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 and the stapedius muscle was electrocoagulated in one or both middle ears. Rat hearing was evaluated by measuring cubic distortion otocoustic 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 efficacy. 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 exposure in animals (Lataye et al., 2000) and humans (Morata et al., 1994; Sliwinska-Kowalska et al., 2003). However, neither the European directive (2003/10/EC) nor the American noise standards (OSHA) take complex exposures including chemicals into consideration.

Recently, our research team showed that toluene can affect the auditory nervous system involved in ear-protective reflexes, in particular the middle ear reflex. This reflex arc is elicited by sound-evoked efferent feedback and provokes contraction of the stapedius and tensor tympani muscles, reducing the acoustic energy penetrating the cochlea. Our previous results showed 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 constituted by efferent neurons originates 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-lès-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 was 22 ± 2°C and 55 ± 10%, respectively. The animal facilities have full accreditation, and experiments were performed according to 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 intracarotid catheter. To minimize stress in animals, levomepromazine (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 initial anesthetic dose (i.e., 5 mg/kg ketamine and 0.75 mg/kg xylazine), were given when necessary. Anesthesia was maintained by injection of a mixed anesthetic containing ketamine (50 mg/kg) and levomepromazine (1.25 mg/kg) 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 Etymotic Research ER4 B earphone. DPOAEs were measured prior to and during delivery of the NS.

Catheter Implantation and Solvent Injections. A ventral 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 heparin (50 U/ml) was inserted into the 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 soy 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 mM 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<sub>noNS</sub> − DPOAE<sub>NS</sub>.

FIG. 1. Study schedule. Hearing tests were carried out with Long-Evans rats using 2f1-f2 DPOAEs. Primaries: f1 = 8 kHz at 65 dB SPL and f2 = 9.6 kHz at 60 dB; f1/f2 = 1.2. The contralateral NS was an 800-Hz BN 4 kHz. The scales for the three different time lines are different.
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: $F(df_b, df_i) = F$-ratio; $p = p$ value, in which $df_b$ is the number of degrees of freedom between groups and $df_i$ 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 analyses were 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 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 0mM (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 116mM toluene did not significantly change the average amplitude of the inner ear reflex (Bonferroni test before vs. during injection [0.014 ± 0.218]) (Fig. 3B).

| TABLE 1 | $f_1$-$f_2$ Amplitudes as a Function of Level of Sound Pressure Delivered to the Contralateral Ear. Noise Spectrum: BN 4 kHz; Burst Duration: 2.5 s |
|---------|---------------------------------------------------------------|
| NS, intensity dB | 90 | 100 | 120 |
| Severed muscles | 0 | 0.5 | 2 |
| Severed muscles | 0 | 0.3 | 1.2 |
| Severed muscles | 0.5 | 0.7 | 1.2 |
| Mean ($n = 4$) | 0.15 | 0.55 | 1.6 |
| SD | 0.15 | 0.15 | 0.15 |
| Coefficient of variation | 158 | 35 | 29 |
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 ± 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 2f1-f2 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 ± 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 f1 = 8 kHz and f2 = 9.6 kHz were emitted at L1 = 65 dB SPL and L2 = 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.
involved in the contralateral sound-evoked middle ear reflex, the immediately suspected an effect on the central nuclei otoacoustic emissions (Fig. 3B). These findings indicate that any of the elements constituting the OC reflex: be it the auditory receptor, the cochlea, or the medial OC nucleus. Carotid trunk significantly increased the OC reflex efficiency of the cubic distortion otoacoustic product measured at 6.4 kHz. 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 stimulation (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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