Human and animal studies have shown that certain aromatic solvents such as toluene can cause hearing loss and can exacerbate the effects of noise. The latter effects might be due to a modification of responses of motoneurons controlling the middle-ear acoustic reflex. In the present investigation, the audition of Long-Evans rats was evaluated by measuring cubic ($f_1 = 8000$ Hz; $f_2 = 9600$ Hz; $f_1/ f_2 = 1.2$) prior to, during, and after activation of the middle-ear acoustic reflex. A noise suppressor was used to modify the amplitude of the $2f_1 – f_2$ distortion otoacoustic emissions. It was delivered either contralaterally (band noise centered at 4 kHz), or ipsilaterally (3.5 kHz sine wave) to test the role played by the central auditory nuclei. This audiometric approach was used to study the physiological efficiency of the middle-ear acoustic reflex during an injection of a bolus of Intralipid (as a vehicle) containing 58.4, 87.4, or 116.2 mM toluene via the carotid artery. The results showed that toluene could either increase or decrease middle-ear acoustic reflex efficiency, depending on the toluene concentration and the ear receiving noise suppressor. A new neuronal circuit of the middle-ear acoustic reflex has been proposed to explain findings obtained in this investigation. Finally, the depressing action of toluene on the central auditory nuclei driving the middle-ear acoustic reflex might explain the synergistic effects of a co-exposure to noise and aromatic solvents.

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

While 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, 2010; Morata and Campo, 2001). 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 take complex exposures including chemicals into consideration.

Recently, our research team showed that toluene can act as an antagonist of neuronal acetylcholine receptors (Bale et al., 2002, 2005; Lataye et al., 2007), and more specifically as a blocker of neuronal voltage-dependant Ca$^{2+}$ channels (Maguin et al., 2009). Because of these effects, toluene might be capable of depressing the auditory nervous system driving the ear-protective reflexes and in particular the middle-ear reflex. The main physiological functions of the middle-ear acoustic reflex are to protect the inner ear from high-intensity noises by reducing the penetration of acoustic energy into the cochlea, and to reduce acoustic masking. To perform its protective role, high-intensity noises presented to either ear cause middle-ear acoustic reflex activation on both ears. This bilateral characteristic is well known, and is used by clinicians to diagnose ear or even facial-nerve pathologies (Prasher et al., 2005; Silman et al., 1988; Wilson and McBride, 1978).

In mammals, the auditory efferent system is a descending centrifugal pathway, with its source in the vicinity of the superior olivary complex (SOC). In the brainstem, this system is divided into two main subsystems: the medial and lateral olivocochlear pathways (White and Warr, 1983). It is composed of crossed and uncrossed neurons constituting the efferent innervations of the organ of Corti. The majority of these neurons terminate on either the outer hair cells (OHCs) or the eight nerve ganglion cells beneath the inner ear cells (Pujol and Lenoir, 1986). While some neurons located outside the SOC are involved in the reflex arc of the middle-ear muscles (Rouiller et al., 1989), most of the motoneurons controlling contraction of the stapedius and tensor tympani muscles are located in the vicinity of the facial or trigeminal nerve nuclei (Lee et al., 2006). Because of the structure of the efferent system, the middle-ear acoustic reflex can be elicited by sound-evoked efferent feedback (Murata et al., 1986). It has been demonstrated that contralateral acoustic stimulation of the efferent olivocochlear pathway is capable of decreasing the activity of auditory-nerve afferent fibers, even in anesthetized animals (Warren and Liberman, 1989). Thus, through stimulation of the facial and trigeminal nerve nuclei involved in the middle-ear acoustic reflex, a contralateral sound may decrease the activity in the opposing cochlea (Campo et al., 2007; Maguin et al., 2009).
Today, a noninvasive approach, distortion product otoacoustic emissions (DPOAEs) can be used to evaluate middle-ear acoustic reflex efficiency and the effects of aromatic solvents on the middle-ear acoustic reflex. DPOAEs are low-level sounds emitted by the cochlea through the middle-ear system, when the auditory receptor is stimulated by two tones: f1 and f2. They can be measured by a sensitive microphone fitted into the outer-ear canal. As a result, they allow measurement of even slight modifications of middle-ear impedance. Among the different types of DPOAEs, cubic (2f1 − f2) DPOAEs can be recorded in anesthetized rats and in humans. They are reliable indicators of outer hair cell (OHC) function (Lim, 1986) and are also very sensitive to changes in middle-ear impendence. Consequently, 2f1 − f2 DPOAEs are highly suitable for studying solvent effects on the middle-ear acoustic reflex. For almost two decades, 2f1 − f2 DPOAEs have been used in humans (Bonfils et al., 1994) and more recently they have been adapted for animal investigations (Pouyatos et al., 2002).

