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Brain Met-Enkephalin Immunostaining after Subacute and Subchronic Exposure to Benzene

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Benzene is used in a wide variety of domestic and occupational activities, and due to its lipophilic nature, it accumulates in lipid-rich tissues like the brain. In this sense, neurotoxic action has long been associated with organic solvent exposure and it has been shown that benzene, injected in a single dose or during a prolongued administration, modifies the content of dopamine, noradrenaline (Paradowski et al. 1985), serotonin and its main metabolite 5-hydroxy indolacetic acid (Paradowski et al. 1984), in several brain regions of the rat, then revealing a stimulating action on brain monoamine synthesis and turnover (Hsieh et al. 1989).

However, information concerning neurotoxic action of benzene exposure *in vivo* on peptidergic neuromodulatory systems is still lacking. Nevertheless, it has been recently described that subacute benzene exposure in rats generates regional changes in brain aminopeptidase activity (Gandarias et al. 1992). These proteolytic enzymes have been widely associated with metabolic control of neuropeptides (Turner 1987) and it has been suggested that they could play a role in benzene neurotoxic mechanism by hypothetically changing regional neuropeptide levels (Gandarias et al. 1992). This being the case, we focused on analyzing met-enkephalin immunostaining in different brain regions of the rat after subacute and subchronic administration of benzene.

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MATERIAL AND METHODS

Male Sprague-Dawley rats (n = 12), 3 months old, bred in our colony and maintained under conditions of controled light (12 h) and temperature (24 ºC), with food and water *ad libitum*, were used in this investigation. Analytical reagent grade benzene (99% purity, Quimon Chem. Co., Barcelona, Spain) was used in this investigation. The animals of the acute experimental group were administered by intraperitoneal injection with benzene in a single dose per day, over three consecutive days. Subchronic treatment group animals were administered with the same dose for two weeks. The selected dose was 1/2 of the LD_{50} per day (0.5 ml/kg/day). This kind of administration is usually employed to carry out studies about the adverse effects of benzene (Gaido and Wierda 1985). The LD_{50} in rats has been found in our laboratory to be 1.0492 $\pm$ 0.30 ml/kg/day, calculated by the Bliss method. The control acute and subchronic groups were given 0.9% NaCl solution in the same volume and duration of the experimental groups. After the last treatment, the animals were anesthesized with Equithensin (0.2 ml/kg) intraperitoneally and perfused transcardially under deep anesthesia with saline followed by 4% paraformaldehyde. The brains were removed, cut into smaller pieces and then immersed in the same fixative medium overnight. 60 micrometer sections were cut using a microtome (Leitz) with an stereotaxic atlas guide and immunostained for met-enkephalin with polyclonal antisera raised in rabbits. The antigens were detected by the avidin-peroxidase technique, using 3,3'-diaminobenzidine as chromogen. Following reduction of endogenous peroxidases with 1% Hydrogen peroxide and blocking of nonspecific background staining with 5% normal goat serum (NGS) the sections were incubated with the following immunoreagents: 1-primary antiserum (rabbit anti met-enkephalin, Chemicon, dilution 1:3.000, 4ºC, 48 h); 2-goat anti rabbit immunoglobulin (goat antirabbit biotinilated, Chemicon, dilution 1:200, room temperature, 2 h); 3-avidin peroxidase complex (strept ABC complex HRP, Dakopatts, room temperature, 2 h); 4-chromogen (3,3'-diaminobenzidine, Sigma, room temperature, 10 min.). Each step was followed by an appropiate wash per triplicate in phosphate buffer saline.

Sections were carefully extended and mounted (DPX mounted for histology, Fluka), examined with a Zeiss photomicroscope and further analyzed with a Kontron image analysis system, a

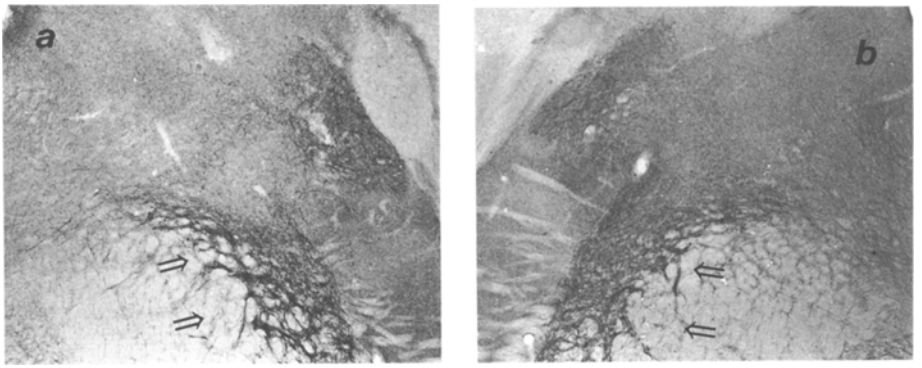

FIGURE 1.- Photomicrographs showing a dense accumulation of met-enkephalin immunoreactive fibers in the central amygdaloid nuclei: a) control; b) acute treatment with benzene (Zeiss photomicroscope. Green filter 10x).

