Inhalational Exposure to Carbonyl Sulfide Produces Altered Brainstem Auditory and Somatosensory-Evoked Potentials in Fischer 344N Rats

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Carbonyl sulfide (COS), a chemical listed by the original Clean Air Act, was tested for neurotoxicity by a National Institute of Environmental Health Sciences/National Toxicology Program and U.S. Environmental Protection Agency collaborative investigation. Previous studies demonstrated that COS produced cortical and brainstem lesions and altered auditory neurophysiological responses to click stimuli. This paper reports the results of expanded neurophysiological examinations that were an integral part of the previously published experiments (Morgan et al., 2004, Toxicol. Appl. Pharmacol. 200, 131–145; Sills et al., 2004, Toxicol. Pathol. 32, 1–10). Fisher 334N rats were exposed to 0, 200, 300, or 400 ppm COS for 6 h/day, 5 days/week for 12 weeks, or to 0, 300, or 400 ppm COS for 2 weeks using whole-body inhalation chambers. After treatment, the animals were studied using neurophysiological tests to examine: peripheral nerve function, somatosensory-evoked potentials (SEPs) (tail/hindlimb and facial cortical regions), brainstem auditory-evoked responses (BAERs), and visual flash–evoked potentials (2-week study). Additionally, the animals exposed for 2 weeks were examined using a functional observational battery (FOB) and response modification audiometry (RMA). Peripheral nerve function was not altered for any exposure scenario. Likewise, amplitudes of SEPs recorded from the cerebellum were not altered by treatment with COS. In contrast, amplitudes and latencies of SEPs recorded from cortical areas were altered after 12-week exposure to 400 ppm COS. The SEP waveforms were changed to a greater extent after forelimb stimulation than tail stimulation in the 2-week study. The most consistent findings were decreased amplitudes of BAER peaks associated with brainstem regions after exposure to 400 ppm COS. Additional BAER peaks were affected after 12 weeks, compared to 2 weeks of treatment, indicating that additional regions of the brainstem were damaged with longer exposures. The changes in BAERs were observed in the absence of altered auditory responsiveness in FOB or RMA. This series of experiments demonstrates that COS produces changes in brainstem auditory and cortical somatosensory neurophysiological responses that correlate with previously described histopathological damage.

Key Words: carbonyl sulfide; evoked potentials; BAER; SEP; CNAP; NCV.

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COS was listed in the Clean Air Act of 1990 as a Hazardous Air Pollutant. In 1999, COS was nominated by the USEPA to the National Toxicology Program for additional toxicological investigation, including neurotoxicity.

The potential neurotoxicity of COS is of concern. COS is a metabolite of the known neurotoxicant, carbon disulfide (CS₂) (Beauchamp et al., 1983; Bus, 1985; Dalvi et al., 1975). Evidence of neurotoxicity of COS itself has also been reported. Acute (4 h) inhalation of 804–1189 ppm COS by Sprague-Dawley rats produced neurological signs such as hypoactivity, tremors, and convulsions at 1062 ppm or greater (Monsanto Agricultural Company, 1985). A subsequent study used nose-only exposure of Fischer 344 rats for up to 4 h to 250–1400 ppm COS (Nutt et al., 1996). Animals surviving > 500 ppm COS were reported to have a motor impairment, and histopathology showed swelling of myelin sheaths in the cerebellum, corpus callosum, and pyramidal tracts. Exposure of Fischer 344 rats to up to 500 ppm COS for 2 weeks produced brain lesions in the frontoparietal cortex, putamen, thalamus, red nucleus, and auditory structures (posterior colliculus and anterior olivary nucleus) (Morgan et al., 2004). When exposed to 400 ppm COS for 12 weeks, the most consistent lesions were found in the posterior colliculus and parietal cortex (Morgan et al., 2004). These lesions were also identified in a parallel study using magnetic resonance microscopy (MRM) (Sills et al., 2004). Additionally, a 2-week satellite study of the longer term 12-week investigation found decreased amplitudes of brain-stem auditory-evoked responses (BAERs) in male animals exposed to 400 ppm COS (Morgan et al., 2004).

The current work provided further characterization of the neurophysiological changes following 12 weeks of inhalational exposure to COS (Study 1). These animals were exposed as a subset of the previously reported study (Morgan et al., 2004). A battery of neurophysiological measures was used to study peripheral nerve function, somatosensory system responses, and BAERs in animals exposed to COS at concentrations up to 400 ppm. This experiment extended the previously limited study of auditory effects found after 2-week exposure to COS (Morgan et al., 2004) and included a neurophysiological examination of peripheral nerves and the somatosensory system. The neurophysiological assessment complemented the previously reported results from a functional observational battery (FOB) conducted at 6 and 12 weeks of COS exposure (Morgan et al., 2004). The somatosensory end points included as lesions in the region of the somatosensory cortex were found at the conclusion of the 2-, 4-, and 8-week substudies of the 12-week exposure (Morgan et al., 2004; Sills et al., 2004). These techniques allowed examination if neurophysiological changes could be detected in the absence of histopathological damage, and whether changes in peripheral nerves contributed to altered responses recorded from the somatosensory cortex. A second study (Study 2) was performed after the conclusion of the 12-week experiment to replicate the neurophysiological auditory effects previously found after 2 weeks of COS exposure, and extend the investigation by including behavioral measures (especially tests of auditory function), allowing comparison of the results in the same rats. A FOB was included to provide a general profile of neuro-behavioral status, and response modification audiometry (RMA) was used to assess the ability of a prepulse stimulus to inhibit the behavioral response to a suprathreshold auditory stimulus. Additionally, the specificity of the auditory effects produced by 2 weeks of COS exposure was assessed using a neurophysiological measure of visual system function.

MATERIALS AND METHODS

Study 1

Animals, Exposure, and Surgery

The animals and exposure conditions used in these studies have been previously described in detail (Morgan et al., 2004; Sills et al., 2004). Briefly, male and female Fischer 344 rats (Charles River Laboratories, Raleigh, NC) were obtained at 6–7 weeks of age. After confirmation of absence of disease and acclimation to the exposure chambers, treatment was initiated at 8–9 weeks of age. Exposure to COS occurred in Hazleton 2000 inhalation chambers. These experiments were conducted under Federal guidelines for the use and care of laboratory animals and were approved by the NICHS and USEPA Animal Care and Use Committees which require compliance with National Institute of Health guidelines.

Animals were exposed to 0 (conditioned air), 200, 300, or 400 ppm COS for 6 h/day (approximately 0700 h–1300 h), 5 days/week (weekends excluded) for 12 weeks. COS (CAS# 463-58-1) was purchased from Tex-La Gases (Houston, TX), with chemical purity determined to be > 98.1% (Morgan et al., 2004). The rats used in Study 1 were exposed in parallel with a previously described experiment (Morgan et al., 2004). Concentrations of COS were chosen based on previous range-finding studies that indicated that 400 ppm COS would be expected to produce brain lesions with minimal acute toxicity. Other groups were included to determine if effects could be observed at lower concentrations with longer (12-week) exposure durations (Morgan et al., 2004; Sills et al., 2004). Immediately following the last exposure, 64 male and 64 female animals (16 per concentration) were transferred from NIEHS to the USEPA. Upon arrival, the rats were housed singly in animal colonies with a 12-h light-dark cycle (lights on 0600 h; 22 ± 2°C; 40 ± 20% relative humidity) with food (#5001, Purina Lab Chow, St Louis, MO) and tap water provided ad libitum.

At 34–40 days following the last exposure to COS, animals were surgically implanted with epidural screw electrodes using previously described methods (Herr and Boyes, 1997; Herr et al., 1992, 1994, 2004). The electrode locations are listed in Table 1 (and Herr et al., 2004) and were located to record from the cortical S1 hindlimb/tail region (SEP1cortex), the cortical S1 facial region cortex (SEP2cortex), and over the cerebellum (BAERs and cerebellar somatosensory-evoked potentials [SEPcerebellum]). Several animals were eliminated from this study due to surgical complications, which were not related to COS exposure. The group sizes for the male rats were as follows: 0 ppm = 12, 200 ppm = 16, 300 ppm = 15, and 400 ppm = 16. The group sizes for the female rats were as follows: 0 ppm = 13, 200 ppm = 14, 300 ppm = 12, and 400 ppm = 12. The animals were allowed approximately 1 week to recover prior to testing.

