Toxicology 287 (2011) 145–152

Contents lists available at ScienceDirect

Toxicology

journal homepage: www.elsevier.com/locate/toxicol

Effects of prenatal and postnatal exposure to amitraz on norepinephrine, serotonin and dopamine levels in brain regions of male and female rats

J. Del Pino, M.A. Martínez, V.J. Castellano, E. Ramos, M.R. Martínez-Larrañaga, A. Anadón*

Department of Toxicology and Pharmacology, Faculty of Veterinary Medicine, Universidad Complutense de Madrid, 28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 11 April 2011
Received in revised form 23 May 2011
Accepted 11 June 2011
Available online 17 June 2011

Keywords:
Amitraz
Developmental neurotoxicology
Prenatal and postnatal pesticide exposure
Male and female offspring rats
Biogenic amines

ABSTRACT

The effects of maternal exposure to amitraz on brain region monoamine levels of male and female offspring rats at 60 days of age were observed. Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to amitraz (20 mg/kg bw, orally on days 6–21 of pregnancy and 1–10 of lactation). Male and female offspring were sacrificed at 60 days of age and possible alterations in the content and metabolism of NE, DA and 5-HT were determined in brain regions by HPLC. The results showed that all these neurotransmitter systems were altered in a brain regional-related manner. In male and female offspring, amitraz induced a significant decrease in the prefrontal cortex 5-HT and its metabolite 5-HIAA and DA and its metabolites DOPAC and HVA levels with interaction of sex. Nevertheless, we verified that striatum DA and 5-HT and corresponding metabolite contents decreased in male and female offspring without statistical distinction of sex. In contrast, amitraz did not modify 5-HT content, but caused an increase in 5-HIAA content in the medulla oblongata and hippocampus in male and female offspring. Alterations in the hippocampus DA, DOPAC and HVA levels after amitraz exposure were also observed displaying a sex interaction. NE levels also showed a decrease after amitraz treatment in the prefrontal cortex and striatum without statistical sex interaction, but MHPG levels decreased in both regions with a sex interaction. Amitraz evoked increases in 5-HT turnover in the prefrontal cortex as well as in DA turnover in the striatum and hippocampus but decreases in NE turnover in the hypothalamus, prefrontal cortex and striatum. The present findings indicated that maternal exposure to amitraz altered noradrenergic, serotonergic and dopaminergic neurochemistry in their offspring in the prefrontal cortex, striatum and hippocampus, and those variations could be related to several alterations in the functions in which these brain regions are involved.

1. Introduction

Amitraz, 1,5 di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triaza-penta-1,4-diene is a member of the formamidine pesticides, widely used in veterinary medicine for the control of external parasites (Müller, 1983), and in agriculture for the protection of fruits, vegetables and cotton against phytophagous (Hollingworth, 1976; Harrinson et al., 1973). Monoamine oxidase (MAO) inhibition was among the first biochemical actions of the formamidines to be reported (Aziz and Knowles, 1973; Beeman and Matsumura, 1973; Benezet et al., 1978). This action was considered novel since previous pesticides (e.g., carbamates and organophosphates) were known to affect primarily cholinergic systems. Since neuronal MAO participates in metabolic inactivation of biogenic monoamines, including the neurotransmitters serotonin, norepinephrine, and dopamine, an aminergic mechanism of action of amitraz was quickly postulated and adopted. In addition, amitraz is a potent inhibitor of acetylcholinesterase (Wang et al., 1975), alters prostaglandin synthesis (Yim et al., 1978), exerts local anaesthetic effects (Pfister et al., 1977; Chinn et al., 1977) and has α_2 receptor agonist properties (Costa and Murphy, 1987; Costa et al., 1988; Altobelli et al., 2001). It has long been known that exposure to amitraz can induce neurotoxic signs such as sedation, loss of righting reflex, motor incoordination and coma between others (Folz et al., 1984; Hsu and Schaffer, 1988) as well as behavioural effects in rodents (Flório et al., 1993; Palermo-Neto et al., 1994, 1997). However, there are few studies on the consequences of perinatal exposure in relation to neurochemical aspects.

Developmental neurotoxicity involves alterations in behaviour, neurochemistry, neurohistology and/or gross dysmorphology of central nervous system occurring in the offspring, as a result of chemical exposure of the mother during pregnancy or lactation. It is known that exposure to pesticides during development may interfere with the normal development of neurotransmitter systems and cause direct damage on them (Richardson et al., 2006). The central nervous system (CNS) during development is particu-

* Corresponding author. Tel.: +34 91 3943834; fax: +34 91 3943840.
E-mail address: anadon@vet.ucm.es (A. Anadón).

