Toluene Alters Brainstem Enkephalinergic System in Rats

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Abstract: Acute exposure to high doses of toluene can generate respiratory depression. However, neurotoxic mechanism of its action in the brainstem is not completely clear. In this work, acute, but not subchronic, exposure of rats to toluene increased leu-enkephalin immunostaining in several myelencephalic nuclei implicated in cardiorespiratory control. Due to the physiological role of enkephalins in the central regulation of breathing, it is suggested that the enkephalinergic system could play a role in neurotoxic respiratory depression induced by high dose acute toluene exposure.

Key words: Toluene, Enkephalin, Rat brain

Introduction

Toluene is a widely used organic solvent1. Acute exposure to this toxicant occurs through the practice of deliberate inhalation (glue sniffers)2, 3 and occasionally among painters and workers in the chemical industry4-9 and can generate neuropathological changes including respiratory depression10-11. It is detected in all brain areas after administration, with the highest concentrations in the brainstem11. Although little is known concerning the neurotoxic mechanism of toluene-induced respiratory depression, it is well known that enkephalin immunoreactive perikarya and fibers are present in myelencephalon and are abundant in respiration regulatory regions12. Hence, the participation of endogenous opioid peptides in respiratory regulation is strongly supported by their anatomic and biochemical localization in the brainstem respiratory area13. Therefore, it has been shown that excitatory responses of brainstem respiratory neurons to iontophoresis of glutamate are reduced after local application of opioids14, 15. It has been demonstrated that opioids can interfere with glutamatergic excitation16 and they can depress ventilation by reducing glutamatergic transmission. On the other hand, phasic respiratory neurons in rats have been located in the ventrolateral medulla, nucleus ambiguus and lateral reticular nucleus17. A large proportion of these neurons have shown to be cholinceptive12 and changes in rat brain acetylcholine metabolism after acute exposure to toluene have been shown18. However, although changes in catecholamine levels and turnover in the striatum, hypothalamus and cortex and also changes in telencephalic and diencephalic monoaminergic receptor affinities after toluene administration in rats have been described19-21, little is known concerning the effect of toluene on neuromodulatory systems. Based on analogy with other neuropeptides, it has been assumed that the enkephalins are hydrolyzed by specific enzymes leading to their inactivation22, 23 and it has been demonstrated that a major pathway of enkephalin degradation occurs via cleavage of the Tyr1-Gly2 amide bond by several aminopeptidases. Alterations in brain regional aminopeptidase activity24 and changes in brain enkephalin immunostaining pattern, which are partially reversed by ganglioside treatment, in central amygdaloid nuclei have been recently described25. Due to the regulatory role of enkephalins in brainstem cardiorespiratory functions, we focused on analyzing enkephalin immunostaining in several regions of the rat brainstem involved in cardiorespiratory and other autonomic functions after high dose acute and subchronic toluene exposure.

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Materials and Methods

Male Sprague-Dawley rats (n=24), 3 months old, bred in our colony and maintained under conditions of controlled light (12 h) and temperature (24°C), with food and water ad libitum were used in this investigation. 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 two experimental groups of animals. The first group (n=6) was given toluene at a dose of 1.3 ml/kg/day for 5 consecutive days and the second (n=6) was given the same dose of toluene for 12 consecutive days. The selected dose was 1/2 of the LD_{50} per day. This dose and this kind of administration have been employed to carry out studies on the adverse effects of organic solvents. The LD_{50} 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 groups. Two hours after the last treatment, the animals were anesthetized with Equithensin (0.2 ml/kg), an alcoholic solution of nembutal and chloral hydrate (Sigma-Aldrich Quimica S.A., Spain), and perfused transcardially under deep anesthesia with saline followed by 4% paraformaldehyde (Sigma-Aldrich Quimica S.A., Spain), and perfused transcardially under deep anesthesia with saline followed by 4% paraformaldehyde (Sigma-Aldrich Quimica S.A., Spain). Simultaneous processing of control and toluene-treated animals was made. The brains were removed, cut into smaller pieces and then immersed in the same fixative medium overnight. Sixty micrometer sections were cut using a cryostatic microtome (Cryocut 3000, Leica Espana S.A., Spain). Due to the fairly long rostrocaudal course of several reticular nuclei in the brainstem, one of every two sections was selected. Samples were immunostained for leu-enkephalin with a commercially available, high quality polyclonal antisera raised in rabbits. Following reduction of endogenous peroxidases with 1% hydrogen peroxide (Sigma-Aldrich Quimica S.A., Spain) and blocking of non specific background staining with 5% normal goat serum (NGS) the sections were incubated with the following immunoreagents: 1-primary antiserum (rabbit anti leu-enkephalin, dilution 1:2000, 48 h, Chemicon International Inc., U.S.A.); 2-goat anti rabbit immunoglobulin (goat antirabbit biotinilated, dilution 1:200, room temperature, 2 h, Chemicon International Inc., U.S.A.); 3-avidin peroxidase complex (strept ABC complex HRP, room temperature, 2 h, Dako A/S, Denmark); 4-chromogen (3,3'-diaminobenzidine, room temperature, Sigma-Aldrich Quimica S.A., Spain). Each step was followed by triplicate washes in phosphate buffer saline and long incubation periods with continuous agitation and triton X-100 (0.5%) were used in order to increase penetration of the reagents. Sections were carefully extended and mounted (DPX mountant for histology, Fluka Chemie A.G., Switzerland), examined with an Olympus BX50 optical microscope (Olympus Optical Co. Ltd., Japan) and further analyzed with a Leica image analysis system (Quantimet 500 MC, Leica España S.A., Spain), a high speed digital image processing system, equipped with a pipeline structured image array processor and large video memory for the automatic measurement of densitometric parameters as grey tone and several grey image processing functions. The microscope image was taken with a video camera (Sony) and digitized. Optical density, or grey level in each point or pixel, was represented by a number from 0 (black) to 255 (white). Integrated optical density for a particular area was performed by tracing contours with a cursor. Light intensity for the microscope was continuously controlled and measurements of integrated optical density in the inferior cerebellar peduncle were made on every slice analyzed as a control, since this zone showed no staining. Values of integrated optical density were defined as the difference between the control zone and the selected structures on every slice analyzed. Results were referred as increments of optical density in every brain region studied with respect to the inferior cerebellar peduncle, in treated animals and controls (mean ± SEM). Differences between means were calculated by the Student’s T test. Statistically significant differences were considered at p<0.05* and p<0.01**.