In the present study, DPOAEs were recorded while the middle-ear acoustic reflex was activated by a noise suppressor tone emitted in the ipsilateral (IPS) or contralateral (CONTRA) ear of the rat under investigation. Using this experimental approach, we were capable of evaluating the function of the peripheral auditory system in its entirety (namely: middle ear + inner ear), and completing the results obtained with electrophysiological measurements in previous experiments (Campos et al., 2007; Maguin et al., 2009). The aims of the current project were: firstly, to develop a noninvasive audiometric tool to studying middle-ear reflex physiology; second, to confirm the depressant effects of toluene on motoneurons; and thirdly, to better understand the physiological meaning of the N-component identified in our previous studies (Campos et al., 2007; Maguin et al., 2009). Determining the anatomical relationship between the afferent and efferent pathways became of special interest with regard to the findings found from these series of toxicological investigations. In addition to DPOAE measurements, cochlear morphological studies were used to evaluate the role played by the auditory receptor relative to that played by the auditory nervous centers located in the brainstem. Results obtained over the course of the study led us to consider the relationship between the afferent and efferent pathways in more details. These considerations allow us to formulate a hypothesis as to how the input from the various nerve centers is integrated to generate an appropriate middle-ear acoustic reflex.

MATERIAL AND METHODS

Animals

Adult Long-Evans rats weighing over 350 g were used in the present study (n = 21). The route taken by the different bolus injections from the carotid trunk up to the brainstem was traced in three additional animals. Animals were purchased from Charles River breeder (Saint Aubin-les-Elbeuf, France) 2 weeks before the start of the experiments. They were housed in individual cages (350 × 180 × 184 mm) with irradiated pinewood bedding (supplier: Special Diets Services, France; ref. Gold kob 891180). Food and tap water were available ad libitum. The animals were on a normal day/night cycle: 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 while conducting the research described in this article, the investigators adhered 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. Levomepromazine (12.5 mg/kg) was given to animals by ip injection 15 min prior to the measurements. The aim of this premedication was to minimize stress in the subjects. Deep anesthesia was induced by injection of a mixture of ketamine (50 mg/kg) and xylazine (6 mg/kg). Supplemental doses of anesthesia of 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 at 38°C. Otoscopic examination was carried out to verify that the tympanic membranes were exempt from any obstruction, infection, or other abnormalities before recording DPOAEs.

In complementary experiments, three animals were used to identify the tissue targeted within the brainstem. These experiments were carried out on animals anesthetized with a high dose of ketamine (75 mg/kg).

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 SPL, respectively, and delivered to the ipsilateral (left) ear. The level of the highest intensity sound was limited to 65 dB SPL so as not to activate the middle-ear acoustic reflex. The ratio of f1/f2 was 1.2, which is suitable both for rats (Henley et al., 1989) and humans (Gaskill 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). These elicitor sounds were considered as the ipsilateral (IPS) acoustic stimulation in our protocol, as illustrated in Figure 1. The frequencies of the primary tones were chosen to yield what is commonly referred to as cubic difference tones: 2f1 − f2.

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

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

Contralateral and Ipsilateral Acoustic Stimulations

Contralateral. The contralateral noise (noise suppressor) was an 800-Hz band noise centered at 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. Noise suppressor intensity for contralateral experiments was 100 dB SPL. The signal

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was synthesized by a B&K Pulse 3109 and emitted by an Etymotic Research ER4 B earphone. DPOAEs were measured prior to and during the delivery of the noise suppressor.

