Chronic exposure to toluene changes the sleep-wake pattern and brain monoamine content in rats

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Toluene, found in glues and cleaners, is among the inhalants most commonly abused by workers and young drug addicts. In
this study, we examined the changes in sleep patterns and monoamine content induced by chronic toluene exposure. Rats
were chronically exposed to toluene vapors beginning at 30 days of age for a duration of 30 days. Experiment I was
performed in a control group (*n*=10) and a chronic toluene exposure group (*n*=10). Rats were implanted with bipolar stainless
steel electrodes for electroencephalographic recording (EEG). In experiment II, conducted in two other groups (control and
exposed to toluene, *n*=10 each), animals were sacrificed by decapitation prior to chromatographic analysis. We found that
chronic toluene administration affected the organization of sleep patterns and monoamine content. Dopamine (DA) and
noradrenaline (NA) increased in the midbrain and striatum. 3,4-dihydroxyphenylacetic acid (DOPAC) increased only in the
striatum. Midbrain levels of serotonin (5-HT) increased in the pons and decreased in the hypothalamus and striatum.
5-hydroxyindoleacetic acid (5-HIAA) increased in the pons, midbrain and striatum and decreased in the hypothalamus.
Chronic toluene exposure induced changes in the serotonergic and dopaminergic systems and increased SWS and PS deficits.
We conclude that toluene exposure disrupts the sleep-wake cycle by affecting the monoaminergic response in cerebral areas
related to sleep.

Key words: brain, chronic exposure to toluene, monoamines, rat, sleep

INTRODUCTION

In recent decades, inhalant abuse has increased dramatically throughout the world, especially among young people. Toluene, one of the most commonly abused organic solvents, is present in paints, glues, gasoline, and cleaners (Echeverria et al. 1991, Lucchini et al. 2000, Anderson and Loomis 2003). Toluene abuse leads to symptoms such as headache, heaviness of the head, giddiness, forgetfulness, fatigue, lassitude, loss of appetite, insomnia, and sleep disturbance. These psychotropic effects affect a wide variety of functions in humans, including the capacity to pay attention to and respond to environmental stimuli and the ability to estimate time.

Toluene modifies lipid composition and interacts with membrane proteins, directly increasing membrane fluidity and altering receptor binding and neurotransmitters (Calderón-Guzmán et al. 2005a). Previous data have shown that toluene alters the function of a variety of ion channels, including ligand-gated channels activated by ATP, acetylcholine, GABA, glutamate and serotonin (5-HT), as well as voltage-dependent sodium and calcium channels (Bale et al. 2005, Williams et al. 2005, Liu et al. 2007). In behavioral states, repeated administration of toluene severely reduces alertness in animals (Takeuchi and Hisanaga 1977, Chen and Lee 2002). Several mechanisms have been proposed to explain the toxic effects of toluene and include the hypothesis that toluene disrupts neu-

romembrane function by a non-specific partitioning within the lipid bilayer by binding at hydrophobic pockets on integral membrane proteins (Von Burg 1981). Toluene-induced partial insomnia and hyperactivity are associated with decreased concentrations of 5-HT, as well as increases in cortical noradrenaline (NA) and 5-hydroxyindoleacetic acid (5-HIAA; Yamawaki et al. 1982, Arito et al. 1985, Von Euler et al. 1988). Chronic exposure to high concentrations of toluene affects dopamine (DA; Riegel et al. 2007, Lo et al. 2009) and 5-HT activity (Castilla-Serna et al. 1993, Calderón-Guzmán et al. 2005a, b). Moreover, industrial solvents applied to the central nervous system (CNS) have demonstrated the toxic effect of such compounds on human and animal EEGs (Depoortere et al. 1983, Compton et al. 1994, Halifeoglu et al. 2000).