Neurophysiological Testing

Animals were transferred to the laboratory and allowed to acclimate for at least 15 min prior to initiating testing. Unanesthetized rats were restrained and placed in a testing apparatus as previously described (Hamm et al., 2000; Herr et al., 1996a, 1998, 2004). Stainless steel syringe needles (25-gauge) were used for stimulation of the ventral caudal tail nerves and recording the compound nerve action potential (CNAP). The electrodes, temperature probe, and thermometer were located as previously described (Table 1 and Herr et al.,...
TABLE 1  
Stimulus and Recording Parameters  

<table>
<thead>
<tr>
<th>Evoked potential</th>
<th>Stimulus</th>
<th>Stimulus rate (Hz)</th>
<th>Filter bandpass (Hz)</th>
<th>Collection: baseline + data (ms)</th>
<th>ADC rate (kHz)</th>
<th>Trials</th>
<th>Active electrode</th>
<th>Reference electrode</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAER</td>
<td>Click and tone pips</td>
<td>5.56</td>
<td>0.1–20,000</td>
<td>1.5 + 14.99</td>
<td>81.967</td>
<td>500</td>
<td>3 mm PL, 0 mm LL</td>
<td>7 mm AB, 2 mm LL</td>
</tr>
<tr>
<td>SEP&lt;sub&gt;1&lt;/sub&gt;cortex</td>
<td>Electrical</td>
<td>0.91&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.1–10,000</td>
<td>25 + 250</td>
<td>40</td>
<td>100</td>
<td>2 mm PB, 2 mm LL</td>
<td>7 mm AB, 2 mm LL</td>
</tr>
<tr>
<td>SEP&lt;sub&gt;2&lt;/sub&gt;cortex</td>
<td>Electrical</td>
<td>0.91</td>
<td>0.1–10,000</td>
<td>25 + 250</td>
<td>40</td>
<td>100</td>
<td>0 mm PB, 6 mm LL</td>
<td>7 mm AB, 2 mm LL</td>
</tr>
<tr>
<td>SEP&lt;sub&gt;cerebellum&lt;/sub&gt;</td>
<td>Electrical</td>
<td>0.91</td>
<td>0.1–10,000</td>
<td>25 + 250</td>
<td>40</td>
<td>100</td>
<td>0 mm PL, 0 mm LL</td>
<td>7 mm AB, 2 mm LL</td>
</tr>
<tr>
<td>CNAP</td>
<td>Electrical</td>
<td>0.91</td>
<td>0.1–1,000</td>
<td>0.95 + 10.05</td>
<td>40</td>
<td>100</td>
<td>4 mm PH</td>
<td>1 mm PH</td>
</tr>
<tr>
<td>FEP</td>
<td>Flash</td>
<td>0.32</td>
<td>0.1–1,000</td>
<td>50 + 500</td>
<td>4</td>
<td>75</td>
<td>1 mm AL, 4 mm LL</td>
<td>2 mm AB, 2 mm LL</td>
</tr>
</tbody>
</table>

<sup>a</sup><sup>/<sup>b</sup>d</sup> = 0.5, 1.0, 2.0, and 3.0 mA (each polarity) and were presented in a randomized order between animals. Generation and calibration of the stimuli have been previously described. All signals were amplified 10,000<sup>x</sup>, averaged, and analyzed using the evoked potential system previously described (Hamm et al., 2000). All evoked potentials were recorded during a single test session in the following order: CNAP, SEP<sub>1</sub>cortex, SEP<sub>2</sub>cortex, SEP<sub>cerebellum</sub>, nerve conduction velocity (NCV), and BAER. The total test session was approximately 35 min. Multiple stimulus intensities were used in order to produce intensity-response functions for the evoked potentials. This procedure is recommended in EPA test guidelines (USEPA, 1998), and demonstrates that the evoked potentials are under stimulus control and that recording conditions are adequate to quantify changes in the biological responses of the magnitude produced by the different stimulus intensities.

**CNAP, NCV, SEP<sub>cerebellum</sub>, SEP<sub>1</sub>cortex, and SEP<sub>2</sub>cortex**  
The electrical stimulus for the ventral tail nerve was a constant current 100-μs biphasic square wave (anodal followed by cathodal, 50 μs each polarity). Current intensities were 1, 2, and 3 mA (each polarity) and were presented in a randomized order between animals. Generation and calibration of the stimuli have been previously described. Stimuli were alternately applied to the first and second pairs of stimulating electrodes. A CNAP, SEP<sub>1</sub>cortex, SEP<sub>2</sub>cortex, and SEP<sub>cerebellum</sub> were simultaneously recorded following stimulation from the first pair of electrodes. Following stimulation from the second pair of electrodes, only a CNAP was recorded to allow calculation of NCV. The tail temperature was recorded following stimulation with each different intensity. A broken electrode over the cerebellum was found for one male animal in the 300-ppm group. Thus, there were only 14 animals in the SEP<sub>cerebellum</sub> and BAER recordings for this treatment condition.

**Brainstem auditory-evoked responses.** Stimuli used for recording BAERs were rarefaction clicks and simulated filtered clicks centered at 4 and 16 kHz (all at 50, 65, and 80 dB<sub>peak</sub> SPL; re: 20 μPa), and 64 kHz (65, 70, and 80 dB<sub>peak</sub> SPL). Stimuli were generated, calibrated, and presented as previously described. The stimulus duration varied depending on the frequency: 50.0 μs (click), 2.54 ms (4 kHz), 634 μs (16 kHz), and 159 μs (64 kHz). The auditory stimuli were presented in a random order between animals.

**Colonic Temperature**  
Colonic temperature was measured immediately following the animal’s removal from the test chamber. A temperature probe (Model RET-1; Physitemp Instruments, Inc, Clifton, NJ), connected to a thermometer (Model BAT-10, Physitemp Instruments, Inc), was inserted approximately 8 cm rectally, and deep colonic temperature was recorded.

**Study 2**  

**Animals and Exposure**  
Male animals were exposed to 0, 300, or 400 ppm COS (15 animals per concentration) for 2 weeks (10 exposures). The COS and exposures were generated in the same manner as that described for Study 1. This study allowed replication and extension of the previously reported finding that 2-weeks exposure to 400 ppm COS reduced the amplitude of BAER peaks in male rats using a click stimulus (Morgan et al., 2004). It also extended the examination to include behavioral measures of auditory function.

**Functional Observational Battery**  
Testing for neurobehavioral changes using a FOB and motor activity was conducted 5 days after the last exposure. Procedural details and scoring criteria for the FOB protocol are provided elsewhere (McDaniel and Moser, 1993). Briefly, rats were evaluated for changes in general appearance, lacrimation, salivation, as well as a ranking of the rat’s reactivity. Open-field measurements included ranking the rat’s arousal and activity level, number of rears, as well as any tremorigenic activity. Reactions to a click stimulus, tail pinch, toe pinch, or penlight were ranked. Palpebral, pinna, and proprioceptive placing (both forelimb and hindlimb) responses were also tested. Forelimb and hindlimb grip strength, landing foot splay, and rectal temperature were quantified. Motor activity data were collected shortly after FOB testing, using a feature-eight maze (Reiter, 1983). Photobeams were placed within the chamber to record both horizontally and vertically directed activity. Activity counts were recorded in twelve 5-min intervals to evaluate habituation of activity during the session. For all testing, the observer was blind with respect to the treatment levels.