**Ipsilateral.** The ipsilateral noise suppressor was a 3.5-kHz sine wave. Noise suppressor intensity was lower (75 dB SPL) than that used in the contralateral conditions so as not to disturb the $2f_1 - f_2$ DPOAEs measured in the same ear. Each tone burst lasted 2.5 s and was followed by a 9.5-s silent window before the next tone burst (Fig. 1). The signal was synthesized by a B&K Pulse 3110 and emitted by one of the transducers included in the probe. In fact, $f_1$ and $f_2$ were generated by one transducer and the noise suppressor tone by the other one. DPOAEs were measured prior to and during the delivery of the noise suppressor.

**Catheter Implantation and Solvent Injections**

A ventrolateral incision was made in the neck to expose the left carotid artery. The external carotid artery was ligated to drive the bolus toward the brainstem. A circular custom-made catheter (Teflon in polypropylene i.d. = 1.6 mm extended with silicon catheters i.d. = 0.635 mm) filled with a solution of NaCl 0.9% and heparin (50 IU/ml) was fitted into the common carotid trunk. This type of catheter allows normal blood flow to be maintained and the vehicle containing the solvent to be injected. An illustration of the catheter is available in Lataye et al. (2007).

Once inserted, the circular catheter was filled with vehicle, consisting of a 10% fatty emulsion of purified soya oil and essentials fatty acids (Intralipid Ref: 830513161; Fresenius Kabi company). All injections were performed with a syringe pump calibrated to deliver a $266 \mu l$ bolus over 80 s (Fig. 1). Three concentrations of toluene (Prolabo, 20675294) were tested in this investigation: 58.4 mM ($n = 4$), 87.4 mM ($n = 5$) and 116.2 mM ($n = 5$). The 0 mM ($n = 4$) concentration corresponded to an injection of vehicle alone.

It is important to bear in mind that all concentrations are given as the concentrations inside the syringe, not to the effective concentrations reaching the brain and/or cochlea. Each animal was tested with a single concentration.

**Histology**

**Evaluation of the Organ of Corti**

Histological analysis of the cochleae was carried out to evaluate the physiological impact of the solvent and how it related to the DPOAE measurements. The succinate dehydrogenase (SDH) activity of the organ of Corti was estimated by evaluating SDH staining density in hair cells of the left cochlea (injection and ipsilateral side) using a colorimetric assay using nitroblue tetrazolium (NBT) monosodium salt. Stock solutions (0.1% NBT, 0.2M sodium succinate and 0.2M phosphate buffer at pH 7.4) were prepared and stored at 4°C. Working solutions were always freshly prepared by mixing the stock solutions NBT, sodium succinate, and phosphate buffer in a 2:1:1 ratio. At the end of the hearing test, animals were anesthetized with an overdose of ketamine and sacrificed. The cochleae were quickly removed and perfused through the round window with the SDH staining solution. The excess of staining solution was allowed to drain out through the oval window. The cochleae were then incubated for 1 h at 37°C.

After SDH staining, the cochleae were perfused with 4% glutaraldehyde in 10mM phosphate-buffered saline at pH 7.5 and incubated overnight. After fixation, the cochleae were drilled and dissected. The bony wall and spiral ligament were removed before trisecting and examining the organ of Corti.

**Tracing Study**

To delineate the tissue targeted by the bolus of Intralipid containing the solvent, the tracer (OsO$_4$) was injected slowly into the carotid with a microsyringe. Before injecting the tracer, three rats were perfused with a mixture of saline solution and heparin. The tracing study was carried out on dead animals. After tracer injection, the brain was removed and frozen (−60°C). Frozen 20-μm-thick sections were cut in the frontal plane through the relevant segment of the brainstem; −10.04 mm from bregma point.

**Data Recording**

The $2f_1 - f_2$ DPOAE changes measured with and without noise suppressor (NS and noNS) are illustrated in Figure 2. The middle-ear acoustic reflex metric followed the decrease in DPOAE amplitude, and could be modeled as follows:

$$\text{MIDDLE EAR ACOUSTIC REFLEX} = \frac{DPOAE_{\text{noNS}} - DPOAE_{\text{NS}}}{C_0}.$$ 

In Figure 2, the gray squares correspond to middle-ear acoustic reflex efficiency upon noise suppressor tone emission.