Sleep polysomnography and evoked potential techniques have been used as tools to objectively determine the psychopharmacological mode of action of psychotropic agents, including 'typical' (such as minor and major tranquilizers) substances and 'atypical' compounds (such as caffeine, nicotine, and alcohol). Few, if any, studies have systematically applied these techniques to assess the effects of psychotropic volatile inhalants on sleep (Borenstein and Cujo 1974, Fernandez-Guardiola et al. 1984, Vrca et al. 1996).

The aim of the present study was to determine the dysfunction caused by chronic toluene exposure on sleep patterns and neurotransmitters related to sleep, such as 5-HT, 5HIAA, DA, NA, homovanillic acid (HVA) and 3,4-dihydroxyphenylacetic acid (DOPAC).

METHODS

Experimental design

We used 40 male adult Wistar rats (mean weight of 292±12 g). Animals were divided into two groups for each condition. In the experiment I, 20 rats at 30 days of age were randomly distributed into two groups for chronic toluene exposure or air inhalation (control) until 60 days of postnatal life. Rats were then submitted for EEG recording. In the experiment II, 20 rats at 30 days of age were randomly distributed into two groups for chronic toluene exposure or air inhalation (control) until 60 days of postnatal life. The rats were then sacrificed, and tissue was submitted for high performance liquid chromatography (HPLC). Animals were housed in a temperature-

controlled *vivarium*, maintained with a 12-h light/dark cycle (light on at 8:00 h) and provided with food and water *ad libitum*. Rats were weighed daily before the inhalation session. Rats were treated according to the 'Guide for the care and use of experimental animals' (Olfert et al. 1993).

Toluene exposure

Once a day, animals were exposed to vapors for a 15 min period in a closed glass chamber with a volume capacity of 2 740 ml of air maintained at room temperature. The exposure vapors contained 15 000 ppm of liquid toluene, equivalent to 54-57 mg of reagent-degree toluene (Merck Co., Mexico) per liter of air. The desired toluene concentration was obtained by direct introduction of 0.4 ml of liquid toluene into the chamber using a graduated syringe (see Castilla-Serna et al. 1993 for more details).

The rats were chronically exposed to toluene vapors beginning at 30 days of age for a duration of 30 days. The rats were exposed to toluene between 8:30 and 9:30 AM each day. The baseline control group did not receive treatment. These animals were similar in age and body weight and were obtained from animal houses with similar light-dark cycles.

Experiment I

Polygraphic recording

Experiment I was designed to assess the effects of chronic toluene exposure on sleep. Animals in the control group (*n*=10) and chronic toluene exposure group (*n*=10) were anesthetized with sodium pentobarbital (40 mg/kg, i.p.) and mounted in a stereotaxic frame (David Kopff Instruments, Munich). All surgical procedures were performed seven days before chronic exposure to toluene was finished. Rats were implanted with bipolar stainless steel electrodes (Bore 0.010 in., Coated 0.013 in., A-M Systems, Inc., Carlsborg. WA) in the right sensorimotor cortex (2 mm length) for electroencephalographic recording (EEG). Electrodes (50 mm length) were placed in neck muscles for electromyographic recording (EMG). A screw implanted in the skull served as the reference point. The electrodes were then soldered to mini-connectors and secured to the skull with dental acrylic. Seven days after postop-

erative recovery, rats were placed in a soundproof recording cage and allowed free access to food and water under controlled light-dark conditions (8:00 AM - 8:00 PM light; 8:00 PM - 8:00 AM dark), without movement restriction. When the animals were habituated to these environmental conditions, a polysomnographic study was conducted over the course of 24 h for both groups. At the proper time, polygraphic filters were set to a range between 0.1 and 30 Hz for EEG in channel 1, and 10 and 300 Hz for EMG in channel 2. In a few cases, it was necessary to use line filters. Polygraphic ink writing paper speed was set to 30 cm/min so that each epoch consisted of 1 min. We obtained 1440 epochs for each of the rats, which were each continuously recorded for 24 h.

