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Structural neuroplasticity induced by melatonin in entorhinal neurons of rats exposed to toluene inhalation

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Several clinical studies have shown that abusing volatile solvents, mainly toluene, produces neurological, neuropathological and neuropsychiatric disorders. Symptoms of these disorders include loss in impulse control, distractibility and memory deficits, which are associated with mild brain atrophy. The entorhinal cortex is critically involved in mnemonic processes, and memory disorders are the major symptom detected in chronic solvent abusers. Therefore, in the present study, we evaluated (1) whether the entorhinal neuronal morphology was impaired by subchronic toluene exposure and (2) if melatonin protected the neuronal cytoarchitecture, as has been demonstrated in neocortical neurons. Consistent with our previous findings, the present study indicates that the entorhinal cell dendritic arborization was significantly reduced in toluene-exposed animals, and melatonin administration significantly rescued the reduced dendritic branching induced by toluene neurotoxicity.

Key words: entorhinal cortex, toluene inhalation, dendritic impairment, melatonin, rats

Intentional inhalation of volatile solvents is widespread in children and adolescents who live in extreme poverty and chaotic environments (Uzun and Kendirli 2005). Toluene, *n*-hexane, and ethyl acetate are the most frequently abused volatile solvents because they are present in most household products and are, therefore, readily available. Several clinical and pathological studies have shown that chronic solvent abuse, mainly involving toluene, produces several neuropsychiatric disorders, such as distractibility, hallucinations, loss in impulse control, and dementia (Chouanière et al. 2002, Filley et al. 2004). These disorders are associated with structural brain abnormalities, including mild cerebral and cerebellar atrophy, white matter damage, and hypo-/hyperperfusion brain foci (Lazar et al. 1983, Hormes et al. 1986, Kucuk et al. 2000, Aydin et al. 2009). Because the human entorhinal cortical network is often associated with mnemonic dysfunction (Filley et al. 2004, Coutureau and Di Scala 2007), it is likely that toluene abuse causes deterioration in the entorhinal neural cytoarchitecture. In this vein, animal models are useful because they allow us to control for a number of variables (i.e., the use of other substances). In addition, animal models permit a proper morphological analysis of the neuronal structure.

Previous studies have demonstrated that toluene-exposed animals exhibit several behavioral disorders, including a delayed performance in waiting-for-reward task (Bowen and McDonald 2009), increased or decreased locomotor behavior and various learning and memory deficits (Hinman 1987, Pascual et al. 1996). In the hippocampus, animals exposed to toluene exhibited a decrease in neuronal proliferation (Seo et al. 2010), loss of pyramidal neurons (Korbo et al. 1996), gliosis (Gotohda et al. 2000) and changes in the expression of NMDA receptor subunits (Bale et al. 2005). Additionally, in a previous study, we demonstrated for the first time that, post-weaning, toluene inhalation severely impaired the dendritic maturation of neocortical pyramidal neurons located in the frontal, parietal and occipital cortices (Pascual et al. 2010); however, it is unknown whether the entorhinal cortex is also vulnerable to toluene exposure. Therefore, in the present work, we examined the effect of subchronic toluene inhalation on dendritic length and branching of layers II/III entorhinal pyramidal neurons in the rat.

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There is evidence suggesting that the neurotoxicity of toluene can be minimized by melatonin, a powerful antioxidant and neurohormone derived from the amino acid tryptophan. For example, brains of animals exposed to toluene vapors and treated with melatonin had reduced free radical production, lipid peroxidation and gliosis (Baydas et al. 2003). In addition, we recently demonstrated that poor neocortical dendritic arborization in toluene-exposed animals could be recovered by melatonin administration (Pascual et al. 2010). However, it is unknown whether melatonin can compensate for structural damage induced by chronic toluene inhalation in a phylogenetically older cortico-limbic region (i.e., entorhinal cortex). Accordingly, the second objective of this study was to evaluate the ability of melatonin to restore the pyramidal entorhinal neuronal structure lost in rats exposed to chronic toluene inhalation.

Pregnant Sprague-Dawley albino rats were individually housed in standard laboratory cages (50 × 30 × 20 cm) and maintained under controlled conditions of light (12/12 h), temperature (21 ± 2ºC), and humidity.