In order to avoid the edge effects, only the four most central DPOAE measurements (open triangles) were taken into account. Transition values were excluded from the calculation (crosses in Fig. 2).

**FIG. 2.** Decrease in DPOAE amplitude provoked by the protective reflexes. Each noise suppressor (NS) tone lasted 2.5 s and was emitted at 100 dB SPL. NS was emitted in the contralateral ear. Left y-axis: DPOAE amplitude. Right y-axis: DPOAE NS-induced variation. Open circle: DPOAE measured for 500 ms without NS. Black circle: mean of 18 DPOAE measured in the absence of NS. Open triangle: DPOAE measured for 300 ms during NS burst emission. Black triangle: mean of four measurements performed during NS burst emission. Cross: DPOAE measurements excluded from the calculation. Gray squares correspond to the reflex amplitude: difference between the mean of the two black circles and the black triangle for each series of measurements.
Prior to injection, four middle-ear acoustic reflex values were recorded; they are plotted in Figures 3 and 4. The average magnitude of the middle-ear acoustic reflex (avgMER) was calculated for all animals using the four values recorded prior to injection.

During solvent injection, the increase in middle-ear acoustic reflex amplitude was called the N-component, or “negative” component. This corresponds to a decrease in the acoustic energy penetrating into the cochlea as middle-ear acoustic reflex amplitude increases. In contrast, the decrease in middle-ear acoustic reflex amplitude was called the P, or “positive” component, corresponding to an increase in the acoustic energy penetrating into the cochlea. Both components were differences calculated using the middle-ear acoustic reflex values obtained during the injection and the avgMER, obtained before injection.

Statistics
Statgraphics Centurion XV software was used to perform all statistical analyses. A one-way ANOVA was run to test the significance of the 2f1 − f2 variations as a function of the 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 analysis was performed using Bonferroni method. The 95% confidence interval was considered to evaluate the significance of statistical tests.

RESULTS
Toluene Effects on the Contraction of the Muscles in the Middle-ear
Contralateral Acoustic Stimulation
When measuring solvent effect in the contralateral ear, the 2f1 − f2 DPOAE amplitudes were approximately 30 dB SPL (Figs. 3A and 3B). Prior to injection, the avgMER values were 12 dB (30–18) for animals receiving 58mM toluene (Fig. 3A) and 16 (30–14) for animals receiving 116mM toluene (Fig. 3B). Injection of toluene at 58mM elicited no P-component, and only one N-component, whose amplitude was approximately 8 dB (Fig. 3A). The amplitude of the N-component was increased for the first 30 s of the injection, it subsequently remained constant up to the end of the injection of interest.

Injection of toluene at 116mM provoked one N-component (3 dB) followed by one P-component (11 dB) (Fig. 3B). Thus, at this concentration, the toluene injection induced two successive components: first, the N-component, then the P-component. It is therefore clear that the concentration of solvent injected directly affects middle-ear acoustic reflex efficiency.

While the N-component lasted 30 s, as shown in Figure 3A, the P-component interacted with the N-component from about 20 s. As a result, the N-component was masked by the P-component at the 116mM concentration. Amplitude of the P-component peaked at the end of the injection period.

The changes of N-component amplitude as a function of toluene concentration are reported in Table 1. There was

![FIG. 3. Variations of 2f1 − f2 DPOAE amplitude and middle-ear acoustic reflex efficiency prior to, during, and after toluene injection (A) 58mM; (B) 116mM toluene injection. A 266 μL bolus of vehicle containing appropriate concentrations of toluene was injected over 80 s (gray triangles). Primary tones were delivered in the ipsilateral ear: f1 = 8000 Hz and f2 = 9600 Hz at L1 = 65dB SPL and at L2 = 60 dB SPL, respectively. Frequency ratio was 1.2. The central noise suppressor was an 800-Hz band noise centered at 4 kHz and emitted at 100 dB SPL. N-component: decrease of the acoustic energy entering the cochlea. P-component: increase of the acoustic energy entering the cochlea. This single representation is representative of the group (n = 5).](https://academic.oup.com/toxsci/article/119/1/146/1636472)
a significant \[F(3,14) = 3.4; p = 0.048\] difference between the amplitudes obtained at 0 and 58mM. At higher concentrations (87 and 116mM) the N-component amplitudes were masked by the presence of the P-component.