**Reflex Modification Audiometry**  
To determine if behavioral changes in responding to an auditory prepulse stimulus could be detected, RMA was performed 11 days following the last COS exposure. White noise was used as a prepulse stimulus, at the same intensities used for the click (broadband) stimulus in BAER testing. The RMA testing was conducted in eight sound-attenuated chambers, each containing a wire mesh plastic-framed test cage that was mounted on a load cell/force transducer assembly designed to measure vertical force (Ruppert et al., 1984),...
as modified by Crofton et al. (1990). Each rat was placed in a test cage and after a 5-min adaptation period, received a total of 100 trials with an intertrial interval of 10 s. The trials were arranged in four blocks of 25 trials. Each trial contained a white noise prepulse stimulus (S1; 0, 50, 65, 80 dB; 40 ms duration, 2.5 ms rise per fall) that preceded the white noise–eliciting stimulus (S2; 120 dB, 40 ms duration, 2.5 ms rise per fall) by 90 ms. The S1 intensity varied between the 25 trial blocks (each block had the same intensity). S1 intensities were presented in an ascending series from low to high decibel. Data collection began simultaneously with the presentation of the eliciting stimulus and continued for 64 ms. Response amplitudes were calculated as the average of each animal’s response for a given S1 intensity.

Surgery and Neurophysiological Testing

The animals were implanted with electrodes approximately 19 days after the last exposure to COS using the methods previously described. Additional electrodes (Table 1 and Herr et al., 2004) were implanted to record flash-evoked potentials (FEPs). Several animals were eliminated from this study due to surgical complications, which were not related to COS exposure. The resulting group sizes were as follows: 0 ppm = 13, 300 ppm = 13, and 400 ppm = 14. The animals had CNAPs, NCV, SEPs, BAERs, and FEPs recorded about 27 days after the last exposure to COS, using the procedures described for Study 1 and in Table 1.

Flash-evoked potentials. To examine nonspecific effects of COS on cortical function, FEPs were recorded from the visual cortex. The light stimulus was a 10-μs flash produced by a photic stimulator (Model PS22, Grass Instrument Division, Astro-Med, Inc, West Warwick, RI). Stimuli were delivered in the presence of acoustic white noise (80 dB SPL) to mask noise produced by the strobe discharge (Herr et al., 1996b; Shaw, 1992). The FEP stimuli and masking noise were generated, calibrated, and presented as previously described (Hamm et al., 2000; Herr et al., 2004). Two flash intensities, 15 and 146 lux-s (strobe settings 1 and 16) were used. Stimulus intensities were presented in a counterbalanced order between animals and chambers. Ambient illumination in the test chamber was about 15 lux. This resulted in relative flash intensities of 50 and 60 dB (Herr et al., 1991). Two control animals had malfunctioning electrodes for recording FEPs, resulting in the following group sizes: 0 ppm = 11, 300 ppm = 13, and 400 ppm = 14.

Colonic Temperature

Following neurophysiological testing, colonic temperature was recorded from all animals as described for Study 1.

SEP cerebellum, SEP cortex, and SEP cortex after forelimb stimulation. To more directly examine the frontoparietal cortical regions, we also recorded SEPs following forelimb stimulation. Following FEP recording, animals were removed from the test chamber, anaesthetized (sodium pentobarbital, 50 mg/kg IP), and returned to their home cages until the anesthesia became effective. The rats were then placed in the test chambers on a heating pad (37°C). The right forelimb was extended on a piece of polyvinyl chloride plastic and held in place with tape. Platinum-stimulating needle electrodes (E2, Grass Instrument Division) were placed across the ventral side of the forelimb (cathode proximal to the shoulder) in the mid-forelimb region. The electrodes were separated by 5 mm. A ground electrode was placed in the upper forelimb. The electrode wires were taped to the testing chamber to prevent movement artifacts. Stimulation of the median/ulnar nerves was accomplished using the same 3-mA biphasic square wave pulse as used for stimulating the tail nerves. Where necessary, stimulus intensity was attenuated to achieve twitching of the toes, without excessive limb movement. One animal in the 0-ppm group died due to anesthesia complications. Due to an equipment malfunction, the data from two animals in the 400-ppm group were discarded. This resulted in the following group sizes: 0 ppm = 12, 300 ppm = 13, and 400 ppm = 11 (SEP cortex) or 12 (SEP cerebellum and SEP cortex).

Histopathological Processing

In both the studies, animals were euthanized 6 days after completion of neurophysiological testing. The animals were perfused and their brains removed for histopathological analysis, similar to that reported elsewhere (Morgan et al., 2004; Sills et al., 2004).

Statistical Analysis

Peak measures (amplitudes and latencies) were quantified using each animal’s average waveform. Peak amplitudes were calculated as the peak-to-peak amplitude (in microvolts) from the preceding positive or negative peak. In order to maintain compatibility with previously reported analysis, the peak amplitudes for BAERs and FEPs were measured from baseline (defined as the average voltage over the prestimulus period) (Herr and Boyes, 1997; Herr et al., 1996a). Peak latencies (in milliseconds) were calculated from stimulus onset. For CNAPs, the duration and area of the negative peak (N1) were quantified as previously described (Herr et al., 2004). NCV (in meters/second) for the 3-mA stimulus was calculated as the latency difference between the first positive peak of the two CNAPs divided by the distance between the two cathodes (3 cm) (Gagnaire et al., 1986; Herr et al., 2004).

Data were analyzed using a repeated measures analysis of variance (ANOVA; PROC GLM) (SAS Institute, 1989, 1997) using a Greenhouse-Geisser correction factor (ε) (Geisser and Greenhouse, 1958; Greenhouse and Geisser, 1959; Keselman and Rogan, 1980) for degrees of freedom for within-subject effects. A significant main effect of treatment, or significant interactions of gender and/or stimulus condition with treatment, was followed by step-down ANOVAs that examined treatment effects at each stimulus condition and/or gender. The critical α level for the ANOVAs was determined for each evoked response using a Bonferroni correction. Peak amplitudes and latencies had an overall ε = 0.025 (0.05/2), which was further adjusted based on the number of peak amplitudes, latencies, and step-down ANOVAs. While minimizing the number of Type I statistical errors (Abt, 1981; Muller et al., 1983), such procedures may decrease statistical power (Muller et al., 1983). Therefore, where a treatment-related effect failed to reach the corrected significance level (but had an p ≤ 0.05), the actual probability values are reported to allow the readers to apply their own judgment regarding the biological significance of the results. RMA data were analyzed using a repeated measures two-way ANOVA. Group mean comparisons were performed using a Tukey-Kramer multiple comparison test (α = 0.05) (Kramer, 1956). Analyses of the FOB data were conducted using one-way ANOVA for continuous measures, and the Kruskal-Wallis test for ranked (nonparametric) data. All data are reported as mean ± SE. Group-averaged waveforms were calculated from individual animal data and are presented for illustrative purposes.

RESULTS

Study 1

General Health

All animals appeared to be in good health, with no obvious signs of toxicity after 12-week exposure to COS. These conclusions are in agreement with the minimal FOB changes...
previously reported after 12-week exposure to COS at these same concentrations (Morgan et al., 2004). There were no treatment-related differences in body weight for either gender when the animals were tested (Supplementary Table 1 on Internet; \( p \) value = 0.4186).

**Neurophysiological Measures**

Treatment with COS for 12 weeks did not alter peripheral nerve function. In contrast to the peripheral measures, exposure to 400 ppm COS altered evoked responses generated in the central nervous system. Some increases in the amplitudes of SEPs recorded from the cortex were indicated. Representative responses to illustrate waveform morphology and adequacy of recording conditions are presented in Figure 1. The amplitude of BAER peaks associated with brainstem auditory neurotransmission was decreased following exposure to COS. These effects were observed in the absence of changes in body temperature.

**CNAP, NCV, SEP<sub>cerebellum</sub>, SEP<sub>1 cortex</sub> and SEP<sub>2 cortex** After 12-week exposure to COS, there were no significant (critical \( \alpha' \)'s = 0.0125–0.0083; 0.025/2–3 peaks) treatment-related changes in CNAP peak-to-peak amplitudes or peak latencies (all \( p \) values > 0.05; Supplementary Fig. 1 on Internet). Similarly, there were no significant changes in peak N<sub>1</sub> area or duration produced by exposure to COS for 12 weeks in male or female animals.

Twelve weeks of inhalational exposure to COS did not alter the NCV in the ventral caudal tail nerves (Supplementary Fig. 2 and Supplementary Table 1 on Internet) of male or female rats (\( p \) values > 0.05). This conclusion was sustained when tail temperature was used as a covariate in the analysis (\( p \) values > 0.05).