A polygraphic recording was analyzed visually according to the methods set out by Alfaro-Rodríguez and González-Piña (2005). The quantitation was performed by an investigator blind to the treatments (toluene versus air exposure). Briefly, recordings were classified as follows: A) Wakefulness (W), characterized by desynchronization of EEG. During the wakeful phase, cortical EEGs showed a fast and low voltage. The EMG had high amplitude and muscle tone was elevated; the pulses of the waves were rapid. The waking behavior included walking, scratching, eating, and drinking; animals always had their eyes open, even when they were lying quietly. B) Slow wave sleep (SWS), characterized by the presence of sleep spindles, slow waves with voltage higher than 75 μV and a decrease in the EMG voltage. The EMG was small in amplitude and the pulses were slow. The rats lay quietly with eyes closed. C) Paradoxical sleep (PS), characterized by desynchronization of the EEG. During PS, the cortical EEG showed a fast and low voltage. EMG amplitude was reduced almost to the isoelectric line, indicating a total loss of muscular tone, and the pulses were slower than in any other phase. The rat lay with eyes closed and with head and trunk on the floor.

Statistical analysis

Mean values (mean ± S.E.M.) were statistically compared using Student's t-test. $p \leq 0.001$.
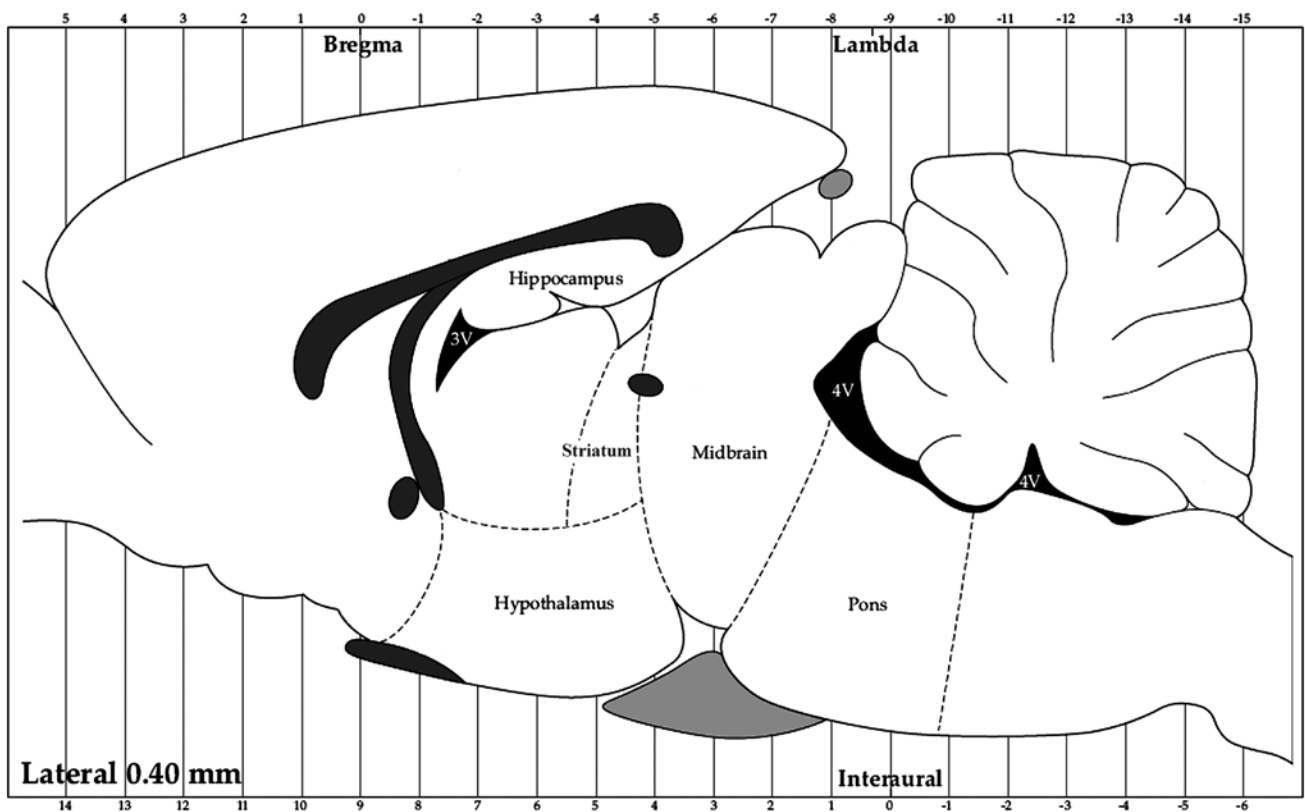