An Ipsilateral Acoustic Stimulation

When measuring solvent effect on the ipsilateral ear, injection of toluene at 58mM provoked no N-component and only one P-component (Fig. 4A). In contrast, 116mM toluene provoked two successive components having a different time-course from that shown in Figure 3B: first, P-component, then, N-component. Thus, depending on the side to which the noise suppressor tone was applied, opposing effects of toluene injections were observed.

The P-component amplitude obtained in the ipsilateral ear was greater than 1 dB \[F(2,14) = 4.98; p = 0.03\] (Fig. 4A). The difference in amplitude between animals receiving no solvent (0mM) and those treated with 58mM toluene was found to be significant by Bonferroni post hoc analysis. The amplitude of the N-component was approximately 2 dB (Table 2). Lower amplitudes were expected in this series of experiments because of the noise suppressor intensities used (100 dB contra vs. 75 dB IPSI). The significant reduction of noise suppressor intensity was required in the ipsilateral side so as not to skew the \(2f_2/C_0\) DPOAEs measurements performed in the same ear.

The N-component amplitude obtained with 116mM toluene (Fig. 4B) was greater than 2.5 dB \(F(2,14) = 20.57; p < 0.001\). Bonferroni post hoc analysis shows significant differences between 116 and [0 or 58mM] (Table 2).

Histology

Cochleae

Cochleae from a representative control subject and a subject having received a 266-\(\mu\)L bolus of Intralipid containing 116mM of toluene were stained for SDH throughout the entire cochlea (Fig. 5). During acoustic stimulation, the toluene-exposed subject showed a clear inhibition of the middle-ear acoustic reflex during the injection; however, histological

<table>
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<th>N</th>
<th>Mean [dB]</th>
<th>SD</th>
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<td>N</td>
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<td>1.6</td>
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Note. Significant differences between homogeneous groups are indicated with a X in bold.
analysis of a control and toluene-exposed organs of Corti revealed no distinct effect of the solvent (Figs. 5A and 5B). Closer examination, using higher magnifications, also showed an absence of cochlear impairments, regardless of the turn examined. Thus, no obvious differences in SDH staining density were observed in apical (Figs. 5C and 5F), medial (Figs. 5D and 5G), or basal turns (Figs. 5E and 5H).

### Brainstem

To determine the route taken by the solvent injected into the ipsilateral carotid artery, a tracer (OsO₄) was used. The tracer was injected into the same artery in controls, and its route of travel to the brainstem observed. OsO₄ injected into the ipsilateral carotid colored the route taken by the solvent on its way to the brainstem. The basilar artery was well delineated at the level of the cerebellar trunk (Fig. 6A). The amount of tracer reaching the ipsilateral brain was greater than that reaching the contralateral brain (Fig. 6B). The two frozen slices show that the basal part of the cerebellar trunk is reached by the tracer (Figs. 6C and 6D). This means that the tracer, and thus presumably the solvent, has reached the SOC in our experimental conditions.

**FIG. 5.** Light micrographs showing a low power view (G × 35) of left cochleae after injection of a 266-μL bolus of (A) Intralip containing toluene (116mM); (B) Intralip alone. The OHC were colored with NBT monosodium salt to evaluate SDH activity. Higher magnifications (G × BRIEGER) of the organ of Corti show the three rows of OHCs in the (C/F) apical, (D/G) middle and (E/H) basal turns.

### Table 2

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DISCUSSION

As expected from previous experiments (Campo et al., 2007; Lataye et al., 2007), a bolus of 116mM toluene injected into the left carotid trunk provoked a biphasic effect regardless of the side of acoustic stimulation. The biphasic effect observed at 116mM could be due to a difference of toluene concentration reaching the targeted tissue. The time-course of the variations of the MER amplitudes in Figure 3D, before and after the P-component, suggests a smooth transition from low-concentration to high-concentration effect.