Exposure to COS for 12 weeks did not produce significant (critical \( \alpha = 0.0083–0.0063; 0.025/3–4 \) peaks) treatment-related changes in SEP<sub>cerebellum</sub> peak-to-peak amplitudes or peak latencies (Supplementary Fig. 3 on Internet).

Exposure to COS for 12 weeks did not significantly (critical \( \alpha = 0.0050–0.0042; 0.025/5–6 \) peaks) alter the peak-to-peak amplitudes or latencies for peaks of SEPs recorded from the S1 hindlimb/tail region (SEP<sub>1 cortex</sub>, Supplementary Fig. 4 on Internet). However, there was a nearly significant increase in peak P<sub>14N<sub>27</sub></sub> amplitude (Fig. 2A) for the 400-ppm COS group (treatment by stimulus intensity interaction: \( F[6,204] = 40.02, \varepsilon = 0.8241, p = 0.0261 \)), for the 3-mA stimulus condition (treatment effect: \( F[3,102] = 4.21, p = 0.0075 \)). Also, there was an indication of an increase in the latencies of peaks N<sub>27</sub> and P<sub>36</sub> produced by 400 ppm COS (treatment effect: \( F[3,102] \geq 4.45, p \leq 0.0056 \)) (Fig. 2B).

When SEPs were recorded from the S1 facial region cortex (SEP<sub>2 cortex</sub>, Supplementary Fig. 5 on Internet), a change in the peak-to-peak amplitude of peak P<sub>16N<sub>21</sub></sub> resulting from 12 weeks of COS exposure was indicated (treatment effect: \( F[3,102] = 7.49, p \leq 0.0001 \)). When averaged over genders and stimulus intensities, 400 ppm COS increased peak P<sub>16N<sub>21</sub></sub> amplitude compared to the control group (Fig. 2C). There were no significant (critical \( \alpha = 0.0083; 0.025/3 \) peaks) effects on SEP<sub>2 cortex</sub> peak latencies produced by COS exposure.

**Brainstem auditory-evoked responses**. Treatment with COS for 12 weeks produced significant (critical \( \alpha = 0.0006; 0.025/4 \) stimuli/11 peaks) changes in the amplitudes, but only minor changes in the latencies, of peaks in BAER waveforms recorded from male and female rats (Fig. 3, Supplementary Fig. 6 on Internet). No gender-related differences in response to COS exposure were indicated, so the data were averaged over male and female rats for statistical analysis.

When BAERs were recorded using a click stimulus, the effects of COS differed significantly between the stimulus intensities for the amplitudes of peaks N<sub>3</sub>, P<sub>1</sub>, N<sub>4</sub>, and P<sub>5</sub> (stimulus intensity by treatment interaction: \( F[6,202] \geq 4.55, \varepsilon \geq 0.9157, p \leq 0.0004 \)) and the effect for peak P<sub>3</sub> nearly met the significance criteria (\( p = 0.0026 \)). The COS-related effects did not differ over stimulus intensities for the amplitudes of peaks N<sub>3</sub> and P<sub>3</sub> (treatment effect: \( F[3,101] \geq 17.61, p \leq 0.0001 \)). Further analysis indicated that changes in peak amplitudes were limited to the 400-ppm COS treatment. Decreased
FIG. 2. Changes in amplitude of peak P_{14}N_{27} (A) using a 3-mA stimulus and latencies of peaks N_{27} and P_{36} (B; averaged over 1, 2, and 3 mA stimulus intensities) recorded from the S1 hindlimb/tail region (SEP1 cortex) and peak P_{16}N_{21} amplitude (C; averaged over 1, 2, and 3 mA stimulus intensities) recorded from the S1 facial region (SEP2 cortex). The data for male and female animals are averaged. Treatment with 400 ppm COS for 12 weeks tended to increase the amplitude of peak P_{14}N_{27} and the latencies of peaks N_{27} and P_{36}, and significantly increased the amplitude of peak P_{16}N_{21}. See text for details (*, significantly different from 0 ppm COS).

FIG. 3. Average BAER waveforms (n = 12–16 rats per waveform) for male animals exposed to 0, 200, 300, or 400 ppm COS for 12 weeks following auditory stimulation with a rarefaction click or tone pip of 4, 16 (80, 65, 50 dB), or 64 kHz (80, 70, 65 dB). Waveforms are plotted with positivity upward. The shaded regions represent the 95% amplitude confidence intervals for the control waveforms and the hatched regions represent the 95% confidence intervals for the 400-ppm COS group waveforms. Exposure to COS decreased peak amplitudes and slightly increased peak latencies (see text for details).
amplitudes for peaks P3 and P4, and increased amplitudes (reduced positivity) for peaks N3 and N4, were indicated for the 65- and 80-dB stimulus intensities. The amplitude of peak P5 was decreased only for the 80-dB stimulus. Additionally, treatment with 400 ppm COS decreased (more positive) the amplitude of peak N5, and increased the amplitude of peak P6 (more positive) compared to the 0-, 200-, and 300-ppm groups, when averaged over the stimulus intensities (Fig. 4). Only the latency of peak P5 was significantly altered by treatment with COS. When averaged over the stimulus intensities, 400 ppm COS decreased (more positive) the amplitude of peak N5, and increased the amplitude of peak P6 (more positive) compared to the 0-, 200-, and 300-ppm groups, when averaged over the stimulus intensities (Fig. 4). Only the latency of peak P5 was significantly altered by treatment with COS. When averaged over the stimulus intensities, 400 ppm COS increased the latency of peak P5 compared to the 0- and 200-ppm groups (treatment effect: $F[3,101] = 8.58, p < 0.0001$; data not shown).

When a 4-kHz stimulus was used, COS significantly altered the amplitudes of BAER peaks N3, P4, and N4 when averaged over stimulus intensities (treatment effect: $F[3,101] \geq 7.06, p \leq 0.0002$) (Figs. 3 and 5, Supplementary Fig. 6 on Internet). COS-related changes in the amplitudes of peaks P3 ($p = 0.0113$) and P5 (0.0288) failed to reach corrected significance levels. Further analysis indicated that treatment with 400 ppm COS increased the amplitude of peak N3 (less positive), and reduced the amplitude of peak P4, compared to the 0-, 200-, and 300-ppm COS groups. Treatment with 400 ppm COS also increased the amplitude of peak N4 (less positive) compared to 0 and 200 ppm COS (Fig. 3, Supplementary Fig. 6 on Internet).

There were no significant effects of COS on peak latencies when BAERs were recorded using a 4-kHz tone pip stimulus.

When using a 16-kHz stimulus (Figs. 3 and 6, Supplementary Fig. 6 on Internet), COS treatment produced intensity-related changes in the amplitude of peaks P4 and N4 (stimulus intensity by treatment interaction: $F[6,202] \geq 4.41, \epsilon \geq 0.9667, p \leq 0.0004$). Additional analysis indicated that 400 ppm COS decreased peak P4 amplitude for the 50-, 65-, and 80-dB peak SPL stimuli (treatment effects: $F[3,101] \geq 7.49, p \leq 0.0001$), and increased the amplitude of peak N4 (less positive) at 65 and 80 dB peak SPL (treatment effect: $F[3,101] \geq 6.64, p \leq 0.0004$), when compared to the 0-, 200-, and 300-ppm groups. Also, treatment with COS altered the amplitudes of peaks N3 and N5 when averaged over stimulus intensities (treatment effects: $F[3,101] \geq 7.23, p \leq 0.0002$). The amplitude of peak N3 was increased (reduced positivity) by treatment with 400 ppm COS, while that of peak N5 was reduced (increased positivity), compared to the 0-, 200-, and 300-ppm groups (Fig. 3, Supplementary Fig. 6 on Internet). Treatment-related decreases in the amplitude of peaks P3 ($p = 0.0022$) and P5 ($p = 0.0392$), and increases in the amplitude of peak P6 ($p = 0.0012$), failed to reach corrected significance levels. As shown in Figure 6, treatment with 400 ppm COS tended to decrease the amplitude of peaks P3 ($p = 0.0076$) and P5 ($p = 0.0017$) when using an 80-dB stimulus, and increase

