

# Toluene Alters Mu-Opioid Receptor Expression in the Rat Brainstem

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**Abstract:** Toluene is an ototoxic organic solvent widely used in industry and could be a cause of sleep apnea. Acute toluene administration in rats induces an increase in the number of neural cells immunostained for mu-opioid receptors in several brainstem nuclei, such as the inferior colliculus, dorsal and lateral periaqueductal gray and dorsal raphe, without changes in the superior colliculus and the interpeduncular and lateral reticular nuclei. These data suggest that mu-opioid receptors could be involved in toluene-induced neurotoxic effects on the physiological regulation of breathing during sleep, and auditive function.

**Key words:** Toluene, Mu-opioid receptors, Rat brain

## Introduction

Glue sniffing<sup>1)</sup> and occupational exposure to toluene<sup>2)</sup> can generate ototoxicity<sup>3,4)</sup> and neuropathological changes<sup>5)</sup>, such as respiratory depression<sup>6)</sup> and sleep apnea<sup>7)</sup>. Toluene is detected in all brain areas after administration, with the highest concentrations in the brainstem<sup>8)</sup>.

Mu-opioid receptors are widely distributed throughout the central nervous system, including several brainstem regions, such as the dorsal raphe, involved in physiological regulation of sleep<sup>9)</sup>, the inferior and superior colliculus, that play a role in sensory integration, the periaqueductal gray lateral and dorsal, that exert a modulatory action on the basic brainstem respiratory rhythm<sup>10)</sup>, the lateral reticular nucleus and the interpeduncular nucleus<sup>11–13)</sup>.

It has been recently shown that chronic toluene exposure alters enkephalin immunostaining in the rat brainstem<sup>14)</sup>. However, little is known concerning the effects of toluene exposure on mu-opioid receptors. This being the case, we focused on analyzing regional mu-opioid receptor immunostaining in the rat brainstem.

## Materials and Methods

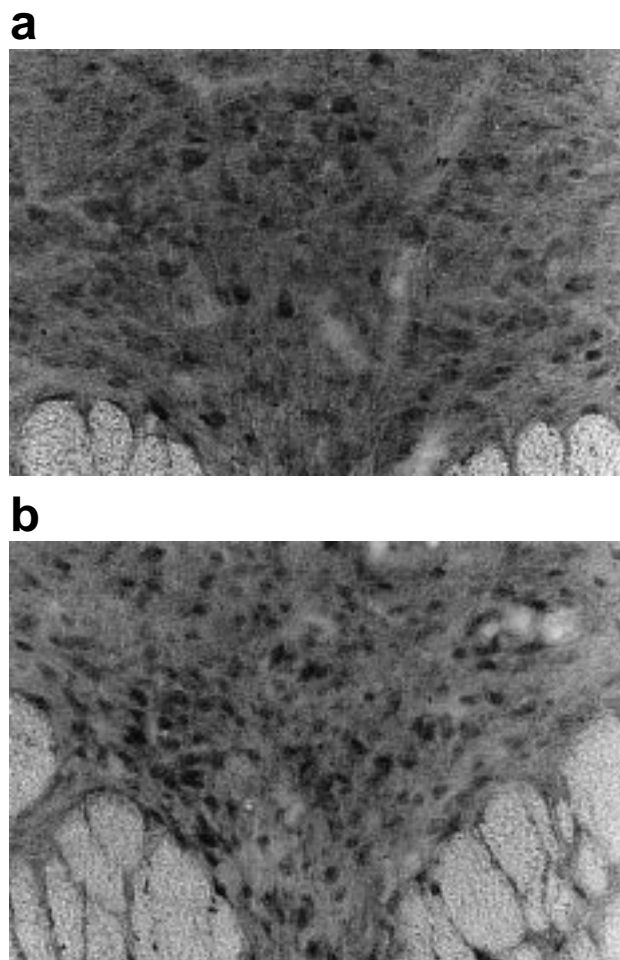
The experimental animals were carefully handled and all experiments were carried out in accordance with the law, avoiding animal suffering. Toluene with purity of 99% (Analytical reagent grade, Quimon Chem. Co., Spain) was used. Toluene was diluted with olive oil at a concentration of 1 ml/ml and administered intraperitoneally to the experimental group (n=6) at a dose of 1.3 ml/kg/day for 5 consecutive days. The selected dose was 1/2 of the LD<sub>50</sub> per day. This dose and this kind of administration have been employed to carry out studies on the adverse effects of organic solvents<sup>15)</sup>. The LD<sub>50</sub> in rats has been found in our laboratory to be 2.61 ± 0.41 ml/kg/day, calculated by the Bliss method. The control group (n=6) was given 0.9% NaCl solution in the same volume and duration as the experimental group. Two hours after the last treatment, the animals were anaesthetized with Equithensin (2 ml/kg), an alcoholic solution of nembutal and chloral hydrate (Sigma-Aldrich Química S. A.), intraperitoneally and perfused transcárdially under deep anaesthesia with saline plus 50 mM phosphate buffer, pH 7.4, followed by 4% paraformaldehyde (Sigma-Aldrich Química S. A.). The brains were removed, cut into smaller pieces and then immersed in the same fixative medium overnight. They were stored for 2 days in 0.1 M phosphate buffer containing