0300-483X/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.tox.2011.06.009

larly susceptible to the toxic effects of xenobiotics (Spyker, 1975; Stanton and Spear, 1990; Winneke, 1992; Tilson, 2000). The mechanism by which these effects occur is not known but currently it is assumed that the monoaminergic neurotransmitters play a role during development, defined as "morphogenetic" (Buznikov et al., 1996; Levitt et al., 1997; Nicotra and Schatten, 1990; Nicotra and Senatori, 1989). Any change in the levels of catecholamines during development could have a profound effect on brain development in both structural and functional (Lakshmana and Raju, 1994). For this reason, and because amitraz crosses the placenta (Palermo-Neto et al., 1994) we performed a study to establish if maternal exposure to amitraz during gestation and lactation affected offspring monoamine levels on adult age; this study was performed on offspring males and females separately in order to identify any gender differences for maternal exposure to amitraz during gestation and lactation. We examined eight brain regions that represent the major areas of catecholaminergic projections and that encompass areas involved in cognitive performance, learning and memory, and motor activity known to be targeted by amitraz. In addition to assessing effects on norepinephrine, serotonin and dopamine levels, we evaluated the neurotransmitter utilization rate (turnover), a measure of presynaptic neuronal activity (Dam et al., 1999a; Seider and Slotkin, 1990).

2. Materials and methods

2.1. Animals

All experiments using live animals were undertaken in accordance with the ethics requirements and authorized by the official ethical committee of our university. Adult male and female Wistar rats (Charles River Inc., Margate, Kent, UK) each weighing 200–210 g were used. The animals were individually housed in polycarbonate cages with sawdust bedding and were maintained in environmentally controlled rooms (22 ± 2 °C and 50 ± 10% relative humidity) with a 12 h light/dark cycle (light from 08.00 to 20.00 h). Food (A04 rodent diet, Panlab SL) and water were available *ad libitum*. For mating, a female was placed into a cage with a male rat overnight. Successful mating was confirmed by the presence of sperm in the vaginal smear, and the following 24 h was designed as day 0 of gestation (GD 0). Six mated females were housed individually in polycarbonate cages and were assigned randomly to two experimental groups: an amitraz treatment group ($n = 3$) and a control group ($n = 3$).

2.2. Test chemical and treatment

Amitraz [Virbac Laboratoires, 06511 Carros Cedex, France; purity 99% (w/w)] was dissolved in corn oil (8 mg/ml) to provide rapid and complete absorption and was administered orally by gavage in a volume of 2.5 ml/kg bw. Dams received daily amitraz at the dose of 20 mg/kg bw [equivalent to 1/30 of the LD_{50} (mean LD_{50} was previously calculated, data do not shown)] on days 6–21 pregnancy (GD 6–21) and on days 1–10 of lactation (PN 1–10); postnatal day 0 was the day of birth (PN 0). Control dams received vehicle (corn oil 2.5 ml/kg bw) on the same schedules. Dose of amitraz was selected based on previous preliminary study that indicated this dose was the higher one that did not cause weight loss or mortality, any reduction of the food or water intake as well as did not induce haematological modifications of other clinical histopathological signs of overt toxicity. Moreover we did not see any changes in suckling of maternal caretaking. None of the prenatal or postnatal treatment evoked a significant change in weight of any of the brain regions on PN 60 (data not shown).

Dams were examined daily throughout the gestation period and lactation periods for mortality, general appearance and behaviour. The maternal body weights were measured on GD 1, GD 5, GD 6, GD 15 and GD 20. Food and water consumption during pregnancy, length of gestation, litter size and sex ratio were also assessed.

All the pregnant rats were allowed to give birth and nurture their offspring normally. On one day after birth (PN 1), all litters were examined externally, sexed and weighed. Only those litters born after a 21-day gestation were used. Litters were organized in groups of twelve pups, six males and six females and the remaining pups were discarded. Litters were weighed at PN 1, PN 7, PN 14 and PN 21. The offspring were weaned on lactation day 21 (PN 21) and were maintained in appropriate conditions, housed individually and without any treatment with full access to food and water until adult age. The study was organized in two treated groups of six males and six females randomly selected respectively from the dams' litters exposed to amitraz, and two control pup groups of six males and six females randomly selected respectively from the control dams' litters.

At PN 60, male and female rats from control and treated groups (pups from control dams, and pups from dams exposed to amitraz, respectively) were killed by decapitation. The brain was removed quickly and the hypothalamus, midbrain, medulla oblongata, cerebellum, brainstem, hippocampus, striatum and prefrontal cortex rapidly dissected out at 4 °C (Glowinski and Iversen, 1966). Tissues were rapidly weighed and stored at −80 °C until analysis. All data were collected by experimenters blind to the treatment condition of the offspring.