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**FIG. 4.** Changes in amplitude of BAER peaks P3, P4, P5, and P6 using a 50-, 65-, or 80-dB peak SPL click stimulus. The data for male and female animals are averaged, and only data for positive peaks are presented. Treatment with 400 ppm COS for 12 weeks tended to decrease the amplitude of peaks P3, P4, and P5, but increase the amplitude of peak P6. Missing error bars either are covered by the symbol or were overlapping and removed for visual clarity. See text for details (*, significantly different from 0, 200, and 300 ppm COS and #, significantly different from 0, 200, and 300 ppm COS averaged over stimulus intensities).
the amplitude of peak P6 for the 50- (p = 0.0021) and 65 (p = 0.0164)-dB stimuli. Treatment with COS also altered the latencies of peaks P1, N2, P4, and P5 (treatment effect: F[3,101] = 6.54, p ≤ 0.0004) and produced stimulus intensity–related changes in peak N5 latency (stimulus intensity by treatment interaction: F[6,202] = 5.29, ε = 0.9412, p ≤ 0.0001) (Fig. 3, Supplementary Fig. 6 on Internet). Peak P1 latency was increased by treatment with 300 and 400 ppm COS compared to 0 and 200 ppm. The latency of peak N2 was increased by exposure to 400 ppm COS compared to 0 and 200 ppm, and treatment with 300 ppm increased the latency compared to 200 ppm COS. Peak P4 latency was increased by exposure to 300 and 400 ppm COS compared to controls, and by 400 ppm COS compared to 200 ppm. The latency of peak P5 was increased by treatment with 400 ppm COS compared to the 0- and 200-ppm groups. Finally, the latency of peak N5 was increased by treatment with 400 ppm COS compared to the 0- and 200-ppm COS groups for the 65-dB peak SPL stimulus, and the 0-, 200-, and 300-ppm COS groups for the 80-dB peak SPL stimulus (data not shown).

Treatment with COS did not produce significant (critical α = 0.0006) changes in the amplitude of BAER peaks recorded using a 64-kHz stimulus (Figs. 3 and 5, Supplementary Fig. 6 on Internet). However, the amplitude of peak P4 (treatment effect: F[3,101] = 4.59, p = 0.0047) showed a slight decrease after treatment with 400 ppm COS, which was largest when using the 80-dB stimuli (p = 0.0011). Also, the amplitude of peak N5 decreased (greater positivity), and P6 was slightly increased, by 400 ppm COS (stimulus intensity by treatment interaction: F[6,202] ≥ 3.11, ε ≥ 0.9609, p ≤ 0.0067). These effects were only observed for the 80-dB peak SPL stimulus condition (treatment effect: F[3,101] ≥ 5.32, p ≤ 0.0019). There were no significant changes in the latencies of BAER peaks when recorded using a 64-kHz stimulus.

Colonic Temperature

There were no treatment- or gender-related differences in colonic temperature at the conclusion of neurophysiological testing (all p values > 0.05). The colonic temperature of male rats was 38.3 ± 0.2 (0 ppm), 38.6 ± 0.1 (200 ppm), 38.3 ± 0.3 (300 ppm), and 38.6 ± 0.2°C (400 ppm), and the temperature of the female animals was 38.4 ± 0.2 (0 ppm), 38.2 ± 0.2 (200 ppm), 37.9 ± 0.4 (300 ppm), and 38.1 ± 0.3°C (400 ppm).

Cortical Lesions

During histopathological processing, it was noted in gross examination that four male animals and one female animal in the 400-ppm COS group had visible cortical lesion, similar to
that previously reported (Morgan et al., 2004; Sills et al., 2004). No cortical lesions were noted in the other treatment groups.

**Study 2**

Treatment with 400 ppm COS for 2 weeks produced minor changes associated with motor function as assessed by the FOB. There were no effects seen using RMA. Similar to Study 1, there were no alterations in peripheral nerve CNAPs or NCV. No changes in SEPs were observed using tail-nerve stimulation. When using forelimb stimulation, there appeared to be an effect of COS on SEPs recorded from cortical regions. The amplitude of BAER peaks associated with brainstem auditory neurotransmission was decreased. No changes in the visual-evoked potentials were observed. Treatment with COS may have elevated colonic temperature at the time of neurophysiological testing.

**Functional Observational Battery**

Changes produced by COS occurred only in the 400-ppm COS group. Motor activity was decreased, with vertical activity ($F[2,42] = 11.09$, $p \leq 0.0001$) showing somewhat greater decreases than horizontal ($F[2,42] = 4.84$, $p < 0.0130$; 51% of control for vertical compared to 79% for horizontal). Forelimb and hindlimb grip strengths (forelimb: $F[2,42] = 17.66$, $p \leq 0.0001$; hindlimb: $F[2,42] = 5.08$, $p < 0.0110$) were decreased equally, to about 80% of control. Slightly abnormal gait was recorded for about half of the group (seven of 15; $X^2 = 16.21$, $p = 0.0003$), and this was described as uncoordinated hindlimb placement and tiptoe gait. A few (three of 15; $X^2 = 6.29$, $p = 0.0430$) rats did not display the forelimb proprioceptive placing response, although the hindlimb response was normal. All other reflexes and responses were normal.

**Reflex Modification Audiometry**

Exposure to COS did not change the baseline startle response or the RMA to a white noise prepulse stimulus (Fig. 7) ($p$ values $> 0.05$). However, all animals could respond to the prepulse stimulus, as indicated by reduced startle amplitudes with increasing $S_1$ intensity (prepulse intensity effect; $p \leq 0.0001$).

**Body Weight**

At the time of neurophysiological testing, there were no significant differences in body weight (Supplementary Table 1 on Internet; $p = 0.9841$).
S1 intensity levels.

prestimulus, as the amplitude of the startle response decreased with increasing

detected after COS treatment. All exposure groups could hear the S1

No differences in baseline startle response, or the RMA to the S1 stimulus, were

exposure to 0, 300, or 400 ppm COS for 2 weeks ({n = 15 rats per treatment}).

resulted in a greater peak P14 latency than the 0- or 300-ppm

F0.025/4 peaks) affected by exposure to COS (treatment effect:

in SEPcerebellum peak-to-peak amplitudes (Fig. 8). However, the

(area or duration following treatment with COS (data not shown).

Similar to the lack of effects on CNAPs, exposure to COS for 2

weeks did not alter the NCV in the ventral caudal tail nerves

of male rats (Supplementary Table 1 on Internet; p = 0.6837).

This conclusion was sustained when tail temperature was used

as a covariate in the analysis (p = 0.7235).

Exposure to COS for 2 weeks did not produce significant

(critical \( \alpha \)'s = 0.0125–0.0083; 0.025/2–3 peaks) treatment-related
changes in CNAP peak-to-peak amplitudes or peak latencies
(all \( p \) values > 0.05) in male animals when using a 3-mA stimu-

lus. Additionally, there were no significant changes in peak N1

area or duration following treatment with COS (data not shown).

Neurophysiological Measures

CNAP, NCV, SEPcerebellum, SEP1 cortex and SEP2 cortex. After

2-week exposure to COS, there were no significant (critical
\( \alpha \)'s = 0.0083; 0.025/3 peaks) treatment-related changes in CNAP peak-to-peak amplitudes or peak latencies
in SEPcerebellum, peak-to-peak amplitudes (Fig. 8). However, the
latency of peak P14 was significantly (critical \( \alpha \) = 0.0063; 0.025/4 peaks) affected by exposure to COS (treatment effect: 
\( F[2,37] = 22.54, p \leq 0.0001 \)). Exposure to 400 ppm COS
resulted in a greater peak P14 latency than the 0- or 300-ppm
groups. The latencies for peak P14 are as follows: 14.61 ±
0.37 (0 ppm), 13.87 ± 0.17 (300 ppm), and 17.08 ± 0.45 (400
ppm).