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30 % sucrose at 4°C. 60 micrometer sections were cut using a cryostatic microtome (Cryocut 3.000, Leica España, Barcelona, Spain) with an stereotaxic atlas guide and immunostained for mu-opioid receptor with polyclonal antisera raised in rabbits. The antigens were detected by the avidin-peroxidase technique, using 3, 3'-diaminobenzidine as chromogen (Sigma-Aldrich Química S. A.). Following reduction of endogenous peroxidases with 1% hydrogen peroxide (Sigma-Aldrich Química S. A.) and blocking of nonspecific background staining with 5% normal goat serum (NGS) (Sigma-Aldrich Química S. A.), the sections were incubated with the following immunoreagents: 1-primary antiserum: rabbit anti mu-opioid receptor (Chemicon International Inc., Temecula, CA, USA), a commercially obtained polyclonal antibody raised in rabbits and directed against mu-opioid receptor 3rd extracellular loop peptide (dilution 1:1.000); 2-goat anti rabbit immunoglobulin: goat antirabbit biotinilated (Chemicon International Inc.), dilution 1:200; 3-avidin-peroxidase complex: strept ABC complex HRP (Dako A/S, Glostrup, Denmark), dilution 1:300; 4-chromogen: 3, 3'-diaminobenzidine (Sigma-Aldrich Química S. A.), 0.3 mg/ml in 0.2 M Tris HCl buffer containing 0.03% hydrogen peroxide. Each step was followed by an appropriate wash per triplicate in phosphate buffer saline and 0.3% Triton X-100 (Sigma-Aldrich Química S. A.) was used. Sections were carefully extended, dehydrated and mounted (DPX mountant for histology, Fluka Chemie AG, Buchs, Switzerland), and examined with an Olympus BX50F optic microscope (Olympus Optical Co. Ltd., Japan). A Leica image analysis system (Quantimet 500 MC, Leica España S. A., Barcelona, Spain) was used to obtain digitized images from brainstem slices and to trace a target area. To valorate the density of positively stained neural cells, random counts were manually made in a particular area that was previously performed by tracing contours with a cursor. At least six random counts of positively immunostained neural cells for an particular area were made in every section analyzed, and no less than 10 sections were selected in every rat (n=6) and brain region. The results obtained were referred as the number of neural cells/mm<sup>2</sup> positively immunostained for mu-opioid receptors, in treated animals and controls (mean ± S.E.M.). Differences between means were calculated by the Student's T test. Statistically significant differences were considered at p<0.01.

## Results

Utilization of a specific polyclonal antibody against mu-opioid receptors showed the presence of abundant round cells intensely immunostained in the dorsal raphe, inferior



**Fig. 1** Light micrographs of brainstem coronal sections immunostained for mu opioid receptors in control (1a) and toluene treated rats (1b).

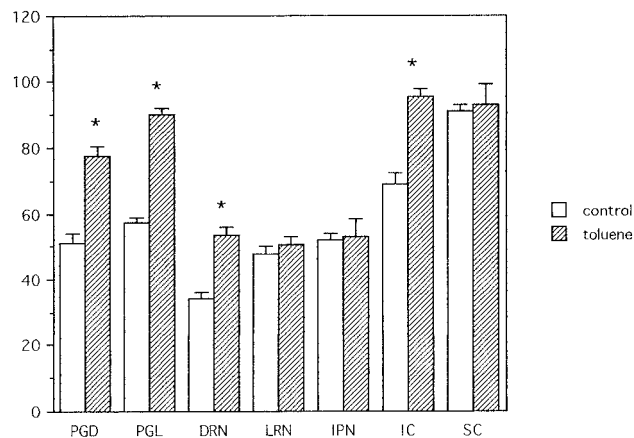
Immunostained neural cells can be seen in the dorsal raphe. 16×.

and superior colliculus, periaqueductal gray lateral and dorsal, interpeduncular nucleus and lateral reticular nucleus.