Fig. 1. Schematic representation of the different regions analyzed in sagittal diagram coordinates. Pons, midbrain, hypothalamus and striatum (according to Paxinos and Watson 1998) **.**

Table I

Sleep parameters (mean±S.E.M.) recorded for 24 h (one day) in rats immediately after chronic toluene exposure for 30 days.

	Control (n=10)	Chronic Toluene exposure (n=10)
W (min)	698.45±16.1	557.2±21.0*
SWS (min)	605.33±18.9	823.34±38.6*
SWS (mean duration; min)	7.25±0.60	8.15±0.65
SWS (latency; min)	39.89±6.5	189.16±12.8**
PS (min)	135.45±7.99	58.84±4.9*
PS (frequency)	55.10±2.1	29.20±1.9**
PS (mean duration; min)	2.7±0.9	1.5±0.2*
PS (latency; min)	48.21±6.8	365.17±24.50**

W - total time spent in waking state, SWS - total time spent in slow wave sleep, PS - total time spent in paradoxical sleep. Statistical analysis was carried out with Student´s t-test: *$p \leq 0.01$, **$p \leq 0.001$.

Experiment II

High performance liquid chromatography (HPLC) procedure

Experiment II was designed to assess the monoamine content. Two other groups of rats without implanted electrodes were included to avoid the monoamine changes produced by mechanical injury due to electrodes on the cortex. Some reports have documented monoamine changes after cortical injury in cerebral regions such as those studied here (Wagner 2005, Bueno-Nava et al. 2008). The control group (n=10) and chronic toluene exposure group (n=10) animals were sacrificed by decapitation. The structures related to sleep (hypothalamus, pons, midbrain and striatum) were dissected out according to techniques described by Glowinski and Iversen (1966), as showed at Fig. 1, and these samples were immediately placed on ice and sonicated in 0.4 N perchloric acid with 0.1% (w/v) sodium metabisulfite, followed by 10 min of centrifugation at 15 000 rpm at 4°C.

Supernatants were stored at -70°C until the chromatographic analysis. The levels of DA, NA, 5-HT and its metabolites HVA and 5HIAA were analyzed by high-performance liquid chromatography (Alltech, HPLC pump, Model: 626) with an electrochemical detector (ESA, Model: Coulochem III) according to Alfaro-Rodríguez and coworkers (2006). Calibration curves for monoamines were constructed using known concentrations of standards prepared in perchloric metabisulfite solution that were injected into the 20 µl loop of the chromatograph. Peaks were integrated with an EZCrom SI (version 3.2.1) program. The monoamine concentrations of samples were obtained by interpolation in their respective standard curves.

We used an Adsorbosphere catecholamine analytical column (Alltech 100X 4.1 mm, 3 µm particle size). The mobile phase consisted of an aqueous phosphate buffer solution (0.1 M, pH 3.2) containing 0.2 mM sodium octyl sulfate, 0.1 mM EDTA and 14% v/v methanol. The flow rate was 1.2 ml/min, and the potential was fixed at +350 mV, E1 = +200 mV and E2 = -200 mV.

Statistical analysis

Monoamine concentration and the metabolite/neurotransmitter ratio were statistically analyzed by one-way analysis of variance (ANOVA), and the subsequent comparisons within groups were performed using the Tukey test ($p \leq 0.05$).