Neither the peak-to-peak amplitudes nor the latencies of SEPs recorded from the S1 hindlimb/tail region (SEP1 cortex) were altered (critical \( \alpha \) = 0.0050–0.0042; 0.025/5–6 peaks) after exposure to COS for 2 weeks when recorded using a
3-mA stimulus (all \( p \geq 0.05 \); Fig. 8).

When SEPs were recorded using a 3-mA stimulus from the
S1 facial region cortex (SEP2 cortex), no changes were indicated
(critical \( \alpha \) = 0.0125–0.0083; 0.025/2–3 peaks) in the peak-to-
peak amplitudes or peak latencies after 2-week exposure to
COS (Fig. 8).

Brainstem auditory-evoked responses. Treatment with
COS for 2 weeks produced significant (critical \( \alpha \) = 0.0008; 0.025/3 stimuli/11 peaks) changes in the amplitudes, but only
minor changes in the latencies, of peaks in BAER waveforms
(Fig. 9).

When BAERs were recorded using a click stimulus, changes
in peak amplitudes produced by COS did not differ over the
three stimulus intensities. Significant changes in the amplitudes
of peaks N3, P4, N4, and P5 were indicated (treatment effect: 
\( F[2,37] \geq 8.78, p \leq 0.0008 \)). Effects produced by COS
treatment on the amplitude of peaks N5 (\( p = 0.0016 \)) and P6
(\( p = 0.0258 \)) failed to reach corrected significance levels.

Further analysis indicated that changes in peak amplitudes
were limited to the 400-ppm COS treatment. The amplitudes
of peaks P4 and P5 were decreased, and that of peaks N3 and N4
increased (less positive) by exposure to 400 ppm COS compared to the 0- and 300-ppm groups (Figs. 9 and 10).

Although failing to reach corrected significance levels, the
amplitude of peak N5 was decreased (more positive) and that of
peak P5 increased, by exposure to 400 ppm COS. There were
no significant COS-induced changes in the latencies of BAER
peaks recorded using a click stimulus.

When an 80-dB 4-kHz stimulus was used, only the amplitude of peak P2 was significantly altered by treatment with
COS (treatment effect: \( F[2,37] = 9.55, p = 0.0005 \)).
Changes in the amplitudes of peaks N3 (p = 0.0284), N4 (p = 0.0057), and P5 (p = 0.0046) failed to reach corrected significance levels. As with the other stimuli, changes in BAER amplitudes were limited to the 400-ppm group. The amplitude of peak P4 was decreased by 400 ppm COS compared to the 0- and 300-ppm groups (Figs. 9 and 10). There were no significant changes in BAER peak latencies produced by COS when recorded using a 4-kHz stimulus.

When an 80-dB 16-kHz stimulus was used, significant changes in the amplitude of peaks P4 and N4 were indicated (treatment effect: F[2,37] ≥ 12.00, p ≤ 0.0001). Changes in the amplitude of peaks N3 (p = 0.0016) and P5 (p = 0.0072) did not reach corrected significance levels. The amplitude of peak P4 was decreased, and that of N4 increased (less positive), by 400 ppm COS compared to the 0- and 300-ppm groups (Figs. 9 and 10). Treatment with COS for 2 weeks did not alter peak latencies when BAERs were recorded using a 16-kHz stimulus.

Flash-evoked potentials. Inhalational exposure to COS did not produce significant (critical α’s = 0.0031; 0.025/8 peaks) changes in FEP peak amplitudes or latencies (Supplementary Fig. 7 on Internet). However, there was a nearly significant decrease in the amplitude of peak N3 in the 400-ppm COS group compared to controls for the 146 lux-s stimulus condition (treatment effect: F[2,35] = 4.09, p = 0.0253). No other peak amplitudes or latencies showed COS-related changes.

SEPcerebellum, SEP1cortex, and SEP2cortex after forelimb stimulation. Exposure to COS did not alter either peak-to-peak amplitudes or peak latencies of SEPcerebellum responses following forelimb stimulation (Fig. 8). However, the waveforms did have a different shape than the SEPcerebellum responses recorded following tail stimulation. This indicates that different populations of cerebellar neurons are activated following tail versus forelimb stimulation.

When SEPs were recorded from the cortical S1 hindlimb/tail region following forelimb stimulation, there were several changes in waveform morphology that appeared to be related to COS exposure (Fig. 8). Due to the extent of the waveform changes, statistical analyses are not presented, but the differences will be described. There was a large increase in waveform negativity in the peak P12-N24 region, and in the region of peak N79 in the animals exposed to 400 ppm COS. This change in the waveform shape resulted in an apparent decrease in the latencies of “peaks P12, N24, and P49” in the 400-ppm COS group. The SEP1cortex waveforms for the 0- and 300-ppm groups were similar in morphology when recorded using forelimb stimulation.
Changes in waveform morphology related to exposure to COS were also noted when SEPs were recorded from the cortical S1 facial region using forelimb stimulation (Fig. 8). There was an increase in waveform negativity over the range from peaks P11–N108 for the animals exposed to 400 ppm COS. This effect was especially noticeable in the regions of peaks P33 and N54. Additionally, the latencies of “peaks P11 and N108” appeared to decrease in the 400-ppm waveforms. The SEP2_cortex waveforms for the 0- and 300-ppm groups were similar in morphology, except for an increase in positivity in the region of peak N54, when recorded using forelimb stimulation.

**Colonic Temperature**

In this study, treatment with COS was associated with an apparent “increase” in colonic temperature recorded following neurophysiological testing (treatment effect: \[F(2,36) = 8.96, p = 0.0007\]). Both 300- and 400-ppm COS groups had significantly greater colonic temperature than control animals. The colonic temperature of the rats was: 37.8 ± 0.2 (0 ppm), 38.3 ± 0.1 (300 ppm), and 38.5 ± 0.1°C (400 ppm).

**Cortical Lesions**

During histopathological processing, it was noted in gross examination that 11 male animals in the 400-ppm COS groups had a visible cortical lesion, similar to that previously reported (Morgan et al., 2004; Sills et al., 2004). No grossly visible cortical lesions were noted in the 0- or 300-ppm COS groups.

**Changes in Evoked Potentials Due to Gender or Stimulus Intensity**

The evoked responses had significant gender- and/or stimulus intensity–related changes that were independent of COS treatment (Figs. 3 and 9, Supplementary Figs. 1,3–7 and Supplementary Table 2 on Internet). In general, females had larger peak amplitudes and areas, and reduced peak latencies, than males. Greater stimulus intensities resulted in larger peak amplitudes, and reduced peak latencies. Specific deviations from these conclusions are detailed in Supplementary Table 2 on Internet. The results show that the evoked responses were under stimulus control and that the study had sufficient power to detect changes of magnitude produced by the different stimulus conditions.

**DISCUSSION**

The data in this manuscript further confirm that the brain is a major target of COS toxicity. Our previous reports show that exposure to 400 ppm COS for 12 weeks resulted in lesions in major neuroanatomical sites such as the posterior colliculus and parietal cortex (Morgan et al., 2004; Sills et al., 2004). Additionally, 2-week exposure to 400 ppm COS decreased the...
amplitude of BAER peaks P\textsubscript{4} and N\textsubscript{5}, and increased the amplitude of (reduced positivity) peaks N\textsubscript{3} and N\textsubscript{4} (using an 80-dB SPL click stimulus) (Morgan \textit{et al.}, 2004). The results of this study confirm these previous findings and present the results of an expanded neurophysiological evaluation of the animals exposed to COS for 2–12 weeks.

The data show that BAERs were altered after exposure to 300–400 ppm COS. The most consistent finding was a decrease in the positivity from approximately peak N\textsubscript{3} to P\textsubscript{5}, and a reduction in negativity over the N\textsubscript{5}–P\textsubscript{6} region (Figs. 3 and 9, Supplementary Fig. 6 on Internet). This resulted in reductions in the amplitudes of peaks P\textsubscript{3}, P\textsubscript{5}, and P\textsubscript{6}, coupled with an increase in the amplitude of P\textsubscript{6}. While some differences in the statistical significance of these conclusions were noted between the different stimuli, examination of these figures shows that the trend was apparent in the 80-dB recordings for all stimuli. When the effects of COS on a peak amplitude differed between stimulus intensities, the effects were the largest for the 80-dB stimulus intensity. This is likely a result of the smaller waveforms produced by the lower stimulus intensities. A stimulus-related reduction in waveform amplitude, coupled with the normal variability in responses, may have resulted in a “floor effect”. This would make detection of COS-induced reductions in BAER amplitudes more difficult to detect.