2.3. Determination of monoamine levels

The eight brain regions analysed in the present study were hypothalamus, midbrain, medulla oblongata, cerebellum, brainstem, hippocampus, striatum and prefrontal cortex. Following sample collections, 300–800 μl of 0.4 M $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ was added to the tissues, and the mixture was homogenized by sonication (Labsonic-U-Braun) before neurochemical evaluation was performed. The homogenates were centrifuged for 15 min at $20{,}000 \times g$ at 4 °C and aliquots of supernatants were taken for analysis of norepinephrine (NE), dopamine (DA) and its metabolites [3,4-hydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA)] and serotonin (5-HT) and its metabolite [5-hydroxy-3-indolacetic acid (5-HIAA)] using a high performance liquid chromatography (HPLC) technique with electrochemical detection (Colado et al., 1993) with modifications in the mobile phase. Also, aliquots of supernatants were taken for analysis of the norepinephrine metabolite [3-metoxy-4-hydroxyphenylethyleneglycol (MHPG)] by HPLC with fluorimetric detection (Artigas et al., 1986). An acid-catalyzed procedure was used to hydrolyze MHPG-sulphate in homogenates of brain region tissues. Volumes of 200–300 μl of the supernatants (in 0.4 M $HClO_4$) were treated for 3 min at 100 °C in a water bath. The samples were then cooled and 30–45 μl of 2 M NaOH were added (final pH: ca. 1.5) and aliquots were injected into a reversed phase HPLC system.

For the analysis of catecholamines NE, DA, DOPAC and HVA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5), 1.6 mM octane sulphonic acid, 0.9 mM EDTA and 10% (v/v) methanol. For the analysis of the indolalkylamines 5-HT and 5-HIAA, the mobile phase consisted of 0.1 M $Na_2HPO_4 \cdot 2H_2O$, 0.1 M citric acid (pH 3.5) and 10% (v/v) methanol. Elution was performed at a flow rate of 1 ml/min and the working electrode potential was set at 0.8 V for catecholamines and 0.7 V for indolalkylamines. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-9A, a 5 μm particle size C_{18}-Nucleosil reversed phase column (4 mm i.d. × 125 mm) proceeded by a C_{18} precolumn, an electrochemical detector (Shimadzu, model L-ECD-6A), a sample injector (20 μl valve) and an integrator (Shimadzu, model C-R6A Chromatopac). For the analysis of the norepinephrine metabolite (MHPG), the mobile phase consisted of 0.06 M $Na_2HPO_4 \cdot 2H_2O$, 0.03 M citric acid and 6% (v/v) methanol. Elution was performed at a flow rate of 1.5 ml/min. Excitation and emission wavelengths of the detector were 275 and 315 nm, respectively. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-10AS, a 25 μm particle size Tracer Extrasil ODS reversed phase column (4 mm i.d. × 125 mm), a fluorescence detector (Shimadzu, model RF-551), a sample injector (20 μl valve) and an integrator (Shimadzu, model C-R6A Chromatopac). All chemicals were of the highest quality grade and obtained from commercial sources.

Peak areas in the sample chromatograms were quantitated by external standard technique using solutions of the catecholamines (NE, DA, DOPAC and HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) reference standards (Sigma Chemical CO., St Louis, MO, USA). For tissue specimens as determined by use of a linear least squares regression procedure, a linear relationship existed in the calibration curve of catecholamines (NE, DA, DOPAC, HVA), indolalkylamines (5-HT, 5-HIAA) and norepinephrine metabolite (MPHG) over the range of 0.002–100 ng/ml, which always yielded a correlation coefficient exceeding 0.9998. Overall mean recovery of catecholamines (NE, DA, DOPAC and HVA), indolalkylamines (5-HT and 5-HIAA) and norepinephrine metabolite (MPHG) from tissues was 100% for every analyte. Within- and between-day variation was <4%. Quantification limit (LOQ) was 2 pg for DA, DOPAC, NE, 5-HT and 5-HIAA and 20 pg for HVA and MPHG in the different tissue matrices. 5-HT, DA and NE turnover were calculated as ratios of metabolites to neurotransmitter.

2.4. Data analysis

Statistical analysis of data was performed using a Statgraphics software, version Plus 4.1 for windows. Values are expressed as mean ± S.E.M. obtained from 12 animals, six males and six females, in each group (control and treated groups). For values combined for males and females, a two-way ANOVA with treatment × sex interaction was the initial test used. Where a significant treatment × sex interaction was detected, a separate Student's t test was carried out for each sex. The results were considered significant at $P < 0.05$. Results significantly different from controls are also presented as change from control (%).

3. Results

Maternal and offspring body weight, physical and general activity development were unaffected by the exposure of dams to amitraz (20 mg/kg bw orally on days 6–21 of pregnancy and 1–10 of lactation). No differences were found between the body weights