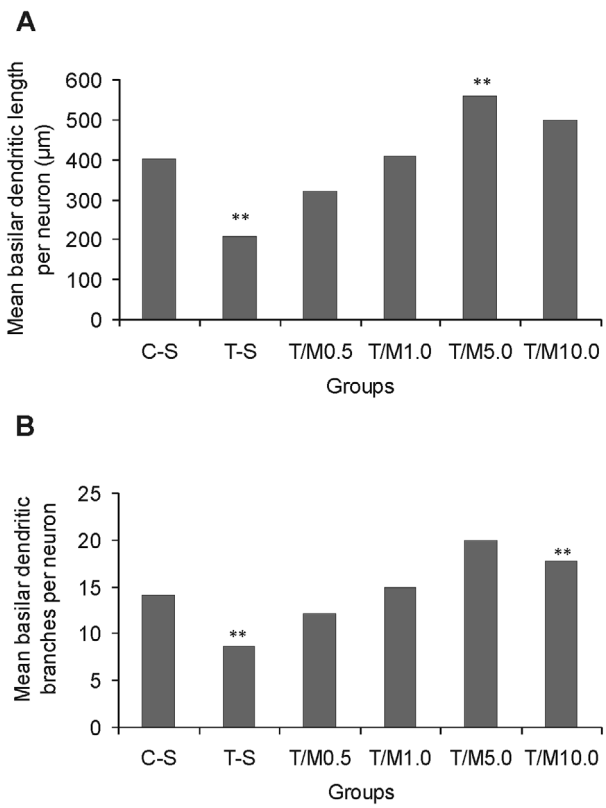

Fig. 1. Basilar dendritic length (A) and branches (B) per neuron of control, toluene-treated and toluene/melatonin (T/M) -treated rats. All comparisons were made in reference to the control group. Data are means ± SEM; ** $P<0.01$ (one-way ANOVA test).

Twelve hours after birth (postnatal day 0, P0), litters were culled to 8 pups per mother (5 males and 3 females) and remained undisturbed throughout lactation. At postnatal 21 days (P21), animals were weaned and divided into two groups counterbalanced by weight: toluene-exposed rats (n=42) and air-control rats (n=10). Both groups had food and water available *ad libitum* and were maintained in the same room with regulated environmental conditions (3–4 rats per cage). At P22, (at 05:00 PM) toluene-exposed male animals were placed, one at a time, into a sealed glass chamber (25 liters) with a vent in the floor and exposed to toluene vapors 10 min/day for ten consecutive days (P22–P32). Toluene (purity 99.5, A.C.S; Merck) was embedded in filter paper and aerosolized by a fan. According to the size of the glass chamber and the physicochemical properties of toluene, animals were exposed to an atmosphere containing about 5 000–6 000 ppm of toluene (Shelton 2007, Shelton and Slavova-Hernandez 2009). It is important to emphasize that, under these conditions, toluene blood concentration was approximately 81 µg/ml (Shelton and Slavova-Hernandez 2009), similar to the level described in toluene abusers (Thiesen et al. 2007). Control animals were handled in a similar way but in a clean-air glass chamber.

Following exposure to toluene (P32), animals were reassigned into 6 experimental groups: (1) control-saline (C–S; n=10); (2) toluene-saline (T–S; n=10); (3) toluene-melatonin 0.5 mg/kg (T–$M_{0.5}$; n=8); (4) toluene-melatonin 1.0 mg/kg (T–$M_{1.0}$; n=8); (5) toluene-melatonin 5.0 mg/kg (T–$M_{5.0}$; n=8); and (6) toluene-melatonin 10 mg/kg (T–M_{10}; n=8). Melatonin (Sigma-Aldrich) was prepared in 10% ethanol-saline (0.9% NaCl and 0.1% ethanol; Merck) and injected intraperitoneally (0.1 ml). The routes of administration and dosages used in this study are similar to those in previous studies that demonstrated neuroprotective effects (Letechipía-Vallejo et al. 2007, Carloni et al. 2008, García-Chávez et al. 2008). Melatonin and saline were administered daily (at 11:00 AM) between P32 and P38. At P40, animals were weighed and sacrificed under deep ether anesthesia. Brains were carefully dissected out and immersed in a Golgi-Cox-Sholl solution for 30 days (Sholl 1953). The brains were then dehydrated, embedded in celoidin, cut coronally at 120 µm and mounted on slides with coverslips for histological studies. To ensure a uniform neuronal sampling in all brains, selected cells had to meet the following criteria: (1) possession of a well-defined