An increase in the density of neural cells immunostained for mu-opioid receptors in the periaqueductal gray dorsal and lateral, dorsal raphe (Fig. 1) and inferior colliculus, without changes in superior colliculus, interpeduncular nucleus and lateral reticular nucleus was found, with respect to controls (Fig. 2).

## Discussion

Toluene is an ototoxic organic solvent that increases free intracellular calcium levels in the spiral ganglion and the outer hair cells<sup>16</sup>. Although its action focuses preferently on cochlear middle and mid-apical turns<sup>17</sup>, electrocochleographic recording of auditory evoked



**Fig. 2** Graphic representation of variations in the density of neural cells immunostained for mu-opioid receptors in the selected brain regions, expressed in cells/mm<sup>2</sup>, in control and toluene treated rats. The key for the representation is as follows: PGD (periaqueductal gray dorsal), PGL (periaqueductal gray lateral), DR (dorsal raphe), IPN (interpeduncular nucleus), LRN (lateral reticular nucleus), IC (inferior colliculus), SC (superior colliculus).

potentials from the round window show a hearing deficit also in the mid-low frequency region in rats, after toluene exposure<sup>18</sup>). Thus, it has been confirmed, through recording of inferior colliculus auditory-evoked potentials, that chronic exposure to toluene alters auditory function<sup>19</sup>).

Although it is well known that cochlear damage by acoustic overstimulation impairs the sensitivity of auditory nerve fibers and reduces the neural output of the cochlea, electrophysiological studies have shown an increased activity in the inferior colliculus after experimental induction of cochlear lesions, suggesting an involvement of this region in the adaptive changes of central auditory pathway function that account to compensate the reduced neural activity generated by the cochlea<sup>20</sup>). Accordingly, in the results here reported, an increase in mu-opioid receptor immunostaining in the inferior colliculus has been observed. This is in agreement with previous reports suggesting a connection between opioid receptors and auditory neural function<sup>21</sup>). Thus, neurons double-labeled for GABA(A) receptors and opioid receptors (mu and kappa) have been described in the inferior colliculus<sup>22</sup>), suggesting that both GABAergic and opioidergic systems could be involved in the functional regulation of central auditory pathway. Due to the physiological role of the inferior colliculus in auditory signal processing, mu-opioid receptors could be involved in toluene-induced neurotoxic effects on auditory function, as well as in the adaptive response of the central auditory pathway against cochlear damage.

It is well known that opioids decrease respiration<sup>23,29</sup>) through

a suppression of brainstem baseline inspiratory neuronal activity<sup>24</sup>), and mu-opioid receptors are involved in respiratory depression after opioid overdose<sup>25</sup>). Thus, it has been described that periaqueductal gray and other suprapontine regions exert a modulatory action on the basic brainstem respiratory rhythm<sup>10</sup>). In the results here reported, a lack of changes in mu-opioid receptor expression in the lateral reticular nucleus, involved in the regulation of breathing, has been observed. However, the number of neural cells immunostained for mu-opioid receptors in the periaqueductal gray dorsal and lateral was increased after toluene administration. These results suggest that mu-opioid receptors in the periaqueductal gray could be involved in the neurotoxic mechanism of toluene-induced respiratory depression.

Occupational toluene exposure can generate sleep apnea<sup>7,30</sup>). Dorsal raphe is involved in the physiological regulation of sleep<sup>9</sup>) and an inhibitory role on REM sleep has been suggested for serotonergic neurons in this region<sup>26</sup>). Thus, alterations in the regulation of respiratory motor output in response to increased levels in non-REM sleep have been described<sup>27</sup>) and mu-opioid receptors in the dorsal raphe are involved in opioid-induced REM sleep inhibition, through activation of G proteins<sup>28</sup>). Accordingly, in the results here reported, an increase in mu-opioid receptor immunostaining in the dorsal raphe has been observed after toluene administration in rats. These results suggest that mu-opioid receptors could be involved in the neurotoxic mechanism of toluene-induced sleep apnea.

Further studies focusing on description of possible alterations in mu-opioid receptor function after toluene exposure are needed to clarify the role of the brainstem opioid system in organic solvent-induced impairments in the regulation of sleep, breathing and auditory function.

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