In a previous publication (Lataye et al., 2007), we succeeded in identifying the nature of the P-component observed during solvent injection, it corresponded to an inhibition of the middle-ear acoustic contraction. With this new experimental approach, we know now that the N-component observed during solvent injection corresponds to an unexpected increase in middle-ear acoustic reflex efficiency. Surprisingly, a low concentration of toluene (58mM) can induce a stronger-contraction of the middle-ear muscles than that observed in physiological (no toluene) conditions (Fig. 3B). Thus, depending on its concentration, toluene can act on the auditory nervous centers to increase or decrease middle-ear acoustic reflex efficiency. However, that does not mean that toluene can have either exciting or inhibiting action on the same targeted tissue as a function of its concentration. Indeed, for similar toluene concentrations, opposing effects on middle-ear acoustic reflex were obtained depending on the side into which the noise suppressor was delivered (IPSI vs. CONTRO). The same toluene concentration cannot play an exciting or inhibiting role on the same targeted tissue. So, the toluene concentration cannot simply explain the findings reported in the present publication. It is clear that the side into which the noise suppressor is delivered appears to be a key parameter in determining the order of N- and P-components, either N then P (Fig. 3D), or P then N (Fig. 4C).

From our previous series of experiments, we knew that toluene could act on the central nervous system (Maguin et al., 2009) although, based on these results, we could not completely discard a cochlear effect. In the present investigation, histological analysis confirmed that the solvent injection did not dramatically affect the cochlea. Indeed, no difference in physiological state was found between cochleae harvested from toluene-exposed rats and those from controls (Fig. 5). Moreover, the fact that no variation of DPOAE$_{\text{noNS}}$ amplitudes can be observed during or after injection proves that cochlea does not seem to be disturbed by toluene in these particular experimental conditions.

Only a disturbance at the central level could explain the toxicological results obtained in the present series of experiments.
A disturbance of the cerebellar trunk was suspected to be at the origin of this phenomenon. A tracer compound was used to determine the path taken by the solvent to reach the brainstem. This was found to be via the basilar and pontine arteries (Fig. 6A). Figures 6C and 6D shows the location of perifacial and medial olivary nuclei in the brainstem. Those nuclei play a major role in the auditory protective reflexes. As shown in the schema illustrated in Figure 7, the middle-ear acoustic reflex is controlled by a feedback loop wired with afferent and efferent auditory neurons. Most motoneurons are spatially distributed over perifacial nuclei (Lee et al., 2006). While the role played by the motoneurons is clear, the physiological role played by the afferent neurons and the interneurons connected to auditory nuclei are still questioned.

The toxicological data obtained through the study described here can explain, at least partly, the key role played by the interneurons linking the nuclei to the cerebellar truck.

Basically the dorsal and ventral cochlear nuclei (CNv; CNv) and the SOC are all involved in the middle-ear acoustic reflex (Borg, 1973; Lee et al., 2006; Rouiller et al., 1989). In our opinion, the interneurons and the neurons from auditory nuclei are capable of interpreting different afferent spikes (I, Figs. 8 and 9), coming from both ears to generate single integrated response (O, Figs. 8 and 9) eliciting the middle-ear acoustic reflex. That means that through the interneurons, the auditory nuclei can adjust the middle-ear acoustic reflex response as a function of the intensities recorded in both ears (Figs. 8 and 9).

We have illustrated this theory in Figures 8 and 9 in which CONTRA stimulation elicits responses from central nuclei in the brainstem directly through the periolivary nucleus (SOC) or indirectly through the facial nucleus (VII). The auditory messages are coded with spikes traveling on crossed and uncrossed afferent fibers originating from ventral and dorsal cochlear nuclei (CNv and CNv). According to this model, the central nuclei have to compute at least three pieces of information in order to determine what sort of middle-ear acoustic reflex response to elicit.