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 by a black and white TV camera and digitalized. 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 an particular area is performed by tracing contours with a cursor. Light intensity for the microscope was continuously controlled, and measurements of integrated optical density in the corpus callosum was made on every slice analyzed as a control, since corpus callosum showed no staining. Values of integrated optical density were defined as the difference between corpus callosum and the studied structures on every slice analyzed. The following brain regions were analyzed: 1-corpus callosum; 2-lateral septum (enkephalinergic zone of the lateral septal nucleus); 3-parietal cortex; 4-caudatus-putamen; 5-hypothalamic medial preoptic area; 6-hypothalamic lateral preoptic area; 7-globus pallidus; 8-lateral and basal amygdala; 9-central nuclei of the amygdala; 10-cortical and medial amygdala. Results were referred as increment of optical density in every brain region studied with respect to corpus callosum, in treated animals and controls (mean ± SDM). Differences between means were calculated by the Student's t-test.

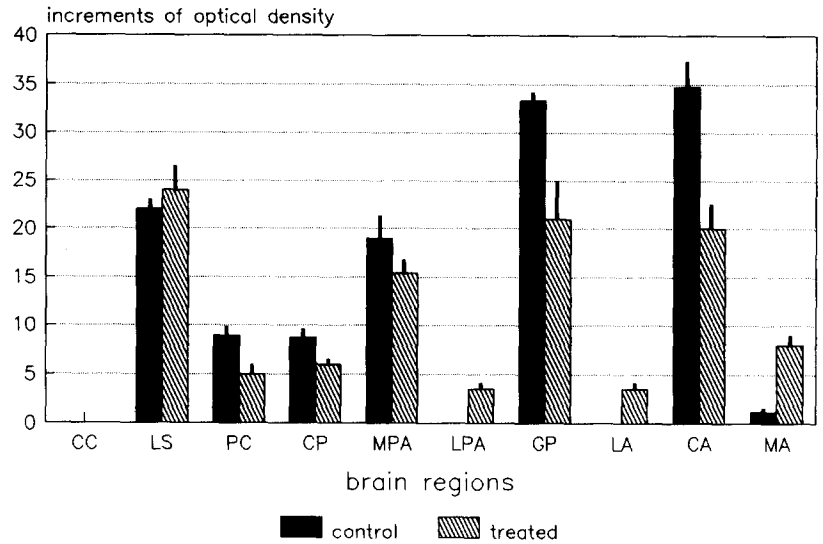

FIGURE 2.- Increments of optical density with respect to corpus callosum in controls and subacute benzene treated animals. The key for the representation is as follows: CC: corpus callosum; LS: lateral septum; PC: parietal cortex; CP: caudatus-putamen; MPA: hypothalamic medial preoptic area; LPA: hypothalamic lateral preoptic area; GP: globus pallidus; LA: lateral and basal amygdala; CA: amygdaloid central nuclei; MA: medial and cortical amygdala.

RESULTS AND DISCUSSION

Intensity of immunostaining in every brain region studied keeps good correlation with grey level in the same zone registered by transforming the microscope image in a black and white TV camera signal, which is analyzed by an appropiate image analysis system. This method allows an indirect but objetive estimation of the rate of immunostaining of fibers for a specific antigen in experimental animals with respect to controls. Background variability was avoided by processing experimental and control animals in identical conditions and all values were referred as increments with respect to copus callosum in every slice analyzed. Direct optical microscopic observation was made showing analogous results, and photomicrographs were obtained (figure 1). A dense accumulation of enkephalin immunoreactive fibers was seen in the basal portion of the lateral septal nucleus and the densest accumulation of enkephalin-containing processes was observed in the globus pallidus, ansa lenticularis and amygdaloid complex, forming a continuous field extending over these areas. This enkephalinergic distribution is coincident with previous reports (Akil et al. 1984; Zamir et al. 1985).