RESULTS

Effects of chronic toluene exposure on sleep

Chronic toluene treatment did not result in differences in body weight compared to control animals. Results showed that chronic toluene exposure affected the organization of sleep patterns. Toluene induced a decrease in W and an increase in SWS. Animals exposed to toluene spent significantly less time in PS, remaining awake during the first 3 h after of the end of 30-day toluene exposure. The amount of wakefulness decreased progressively throughout the 24 hours of sleep recording. There was a complete inhibition of SWS during the first 3 hours of the day (latency of 189.16±12.8 min). Sleep patterns recovered progressively, with SWS reappearing on the second half of the recording day but failing to reach control levels by the end of the 24 hours of sleep recording (see Table I).

PS sleep was affected the most. We observed complete inhibition of PS during the first hours after chronic toluene exposure. Progressive recovery was observed in subsequent hours (latency of 365.17±24.50 min; Table I).
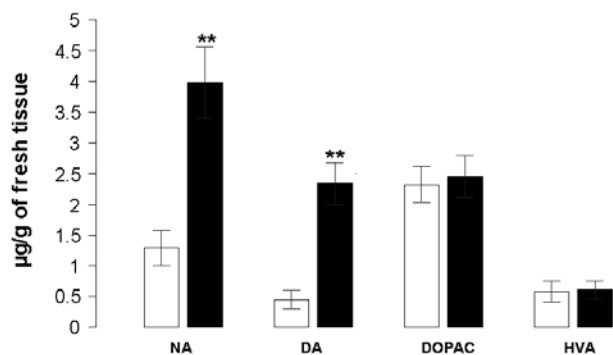
Chronic toluene exposure induced alterations in sleep patterns. Although there was a reduction in the amount of sleep after exposure to toluene, sleep inhibition was not complete because SWS was present throughout the 3 h of recording compared to control (latency of 39.89±6.5 min). Wakefulness decreased by 20% in relation to control values, while SWS increased 25%, and PS was reduced by 37%.

Analysis of noradrenaline (NA), dopamine (DA), homovanillic acid (HVA) and 3,4-dihydroxy-phenylacetic acid (DOPAC).

Total NA, DA, HVA and DOPAC content in the pons and striatum were measured. When the average concentration of monoamines (µg/g of tissue) was analyzed by region, we observed that NA and DA increased in the midbrain (**$p<0.01$). Moreover, this increase in catecholamine levels was related to sleep behavior. In contrast, levels of the DA metabolites DOPAC and HVA levels did not change from control values (Fig. 2).

In the striatum, we found a significant increase in NA (*$p<0.05$), DA (**$p<0.01$) and DOPAC (**$p<0.01$) levels after chronic exposure to toluene. HVA levels did not change after exposure to toluene (Fig. 3).

Analysis of serotonin (5-HT) and 5-hydroxy-indolacetic acid (5-HIAA)

Total 5-HT and 5-HIAA content in the pons, midbrain, hypothalamus and striatum (µg/g of

Fig. 2. Average concentration of monoamines in the midbrain (µg/g of fresh tissue), evaluated during control treatment (white bars) and after chronic toluene exposure (black bars). NA, DA, DOPAC, and HVA were measured by HPLC. Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *$p<0.05$; subsequent comparisons within groups were performed using the Tukey test, **$p<0.01$.
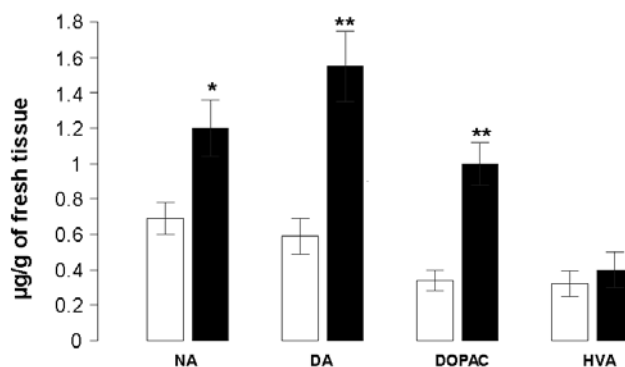
Fig. 3. Average concentration of monoamines in the striatum (µg/g of fresh tissue), evaluated during control (white bars) and after chronic toluene exposure (black bars). NA, DA, DOPAC, and HVA were measured by HPLC. Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *$p<0.05$, and subsequent comparisons within groups were performed using the Tukey test *$p<0.05$, **$p<0.01$.