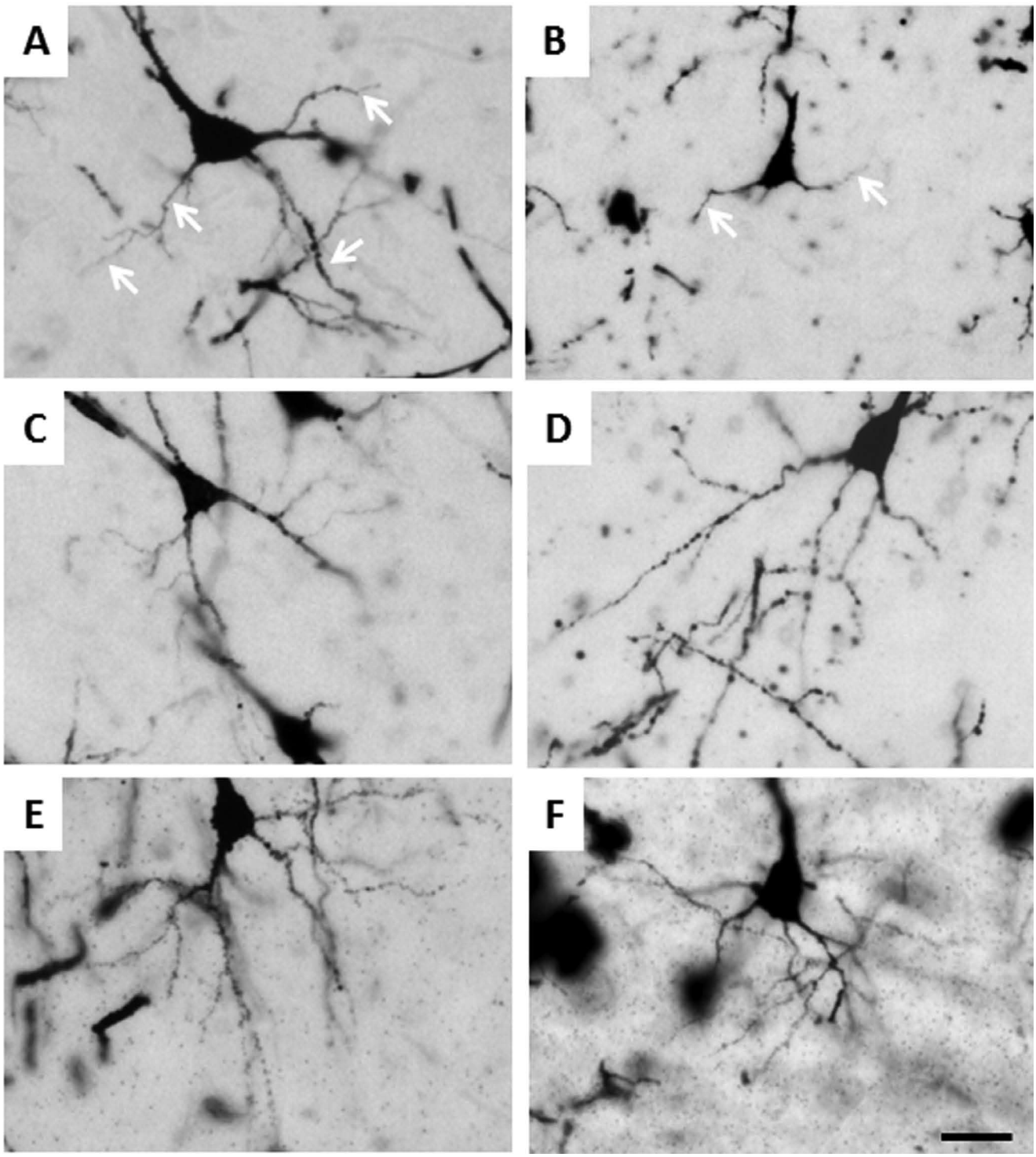

Fig. 2. Photomicrographs of layer II/III pyramidal neurons located in the entorhinal cortex from different experimental groups. (A): typical pyramidal cell found in control animals, where the dendritic processes are clearly more developed than those of animals exposed to toluene (B; see white arrows). (C–F): Dendritic phenotype of entorhinal neurons of animals exposed to toluene and treated with varying doses of melatonin (C – 0.5 mg/kg; D – 1.0 mg/kg; E – 5.0 mg/kg; F – 10.0 mg/kg). Scale bar is 30 μm (same scale for all photomicrographs).