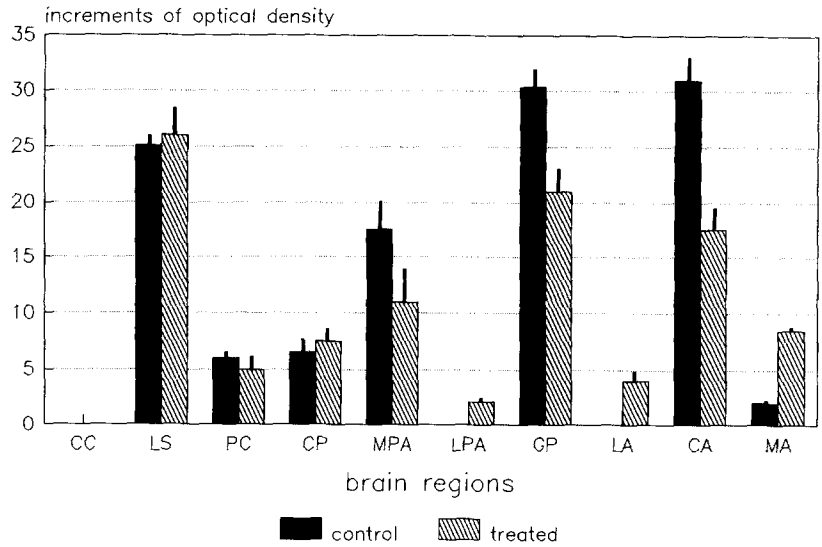

FIGURE 3.- Increments of optical density with respect to corpus callosum in controls and subchronically benzene treated animals. The key for the representation is as follows: CC: corpus callosum; LS: lateral septum; PC: parietal cortex; CP: caudatus-putamen; MPA: hypothalamic medial preoptic area; LPA: hypothalamic lateral preoptic area; GP: globus pallidus; LA: lateral and basal amygdala; CA: amygdaloid central nuclei; MA: medial and cortical amygdala.

Regional integrated optical density levels after immunostaining for met-enkephalin showed not relevant changes in lateral septum, parietal cortex, caudatus-putamen, lateral and medial preoptic areas of the hypothalamus and the lateral-basal amygdala when compared with controls after subacute benzene exposure. However, a marked reduction in met-enkephalin like immunostaining was measured in the globus pallidus and central amygdaloid nuclei ($p<0.01$), with a parallel elevation in central-cortical amygdala ($p<0.005$), after three days subacute benzene exposure (figure 2).

Subchronic benzene intraperitoneal exposure for two weeks generated similar changes in met-enkephalin immunostaining as produced by subacute exposure, except for a more marked reduction in the hypothalamic medial preoptic area. Thus, there were no relevant changes in lateral septum, parietal cortex, caudatus-putamen, lateral preoptic area of the hypothalamus and lateral-basal amygdala, but subchronic exposure to benzene generated reduction in met-enkephalin like immunostaining in globus pallidus ($p<0.05$) and central amygdaloid nuclei ($p<0.01$), with a simultaneous elevation in cortical-medial amygdala ($p<0.005$) (figure 3).

As was pointed out, high concentrations of enkephalins have been described in globus pallidus, amygdala, hypothalamus and striatum, and opioid peptidergic systems have been shown to modulate cognition, learning, memory and other nervous system physiological functions (Zager and Black 1985). The limbic system is usually affected by organic solvent exposure and it has been demonstrated that aromatic hydrocarbons can cause behavioral changes in mood and even addiction. This is the case for the largely studied "glue sniffers" (Schikler et al. 1982; Lazar et al. 1983).

However, information concerning the role of enkephalinergic system in benzene neurotoxicity is still lacking. This being the case, the aim of this work has been the study of the possible changes in met-enkephalin immunostaining in different regions of rat brain after subacute and subchronic benzene exposure. Subacute treatment with benzene generates a marked reduction in met-enkephalin immunostaining in globus pallidus and central amygdaloid nuclei, with a simultaneous elevation in cortical-medial amygdala. Subchronic administration of benzene produces similar changes with a more marked reduction in immunostaining of fibers for met-enkephalin at the level of the hypothalamic medial preoptic area. It has been described that, in spite of the different ways of administration and duration of the stimulus, the alterations in the neurotransmission seem to be produced not only by acute but also by prolonged intoxication with the solvent (Paradowski et al. 1984), which agrees with the results herein reported. Likewise, these changes could coincide with previously described regional alterations of brain monoamine metabolism after benzene exposure (Paradowski et al. 1984; Paradowski et al. 1985; Hsieh et al. 1988), due to the neuromodulatory action of opioids on classical neurotransmitter systems. Thus, it is well known that enkephalinergic neurons interact with dopaminergic neurons, and in the various conditions such as stress, changes in dopaminergic activity were always accompained by changes in met-enkephalin concentrations (Nabeshima et al. 1986).

On the other hand, it has been recently described that subacute exposure to benzene generates changes in aminopeptidase activity in several regions of the limbic system, including the amygdala (Gandarias et al. 1992). These proteolytic enzymes have been proposed as the possible mechanism for the regulation and control of the physiological activity of several

neuropeptides (Turner 1987). The results here reported could be compatible with those above, since we have found regional changes in immunostaining of fibers for met-enkephalin in the cortical and medial amygdala.

The regional specificity of changes in met-enkephalin like immunostaining suggest the involvement of endogenous opioid system in subacute and subchronic benzene neurotoxicity. However, its involvement could be a secondary one, perhaps due to the consequence of a nonopioid disturbance. Further studies, including biochemical measurement of enkephalin levels in the limbic system of the rat after benzene exposure are needed to clarify the real role of opioids in organic solvent neurotoxicity.

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