fresh tissue) were measured. The levels of 5-HT did not change in the midbrain but increased significantly in the pons (*$p<0.05$), while a significant reduction was observed in the hypothalamus (*$p<0.5$) and striatum (*$p<0.05$; Fig. 4). With regard to 5-HIAA content, we observed a significant increase in the pons (**$p<0.01$), midbrain and striatum (**$p<0.01$), with a significant decrease in the hypothalamus (*$p<0.05$; Fig. 5). 5-HT content in the striatum decreased; however, levels of its metabolite 5-HIAA increased after chronic exposure to toluene (Figs. 4–5).

Monoamine ratio analysis

Monoamine ratio analysis of the DOPAC+HVA/DA showed that after toluene exposure, DA increased significantly more than its metabolites DOPAC and HVA both in the midbrain (**$p<0.01$) and in the striatum (*$p<0.05$; Fig. 6). In contrast, 5-HIAA/5-HT ratio analysis revealed that levels of the metabolite 5-HIAA increased more than those of its precursor in both the midbrain (*$p<0.05$) and pons (*$p<0.05$). Statistical significance was reached only in the analysis conducted with samples from the midbrain (Fig. 7).

DISCUSSION

Previous studies have shown that the effects of toluene may involve disruptions in catecholamine levels (Arito et al. 1985, Von Euler et al. 1988). However, there are some important differences that must be noted to discuss the relevance of our results. First, Arito and coauthors (1985) used animals with intraperitoneal toluene administration. We think that this type of administration is not consistent with how toluene exposure occurs in the real world. Toluene is present in a wide variety of industrial products, such as paints, varnish, nail varnish and some adhesives. It is also used in the manufacture of gasoline and in industrial charcoal processes. Thus, the compound is primarily inhaled by industrial workers in a chronic pattern. In addition, in some undeveloped countries, toluene is used as an inhalable drug. Thus, unlike Arito, we administrated toluene via inhalation to mimic a common way by which toluene enters the organism. In the case of Von Euler and coworkers (1988), monoamines were measured using quantitative histofluorimetry, which estimates catecholamine levels in nerve terminals and is not comparable to the levels measured using HPLC. Von Euler also used HPLC, but the catecholamine levels were determined in the frontal cortex and the substantia nigra. We assessed catecholamine levels by means of HPLC in areas related to sleep. The results obtained in the present work indicate that chronic exposure to toluene vapors significantly affects various stages of wakefulness and behavior (resting, tremor, hindlimb abduction, and Straub tail). These results suggest that changes in PS do not occur as compensation after an increase in the total levels of SWS but rather as the result of the breakdown of mecha-
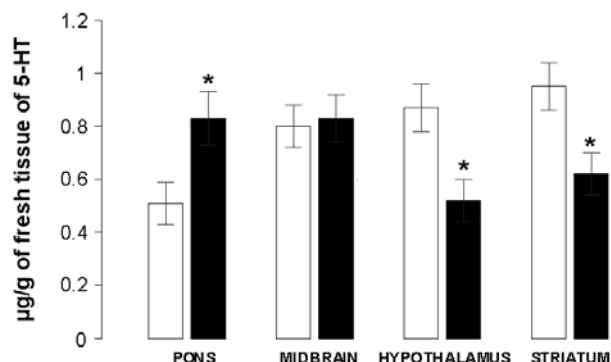