pyramidal silhouette, (2) being the most differentiated neuron in the optical field, (3) being completely impregnated, (4) showing symmetrical dendritic processes, and (5) being located in II/III superficial cortical layers (300–600 μm from the pial surface) of the lateral entorhinal cortex, which were carefully delimited from the coordinates described in the Rat Stereotaxic Atlas (Paxinos and Watson 1998). Preparations were coded to avoid bias during collection of morphological data. Entorhinal sections were analyzed under an Olympus CX-3 light microscope (400×), and selected neurons that met the above criteria were captured with a digital camera attached to the microscope (Olympus CCD 5.0) and analyzed using Micrometrics SE Premium V-2.8 software. The following dendritic parameters were quantified: (1) basilar dendritic length/neuron (μm), (2) basilar dendritic branches/neuron, and (3) number of basilar branches per order. Dendritic branches were classified according to the method of Coleman and Riesen (1968): dendrites leaving the cell soma were cataloged as first order; direct branches from first-order dendrites were designated as second order, and so on. A total of 416 neurons were sampled from the entorhinal cortex (C–S: 80; T–S: 80; T–$M_{0.5}$: 64; T–$M_{1.0}$: 64; T–$M_{5.0}$: 64; T–$M_{10.0}$: 64). Animals were treated and housed in accordance with the Guidelines of the U.S. Public Health Service and NIH "Principles of Laboratory Animal Care". Statistical analysis was performed using a one-way ANOVA test and the *post-hoc* Scheffé test (STATA 9.1 software) when significant differences ($P<0.05$; $P<0.01$) were detected.

Toluene-exposed animals showed a significant reduction in dendritic length and branches/neuron compared to age-matched controls (Figs 1A and 1B, respectively; $P<0.01$). However, the animals exposed to toluene vapors and treated with melatonin recovered that majority of their stunted dendritic processes. Consistent with our previous findings, toluene-exposed animals that were treated with 5–10 mg/kg of melatonin showed more numerous dendritic arbors than were seen in controls, suggesting a trophic-like effect. Figure 2 shows example micrographs of pyramidal neurons located in the entorhinal cortex from different experimental animals. Fig. 2A shows a typical entorhinal neuron found in control animals where the dendritic processes are clearly more developed than those of animals exposed to toluene (Fig. 2B; see white arrows). In contrast, the dendritic morphology of entorhinal neurons in animals exposed to toluene and treated with a range of doses of melatonin were significantly recovered (Fig. 2C–F). Dendritic distal branches were more severely impaired by toluene than were the proximal ones (Fig. 3; 2nd order: ~25%; 3rd order: ~50%; and 4th order: ~79%), and melatonin increased the branching in the more distal dendritic arbors. Finally, no significant differences in body weight were observed between experimental groups.

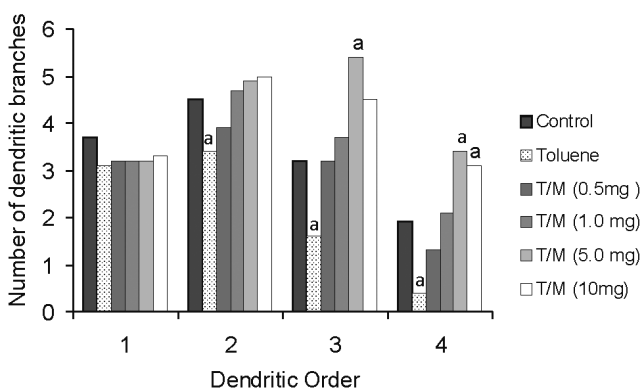

Fig. 3. Number of dendritic branches per dendritic order (see Methods) in control, toluene-treated and toluene/melatonin (T/M)-treated animals. (a) $P<0.01$ (one-way ANOVA test) in reference to control animals.

In the current study, we demonstrated that toluene inhalation significantly altered dendritic length and branching of pyramidal neurons in rat entorhinal cortex. Additionally, melatonin restored the dendritic arborization, which was lost following toluene exposure. These results are consistent with our previous study on neocortical pyramidal neurons (Pascual et al. 2010).

In this study, we performed a detailed morphometrical analysis of individual dendritic orders (Coleman and Riesen 1968). This approach is superior to global dendritic analysis, because it allows us to identify the most vulnerable regions of the neuron to toluene neurotoxicity and the beneficial effect of melatonin. The most profound impact of toluene inhalation was in the distal basilar dendrites (see Fig. 3). Previous studies have demonstrated that dendritic outgrowth follows a proximal-to-distal gradient of vulnerability (Greenough et al. 1973); therefore, it is possible that the greater impairment detected on distal branches can be related to the heterochronic rate of dendritic maturation. In the rat, proximal dendrites arising directly from the soma (1st order dendrites) reach maturity by the 12th postnatal day, but the growth rate in more-distal branches continues slowly until adulthood (Petit et al. 1988).