When animals were exposed to COS for 2 weeks, the changes in BAER waveforms were similar, but fewer peaks were significantly altered, than after treatment for 12 weeks. In both cases, the amplitude of peaks P\textsubscript{4} and P\textsubscript{5} (click stimulus) were reduced, and that of peak N\textsubscript{3} was increased (reduced positivity). In contrast to the 12-week exposure, alterations in the amplitudes of peaks N\textsubscript{3}, P\textsubscript{5}, N\textsubscript{4}, and P\textsubscript{6} failed to reach corrected significance levels after 2-week treatment with COS. These results indicate an exposure duration–related increase in the toxicity to various regions of the auditory system involved in generating BAERs.

Knowledge of anatomical sites of BAER peak generation allows diagnosis of peripheral versus central sites of changes in the auditory pathway. The peaks of the BAER waveform are believed to be generated in the following regions: peak P\textsubscript{1} by the auditory nerve, peak P\textsubscript{2} at the level of the cochlear nucleus, peak P\textsubscript{3} in the region of the olivary complex, peak P\textsubscript{4} in the region of the lateral lemniscus, peak P\textsubscript{5} in the brainstem and posterior colliculus, and peak P\textsubscript{6} in the brainstem and medial geniculate nucleus (Chen and Chen, 1991; Melcher \textit{et al.}, 1996; Møller and Jannetta, 1986; Shaw, 1988; Zaaroor and Starr, 1991). The lack of changes in the amplitude and latency of peaks P\textsubscript{1} and P\textsubscript{2} indicates normal function of the auditory nerve and cochlear nucleus regions. However, the observed reductions in the amplitudes of peaks in the P\textsubscript{3}–P\textsubscript{5} region are consistent with a lesion in the region of the olivary complex—lateral lemniscus region of the brainstem.

These conclusions are supported by histopathological and MRM evaluations performed in subsets of these animals and reported previously (Morgan \textit{et al.}, 2004; Sills \textit{et al.}, 2004). Using MRM, regions of hypointensity were observed in the posterior colliculus and anterior olivary nuclei of animals exposed to 400 ppm COS for 4–12 weeks. These regions of hypointensity correlated with neuronal loss and microglial infiltration using standard histopathological procedures (Morgan \textit{et al.}, 2004; Sills \textit{et al.}, 2004). The reduced amplitudes of BAER peaks, with lesser changes in peak latencies, is consistent with a loss of neurons rather than changes in conduction along remaining brainstem neural pathways (Dyer, 1986; Mattsson \textit{et al.}, 1992). Therefore, there was a high degree of association between functional changes measured using neurophysiological techniques with pathology observed using MRM and standard histopathological techniques.

It is interesting to note that neurophysiological and pathological changes were observed after 2-week exposure to COS, in the absence of behavioral changes quantified using FOB or RMA technique. No changes in the inhibition of the startle response by the prepulse stimulus were observed, indicating that the animals could respond to the white noise prepulse. This prepulse stimulus contains a wide range of frequencies and was chosen to correlate with the click stimulus (where large effects were observed) used in recording BAERs. Additionally, there were no changes in the FOB-orienting response to a stimulus produced by a “clicker” (Morgan \textit{et al.}, 2004; Moser, 1989). These results indicate that despite the pathological abnormalities in the central nervous system, the rats were able to respond to the auditory stimuli.

The behavioral correlates to these brainstem auditory pathway lesions in rats are currently speculative. The anterior olivary nuclei represent the first portion of the auditory pathway that receives bilateral inputs (Aitkin \textit{et al.}, 1984; Suneja \textit{et al.}, 1995). This region of the brainstem auditory system is believed to be involved in sound localization in space due to temporal and phase differences in inputs arriving from opposite sides of the head (Aitkin \textit{et al.}, 1984; Pratt \textit{et al.}, 1998). It is interesting to note the findings of human clinical observations involving brainstem lesions. One observation with lesions in the posterior colliculi is the inability to comprehend spoken words (word deafness) (Johkura, 2002; Johkura \textit{et al.}, 1998; Masuda \textit{et al.}, 2000; Meyer \textit{et al.}, 1996; Vitte \textit{et al.}, 2002). These patients may present only moderately changed pure tone audiograms (Johkura \textit{et al.}, 1998; Masuda \textit{et al.}, 2000; Vitte \textit{et al.}, 2002), with unaltered BAER peak latencies (Masuda \textit{et al.}, 2000; Meyer \textit{et al.}, 1996; Vitte \textit{et al.}, 2002). However, changes in peak latency and amplitude for BAER peak V (similar to peak P\textsubscript{4} in rats) (Herr and Boyes, 1995) have also been reported (Johkura, 2002; Johkura \textit{et al.}, 1998). Thus, it is possible that the altered BAERs that we report in rats exposed to COS represent changes in the animal’s ability to process biaural sounds. These brainstem sites were directly involved in producing the BAER waveforms and were less specifically involved in FOB or RMA measure.

As implied in the previous paragraphs, the animals in these studies did not present obvious toxicological signs. Changes in
colonic temperature were limited to an increase in the 300- and 400-ppm COS groups after 2-week exposure. This change was not observed after treatment with COS for 12 weeks. It is interesting to note that the colonic temperatures were similar in the 300- and 400-ppm groups after both 2- and 12-week exposure, and that it was the control animals that had a slight reduction in colonic temperature in the 2-week study.

The FOB indicated that exposure to COS for 2 weeks produced changes in motor function, as measured by decreased motor activity, altered gait, and decreased grip strength. The only sensorimotor response altered was the proprioceptive placing response, which is a robust response always seen in control animals. For that reason, this finding may have biological relevance, even though it was only observed in a few rats. In a different group of animals, 2 weeks of COS exposure also produced decreased grip strength, gait changes, and hyponxia: without decreases in motor activity (Morgan et al., 2004). This previous study indicated compensation in the motor effects as more deficits were evident after 2 weeks of COS exposure compared to 6-week exposure, and even less after 12 weeks of exposure to COS. However, there were differences between the experiments, as the current study examined the rats 5 days after exposure ended (compared to the day after the last exposure), different motor activity systems were used, and the measure of proprioceptive placing was not used in the previous study (Morgan et al., 2004). However, it is evident that in spite of brain lesions, the animals presented only mild behavioral abnormalities.

The lack of large and consistent changes in behavioral end points involving motor function (Morgan et al., 2004) (and FOB results) is consistent with the neurophysiological evaluations. We did not observe changes in peripheral nerve function as measured by CNAPs or NCV neurophysiological measures. Thus, the data suggest that COS does not produce major changes in the function of peripheral nerves with up to 12-week exposure duration.

Exposure to higher concentrations of COS has been reported to produce cerebellar pathology. Exposure to > 500 ppm COS for 4 h has been reported to produce swelling of myelin sheaths in the cerebellum, corpus callosum, and pyramidal tracts (Nutt et al., 1996). Additionally, exposure to 600 ppm COS for 1 day (6 h) has been found to produce necrosis of the cerebellar roof nucleus and vacuolation of cerebellar medullary white matter. These effects were not observed at lower concentrations of COS (Morgan et al., 2004). In this report, no changes in peak-to-peak amplitudes in SEP cerebellum recordings were noted after either 2- or 12-week exposure to up to 400 ppm COS. An increase in the latency of peak P14 was indicated after the 2-week exposure to 400 ppm COS. This would imply changes in the responsiveness of cerebellar neurons (Carrea and Grundfest, 1954; Morin et al., 1957). The lack of altered latency for peak N10 indicates normal transmission in the spinocerebellar pathways (Carrea and Grundfest, 1954; Dow, 1939). However, the increased latency for peak P14 was not noted after 12-week exposure to COS. This would suggest either recovery from the effects of COS or an isolated finding.

