity, we widened the segment of mechanically modified basilar membrane and thereby the mechanical impact on cubic distortion products.

2f1-f2 distortion product otoacoustic emissions decrease by ~2 dB because of inner ear reflex activity, as compared with a decrease of ~12 dB measured after eliciting the middle ear reflex. This indicated huge differences in terms of ear protection (Venet et al., 2011) and puts the protective effects of both reflexes into perspective: the middle ear reflex decreases the acoustic energy penetrating into the cochlea by modifying compliance of the tympano-ossicular chain, whereas the inner ear reflex modulates only the basilar membrane mechanics to absorb acoustic energy. Given the effectors targeted by each reflex, the scale of effects is not expected to be the same.

The toxicological data obtained during this study revealed that the inner ear reflex was more resistant to toluene (delivered via the carotid artery) than the middle ear reflex. A bolus of 116mM toluene injected into the left carotid trunk did not significantly modify the magnitude of 2f1-f2 distortion product otoacoustic emissions (Fig. 3B). These findings indicate that injection of toluene into the common carotid trunk did not alter any of the elements constituting the OC reflex: be it the auditory receptor, the cochlea, or the medial OC nucleus.

In contrast, the same amount of toluene injected into the right carotid trunk significantly increased the OC reflex efficiency (Figs. 3C and 4B). For memory, the avgIER values were 2 dB before injection and increased to 3 dB during the first 20 s of injection. Based on our previous findings (Venet et al., 2011), we immediately suspected an effect on the central nuclei involved in the contralateral sound-evoked middle ear reflex, the perifacial and medial OC nuclei. This was confirmed by destruction of the middle ear muscles in both sides, which eliminated the increase in avgIER values (Fig. 4C). Thus, the results reported in Figure 3C can be explained by inhibition of the protection offered by the contralateral middle ear reflex, allowing penetration of a higher level of acoustic energy into the right cochlea. The OC reflex is bilateral, and its effects on 2f1-f2 distortion product otoacoustic emissions are intensity dependent. Therefore, the increase in amplitude measured in the ipsilateral ear was due only to an increase in contralateral noise penetrating into the cochlea.

Inner ear reflex resistance to toluene can be explained by differential wiring of the efferent neurons involved in the inner ear and middle ear reflexes if the medial olivary nuclei are involved in both reflexes. First of all, the medial OC system consists of a crossed (COCB) and an uncrossed (UOCB) component (Warr et al., 1986). Existing evidence suggests that the COCB responds best to ipsilateral acoustic stimulation,
whereas UOCB responds best to contralateral acoustic stimulations (Liberman and Brown, 1986). Our toxicological data show that, in anesthetized rat, the COCB and UOCB components of the medial olivary complex system can affect the inner ear reflex to equivalent extents. One of the two components is sufficient to activate “normal” behavior of the inner ear acoustic reflex. In the experiments described here, either the COCB or the UOCB was preserved and was sufficient to ensure an appropriate inner ear reflex. In other terms, the intoxication pathway used in this study did not allow inhibition of the neuronal circuits of the inner ear reflex. Logically, toluene injection into both carotids would simultaneously inhibit the COCB and the UOCB, but it is also likely to be lethal and cannot therefore be used to measure acoustic reflexes. Consequently, it is necessary to develop and test novel injection pathway, which would allow simultaneous intoxication of both central nuclei. This method could be used to reveal the effects of toluene on the inner ear reflex.

In conclusion, like the middle ear reflex response, that of the inner ear reflex depends on noise intensity, even for amplitudes only varying over a narrow range. However, the central elements involved in the inner ear reflex, like the COCB and the UOCB, are less sensitive to solvent than those involved in the middle ear reflex because of their crossed structures. One single intact OCB would be sufficient to maintain inner ear reflex efficacy. Given the toluene effects on the inner ear reflex, it is clear that the synergistic effects of co-exposure to noise and aromatic solvents such as toluene are mainly because of the solvent depressing the central nuclei driving the middle ear acoustic reflex. Depression of the inner ear reflex, if it occurs, should mainly alter the capacity to discriminate between frequencies.

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