In the present study, the concentration and length of toluene exposure (5 000–6 000 ppm/10 min/day) mimics that of solvent abuse in children and adolescents, in which they inhale repeatedly and chronically from a chemical-saturated bag (or other container) through the mouth and nose ("bagging") (Filley et al. 2004, Thiesen et al. 2007).

Although, in the present investigation, we did not evaluate the functional consequences of entorhinal dendritic alterations, it is possible that the clinical disorders described in individuals with a history of chronic toluene abuse (e.g., memory loss) (Filley et al. 2004, Copur et al. 2005) could be related to structural alterations of entorhinal neurons. In fact, layer II/III entorhinal pyramidal neurons make connections not only with hippocampal structures but also with other brain regions, including perirhinal, postrhinal, piriform, prefrontal, and parietal cortices, where dysfunction is closely associated with several mnemonic disorders (Coutureau and Di Scala 2007).

Although the exact mechanisms involved in the neurotoxic effect of toluene on dendritic growth are unknown, a good candidate appears to be oxidative stress. Rats exposed to toluene demonstrated an increase in free radical formation and lipid peroxidation in the hippocampus, cerebral cortex and cerebellum (Baydas et al. 2003). Additionally, because toluene is highly lipophilic, it can alter the physicochemical properties of cell membranes and ganglioside concentrations and, as a consequence, may impair dendritic structure (Walkley et al. 2000, Edelfors et al. 2002). In addition, toluene exposure can interfere with NMDA receptor function, in particular NR2A and NR2B subunits (Bale et al. 2005), and the regulation of calcium influx through NMDA receptors has been found to be a key modulator of dendritic outgrowth. Therefore, it is possible that glutamatergic dysfunction could be another pathophysiological mechanism involved in dendritic abnormalities induced by toluene (Konur and Ghosh 2005, Lei et al. 2006).

In the present study, we show that administration of melatonin produced a significant recovery of dendritic branching in rats exposed to toluene vapors. The distal branches were more responsive than were proximal ones, which was probably due to the differential rate of maturation discussed above. Although the "therapeutic" effect of melatonin was detected at all doses used, the most dramatic effect was observed in the number of terminal segments at 5–10 mg/kg. This dose range of melatonin provided the best neuroprotection, which is consistent with our previous study in neocortical pyramidal neurons (Pascual et al. 2010), as well as in other animal models. For example, rats subjected to acute global cerebral ischemia and treated with melatonin (10 mg/kg), showed less hippocampal neuronal damage than animals receiving vehicle only (Letechipía-Vallejo et al. 2007). Similarly, animals subjected to hypoxic-ischemic stroke and treated with melatonin (5–10 mg/kg) demonstrated a significant reduction in the extent of injury, along with increased dendritic branches and spine densities in both hippocampal and prefrontal neurons (González-Burgos et al. 2007, García-Chávez et al. 2008). Because increased production of free radicals appears to play a key role in neuronal damage following toluene exposure, it is possible that melatonin exerts its neuroprotective or neurorescue effects by neutralizing the free radical overproduction and/or by stimulating the gene expression of antioxidant enzymes. In addition, it has been demonstrated that melatonin receptor agonists can increase the expression of brain derived neurotrophic factor (BDNF) (Imbesi et al. 2008), and BDNF can potentiate the dendritic outgrowth (McAllister et al. 1999). Accordingly, it is likely that the neurotrophic-like effect of melatonin can be indirectly potentiated by melatonin-induced BDNF expression. Finally, another possible mechanism that accounts for the neuroprotective effect of melatonin could be changes in the dynamics of the cytoskeletal network, because melatonin can promote neuritic outgrowth by increasing tubulin polymerization and microfilament redistribution (Benitez-King 2006).

In summary, the present findings indicate that melatonin induces a significant dendritic outgrowth in entorhinal pyramidal neurons, which supports previous reports. In future studies, it will be important to study whether structural alterations observed in the entorhinal cortex correlate with neurobehavioral changes in regards to cognitive and limbic function. Because melatonin has been shown to be effective clinically, even at high doses (Furio et al. 2007), the data obtained in this and other studies could be useful in humans who are frequently exposed to high concentrations of organic solvents, either for occupational reasons or in cases of abuse.

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