Fig. 4. Average concentration of 5-HT (µg/g of fresh tissue), in different brain regions. Control (white bars) and after chronic toluene exposure (black bars). Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *$p<0.05$, and subsequent comparisons within groups were performed using the Tukey test, *$p<0.05$.
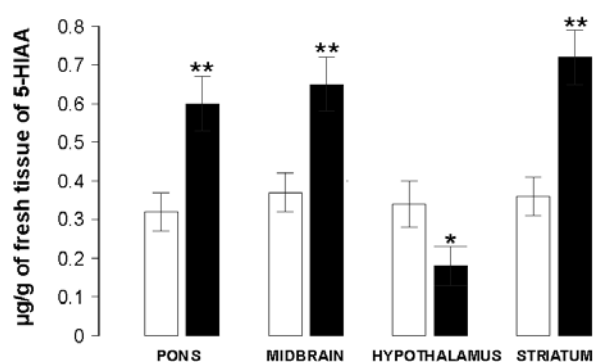

Fig 5. Average concentration of 5-HIAA (µg/g of fresh tissue), in different brain regions. Control (white bars) and chronic toluene exposure (black bars). Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *p<0.05, and subsequent comparisons within groups were performed using the Tukey test, *$p<0.05$, **$p<0.01$.

nisms that participate in PS generation and maintenance. It has been proposed that PS is generated by cholinergic neurons located in the pons and modulated by 5-HT (Kayama and Koyama 2003). We found a significant decrease of PS in total sleep recordings and an increase in SWS in rats exposed chronically to toluene vapors. These rats also showed high levels of 5-HT in the pons. It has been proposed that 5-HT is related to regulation of SWS (Steriade 2004).

Our results also showed that toluene increased DA levels, while those of its metabolite HVA remained unaffected. It is well documented that DA influences acetylcholine release (Stengard 1995, Millan et al. 2007, Tan and Bullock 2008). Additionally, sleep deprivation produces an elevation in SWS related to an increase in the rate of 5-HT turnover (Alfaro-Rodriguez et al. 2006, Senthilvelan et al. 2006a, b). In the present study, we found an increase in the rate of 5-HT turnover in brain regions with the largest neuronal populations of serotonergic cells and an increase in the total amount of SWS. Some consequences of sleep disruptions on humans have been discussed by Oniani (1977)

We found an increase in 5-HT in the pons with a concomitant decrease in this neurotransmitter in hypothalamus. These differences may result from the inhibitory effects that 5-HT exerts on the autoreceptors in the dorsal raphe nucleus, which in turn induced a decrease in the release and metabolism of 5-HT in the suprachiasmatic nucleus (SCN; Steriade 1992).

These results indicate that serotonergic mechanisms play a role in some of the effects of toluene inhalation in rats but cannot explain the absence of tolerance development after chronic exposure to toluene.

The hypothalamus is a structure that is regarded as the primary site of mammalian circadian clock regulation and is known to be under considerable control by the 5-HT system (Glass et al. 2003). Therefore, it is probable that the rate of metabolism of toluene differs with different exposure times because of the circadian rhythm of oxidase activity. These records suggest that the circadian rhythm of enzymatic activity is one of the causes of circadian differences in the susceptibility to toxicity after exposure to toluene (Pouzet 2002).

The levels of the metabolite 5-HIAA increased in the midbrain and striatum. Moreover, DA, NA and DOPAC were all found to be increased in striatum; however, HVA levels were not altered in either the striatum or the midbrain. These results demonstrate that only those metabolites broken down by monoamine oxidase (MAO) displayed markedly increased concentrations. This increase was especially clear in the striatum, where there are both serotoninergic and dopaminergic synaptic boutons. HVA, which requires catechol-O-methyl transferase (COMT) for its synthesis, was not affected by toluene exposure. The results showed that toluene exposure could increase the levels of MAO and decrease the levels of COMT.