Results

Intensity of immunostaining in every brainstem region studied keeps good correlation with grey level in the same zone, registered by transforming the microscope image in a black and white video camera signal, which is analyzed by an appropriate image analysis system. This method allows an indirect but objective estimation of the rate of immunostaining of fibers for a specific antigen in experimental animals with respect to controls. Direct optical microscopic observation was made showing analogous results, and photomicrographs were obtained (Fig. 1). In the control group, neuronal perikarya and a dense and caudally increasing accumulation of enkephalin immunoreactive fibers were seen in the myelencephalic reticular formation, extending ventrally from the nucleus of the solitary tract to the lateral reticular nucleus and nucleus...
ambiguus. This enkephalinergic distribution is coincident with that described in a previous report\(^\text{27}^\text{27}\). Acute toluene exposure alters enkephalin immunostaining (grey level) in several regions of the brainstem. The pattern of leu-enkephalin immunoreactivity in acute toluene-treated rats was similar to the control group except for an increase in grey level that was evident in nucleus ambiguus (p<0.01), paragigantocellular reticular nucleus (p<0.01), lateral reticular nucleus (p<0.05), nucleus of the solitary tract (p<0.05), and spinal trigeminal nucleus (p<0.05), where significant increases were measured (Fig. 2). Pontine reticular nucleus showed a significant reduction (p<0.01). However, subchronic treatment with toluene showed a lack of changes in the above mentioned regions. We could only find a significant increase in grey level in the inferior olivary complex (p<0.05), with a reduction in the substantia nigra (p<0.01) (Fig. 3).

**Discussion**

Although interference with the energy metabolism of the central nervous system and a direct action on glial cells have been suggested\(^\text{28}^\text{28}\), the primary action of toluene could focus...
on membrane fluidity, leading to changes in neurotransmitter release, receptor characteristics and transduction mechanisms\textsuperscript{29,30}. Exposure to this toxicant produced changes in aminopeptidase activity\textsuperscript{24} and enkephalin immunostaining in rats\textsuperscript{25} but failed to generate alterations in neurotensin receptor affinity in rat brain striatal membranes\textsuperscript{30}. It has also been demonstrated that ganglioside pretreatment prevents both toluene toxicity in the rat striatum\textsuperscript{30} and enkephalinergic changes in other regions\textsuperscript{25}. On the other hand, although it is well known that acute exposure to high doses of toluene can generate respiratory depression\textsuperscript{10}, the neurotoxic mechanism of this action is not completely clear. Enkephalinergic neuromodulatory system has been implicated in several brainstem regulatory functions, including respiratory control\textsuperscript{25} and it has been demonstrated that opiates can generate respiratory depression\textsuperscript{32}. Therefore, phasic respiratory neurons in rats are located preferentially in myelencephalic regions with a dense enkephalinergic fiber plexus and abundance of neuronal perikarya immunostained for leu-enkephalin, such as the nucleus ambiguous, nucleus of the solitary tract and several reticular nuclei\textsuperscript{17}, and different studies have shown that the administration of opioids in the pontomedullary area depresses respiration\textsuperscript{33,34} by reducing glutamatergic transmission\textsuperscript{14,16}. In the results here reported, changes in optical density in these regions, previously immunostained for leu-enkephalin, after high dose acute toluene exposure in rats have been described. Therefore, the increase in immunostaining for leu-enkephalin in myelencephalic regions implicated in physiological control of cardiorespiratory functions, such as nucleus ambiguous, nucleus of the solitary tract, lateral reticular nucleus and paragigantocellular reticular nucleus after acute toluene exposure in rats, described here, suggests the possible implication of enkephalins in respiratory depression induced by this toxicant. However, subchronic treatment with toluene generated a lack of changes in enkephalin immunostaining in the above mentioned regions in treated animals with respect to controls. Further studies focusing on description of possible alterations in enkephalin levels and enkephalin receptor function after toluene exposure in brainstem respiratory regions are needed to clarify the role of opioid peptides in neuropathological mechanism of organic solvent-induced respiratory depression.

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**References**

TOLUENE AND ENKEPHALINS