To make the explanations as clear as possible, we will now provide a simple example, to describe what happened to the middle-ear acoustic reflex during solvent injection via the left carotid trunk. The CONTRA acoustic stimulation emitted at 100 dB SPL generated more numerous spikes (I12, Fig. 8) than those generated by the primary tones (I4) whose intensity was 65 dB SPL. Thus, in physiological conditions, the SOC integrates spikes coming from all ventral pathways (uncrossed I4 and crossed spikes I12) to give a balanced response [(I12 + I4)/2 = O6]. Then, the facial nucleus computes the SOC response (I8) with spikes (I4) coming from CND to give a balanced response [(I12 + I4)/2 = O6]. Finally, the output signal (I8 + I4 = O6, output signal 6) eliciting the contraction of the middle-ear muscles through efferent limbs takes all of the acoustic messages coming from both ears into consideration.

Considering O6 as the integrated output signal eliciting the middle-ear acoustic reflex, the toxicological data reported in
this article show that toluene can disturb one or two integration levels depending on its concentration (Fig. 8).

Thus, a bolus containing 58mM toluene disturbs only the first level of the cerebellar integration center: the spikes coming from the CN_{V(IPS)} (Fig. 8). Consequently, the periolivary region receives only spikes coming from the CN_{V(CONTRA)} [I12], and the facial nucleus receives I12 and spikes coming from the CN_{D} regions (I4). As a result, injection of 58mM toluene results in an output signal rated O8. This high value explains why a low-concentration of toluene increases the amplitude of middle-ear acoustic reflex contraction over that observed in the absence of toluene with the same acoustic parameter (O8 > O6; Fig. 3A).

In contrast, a bolus containing 116mM toluene disturbs the SOC in its entirety (Fig. 8, 4th line), leading to an output signal rated O4 (O4 < O6) This explains why the amplitude of middle-ear acoustic reflex contraction was lower at this concentration (Fig. 3B).

As illustrated in Figure 7B, acoustic stimulations emitted on the IPSI side also elicit responses from auditory cerebellar regions. But, in these conditions, the totality of the spikes provoked comes from uncrossed afferents sources (Fig. 8, right column) originating in CN_{IPSI} and CN_{IPSI}. During this time, the CONTRA ear is only stimulated by the background noise. Consequently, we can class the spikes in two categories: I2 coming from the quiet side and I6 coming from the noisy side (Figs. 7B and 8). As previously, we will take a simple example.

Under physiological conditions, the crossed spikes are scarce (I2) because there is no acoustic stimulation on the IPSI side. On the contrary, spikes coming from the CN_{V(IPS)} and CN_{D(IPS)} are numerous: I6. Like in the previous example, the auditory nervous centers deal with spikes at two levels: first, in the vicinity of the SOC leading to spikes rated I4; second, at the facial nucleus generating an output signal rated O5.

A bolus containing 58mM toluene disturbs only the auditory spikes coming from the CN_{IPSI} (Figs. 7B and 8). As a result, the periolivary region transfers I2 spikes to the facial nucleus, resulting in an output signal rated O4. Because O4 < O5, toluene at 58mM decreases the amplitude of the contraction induced by the middle-ear acoustic reflex (Fig. 4A). On the other hand, a bolus containing 116mM toluene disturbs the SOC as a whole, leading to an output signal O6. Because O6 > O5, toluene at 116mM increases the amplitude of the contraction induced by the middle-ear acoustic reflex (Fig. 4B). Consequently, O6 depends only on I6 spikes, which results from CN_{D} IPSI through the facial nucleus.

To conclude, the results obtained in these investigations can be explained by the presence of interneurons between the facial nucleus and the SOC, as suspected by Borg (1973) and Lee et al. (2006). They could analyze the intensity of the noise coming from both ears to adapt the middle-ear acoustic reflex response. Therefore, the neuronal circuits involved in the middle-ear acoustic reflex are more complex than those previously described. A possible organization of the neuronal circuits has been proposed in the present paper. It should help the interpretation of clinical tests hampered by incomplete physiological and anatomical knowledge of the central routes involved in reflex activation for the middle-ear acoustic reflex. Finally, these findings show that toluene can depress the auditory nervous system driving the middle-ear acoustic reflex. That might explain, at least partly, the synergistic effects of a co-exposure to noise and solvent (Lataye and Campo, 1997).

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**REFERENCES**