SEP indicated effects of COS on cortical function. When using tail stimulation and recording from the S1 hindlimb/tail region (SEP1_cortex), there were only minor alterations after treatment with COS. No effects were noted after the 2-week treatment duration. After 12-week exposure, there was a suggestion of an increase in peak P14N27 amplitude and peak N27 and P36 latencies produced by 400 ppm COS. These changes are similar to those observed when recording from the S1 facial region (SEP2_cortex). No changes were noted after 2-week exposure to COS, but after 12-week exposure to 400 ppm COS, an increase in the peak-to-peak amplitude of P14N27 of the SEP2_cortex was observed, with no changes in peak latencies. The early positive-negative deflection of cortical SEPs is believed to be related to the initial depolarization of cortical cells by thalamocortical afferents (Di et al., 1990) and is maximal over the primary (S1) cortical area for the body region (Di et al., 1994; Koyanagi and Tator, 1996). Increases in amplitude of the evoked responses could be related to an increased synchrony of afferent input or reduced inhibitory influences (Dyer, 1986), and also has been reported to be associated with hypothermia (Dyer and Boyes, 1983). Reductions in colonic temperature were not observed in these studies; thus the increases in peak-to-peak amplitudes were not related to hypothermia. It is interesting to note that increases in the first negative peak (N1, N50) of FEPs have been reported following cortical lesions (Dyer et al., 1987b). After 2-week exposure to 400 ppm COS, 11 male animals had visible cortical lesions. In contrast, after 12-week exposure, only four male animals and one female animal in the 400-ppm COS groups had visible cortical lesions. Therefore, there was a disassociation between the presence of the cortical lesions and changes in SEPs produced by stimulating tail nerves. This may be related to the locations of the cortical lesions, which tended to be localized to the parietal cortex (Morgan et al., 2004; Sills et al., 2004). Even though the S1 facial (SEP2_cortex)-recording electrode was closer to region, stimulation of tail nerves may not have provided optimal activation of the cortex to detect abnormalities.

To more directly stimulate the forelimb and facial cortical regions, forelimb stimulation was also used as an adjunct procedure in the 2-week exposure study. Both stereotaxic atlases (Paxinos and Watson, 1998) and recent imaging studies (Brinker et al., 1999; Spenger et al., 2000) indicate that stimulation of the forelimb and facial area activates regions of the cortex that are anterior and lateral to those activated by hindlimb and tail stimulation. As shown in Figure 8, forelimb stimulation produced different waveform morphologies in the SEP cerebellum, SEP1_cortex, and SEP2_cortex than observed after tail stimulation. There were no COS-related changes in the SEP cerebellum after forelimb stimulation, again indicating that exposure to 400 ppm COS for 2 weeks did not produce large alterations in cerebellar neuronal responsiveness. As expected,
The SEP$_1$$_{cortex}$ was smaller (indicating less activation), and the SEP$_2$$_{cortex}$ was larger (indicating greater activation) after forelimb stimulation compared to tail stimulation. Treatment with 400 pm COS produced changes in waveform morphology in both the SEP$_1$$_{cortex}$ and the SEP$_2$$_{cortex}$ waveforms after forelimb stimulation. In general, this was reflected by an increase in negativity over a large portion of the waveform. The changes in the SEP$_2$$_{cortex}$ waveforms were qualitatively greater in the presence of the cortical lesions. The relationship between the changes in SEP waveforms after forelimb stimulation and alterations in the FOB (proprioceptive placing) remains speculative at this time. It is interesting to note that exposure to COS altered both measures after 2 weeks of exposure. However, the data show that activation of cortical areas near the COS-induced lesions produced noticeable changes in SEPs.

The changes in cortical responsiveness produced by exposure to 400 ppm COS for 2 weeks did not generalize to all cortical regions. FEPs are generated in the visual cortex (Barth et al., 1995; Brankačk et al., 1990; Dyer et al., 1987a,b). Changes in FEPs could reflect alterations in the retina, lateral geniculate nucleus, subcortical regions, or cortical processing. The lack of changes in FEPs indicates that not all sensory modalities, nor a general change in the ability of the cortex to respond to inputs, were altered by 2-week exposure to COS. The mechanism by which COS produces neurotoxicity is currently unknown. COS is a metabolite of the neurotoxicant CS$_2$ (Beauchamp et al., 1983; Bus, 1985; Dalvi et al., 1975), which is believed to exert its toxicity via interactions with proteins that result in protein cross-linking (Graham et al., 1995). CS$_2$ neurotoxicity alters visual and auditory responses (Eskin et al., 1988; Herr et al., 1992; Merigan et al., 1988; Rebert and Becker, 1986) and affects peripheral nerves (Gottfried et al., 1985; Graham et al., 1995; Pappolla et al., 1987; Rebert and Becker, 1986; Sills et al., 1998). Neither visual nor peripheral nerve-evoked responses were altered in this study, suggesting that COS-induced neurotoxicity is distinct from that produced by CS$_2$. Additionally, COS has been reported to chelate copper ions which may alter the activity of different enzymes requiring copper as a cofactor (Johansson, 1989), similar to metabolites of CS$_2$. However, it is unknown how chelation of ions might produce the changes in histopathology and neurophysiology found after COS exposure. COS can be metabolized to hydrogen sulfide (H$_2$S) by carbonic anhydrase (Chengelis and Neal, 1979, 1980). Inhibition of carbonic anhydrase has been shown to partially protect Sprague-Dawley rats from COS-induced lethality (20–30 mg/kg, ip, gas form), reportedly due to central respiratory arrest (Chengelis and Neal, 1980). This study suggests that metabolism to H$_2$S may be related to the lethal effects of COS. However, pathological changes to the brain like those reported for COS (Morgan et al., 2004; Sills et al., 2004) have not been reported after exposure to H$_2$S (USEPA, 2003). It is interesting to note that H$_2$S is known to inhibit cytochrome c oxidase which would interfere with energy metabolism.

The histopathological changes in the brainstem (Morgan et al., 2004; Sills et al., 2004) produced by COS are similar to those found in some energy-deprivation toxicities. After exposure to 400 ppm COS (or greater), the major sites of pathology were the S1 cortex associated with the face, vibrissae, and forelimbs, the putamen, and brainstem auditory pathways including the posterior olive, lateral lemniscus, and posterior colliculus (Morgan et al., 2004; Sills et al., 2004). Lesions in these areas (especially the brainstem auditory nuclei) are often observed with acute thiamine deficiency (Cavanagh, 1993, 1998). Treatment with 1,3-dinitrobenzene produced lesions in the anterior olivary nucleus, posterior colliculus, and cerebellum, that appeared to be associated with metabolic activation of these brain regions (Mulheran et al., 1999; Philbert et al., 1987; Ray et al., 1992). These same areas of the brain, especially the auditory structures, have been shown to have high metabolic activity as measured by $^{14}$C 2-deoxyglucose uptake (Schwartz and Sharp, 1978; Sokoloff et al., 1977). Exposure to 200–400 ppm COS has been shown to inhibit cytochrome oxidase in the posterior colliculus, and parietal cortex, in an exposure duration–related manner (Morgan et al., 2004). While not conclusive, the existing data suggest that changes in energy balance in metabolically active regions of the rat brain may be involved in COS-induced neurotoxicity (Sills et al., 2005).

In summary, exposure to COS has been shown to alter neurophysiological measures of cortical somatosensory and brainstem auditory function in a dose- and time-related manner. The results of neurophysiological testing identified specific brainstem auditory regions that were altered by COS exposure and showed that changes in cortical responses are not widespread. Alterations in peripheral nerve and visual function were not observed. These neurophysiological techniques suggest a no-effect level of 200 ppm COS, although cytochrome oxidase has been shown to be inhibited at 200 ppm COS (Morgan et al., 2004). However, the application of these findings to a human risk determination for COS remains to be determined. Future studies will focus on delineating changes early in the course of COS exposure, and further investigating the role of altered energy balance in COS-induced neurotoxicity.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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