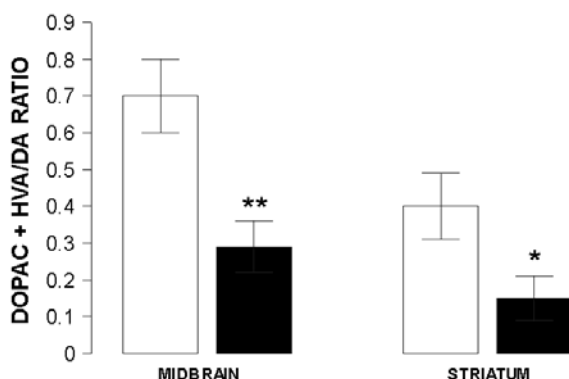Subchronic exposure to 40 ppm toluene in rats elicited sensitization to toluene-induced narcosis and noise

Fig. 6. DOPAC+HVA/DA ratio analysis in the midbrain and striatum. Control (white bars) and after chronic toluene exposure (black bars). Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *$p<0.05$, and subsequent comparisons within groups were performed using the Tukey test, *$p<0.05$, **$p<0.01$.
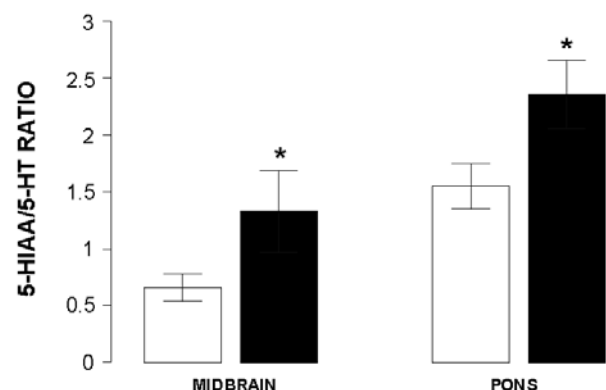

Fig. 7. 5-HIAA/5-HT ratio analysis in the midbrain and pons. Control (white bars) and after chronic toluene exposure (black bars). Mean ± S.E.M. Values were statistically compared using one-way ANOVA, *$p<0.05$, and subsequent comparisons within groups were performed using the Tukey test, **$p<0.01$.

and decreased rearing activity. Certain reports have provided evidence that adverse changes in neurobehavioral and neurochemical functions may result from alterations in DA and 5-HT transmission (Berenguer et al. 2003, 2004). In line with our results, other studies have demonstrated that toluene inhalation induces abnormal behavior states resembling the serotonergic syndrome in rats: resting, tremor, hindlimb abduction, Straub tail, head weaving and rigidity. These results indicate that 5-HT syndrome may be a consequence of altered serotonergic mechanisms (Yamawaki et al. 1982, Castilla-Serna et al. 1993, Von Euler et al. 1998). Riegel and others (2004) proposed that toluene treatments increase DA and 5-HT levels in the caudate nucleus, substantia nigra and nucleus accumbens. Measurements of the metabolite DOPAC further suggested a change in turnover. Such altered monoamine activity suggests that toluene-induced neurotoxicity may result from the generation of free radicals by the pathway suggested by Calderón-Guzmán and colleagues (2005a), in which the hydroxyl group (OH) moves away from the methyl group (CH_3) attached to the aromatic ring of solvents.

The SWS increase and PS deficit observed in this study suggest an enhancement of inhibitory processes that induced excitation following exposure to solvents such as toluene. Our results are supported by studies suggesting that EEG synchronization results from the active inhibition of thalamocortical neurons (Steriade et al. 1990, Contreras and Steriade 1995). Chronic toluene exposure induces changes in the serotonergic and dopaminergic systems with consequences on sleep and possibly on other behavioral areas such as working memory (Wilkość et al. 2010) Regarding that regional differences in the vulnerability of dopaminergic neurons have been described (Marti et al. 2009), discrete effects of toluene on this system must not be discarded.

CONCLUSION

We conclude that toluene exposure disrupts the sleep-awake cycle by affecting the catecholaminergic response in cerebral areas related to sleep. These observations have important implications for human health because sleep is relevant to learning and memory. Additional studies are needed to elucidate the mechanisms underlying sleep disturbance in workers and young individuals who are exposed to such solvents.

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