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Effects of exposure to amitraz on noradrenaline, serotonin and dopamine levels in brain regions of 30 and 60 days old male rats

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ABSTRACT

The effects of amitraz oral exposure (20, 50 and 80 mg/kg bw, 5 days) on brain region monoamine levels of male rats at 30 and 60 days of age were examined. The amitraz-treated rats at the oral doses of 20 and 50 mg/kg bw had no visible injury, i.e., any clinical signs of dysfunction observed in any of the animals. However, rats treated with amitraz at the highest dose (80 mg/kg bw, 5 days) showed a slight motor incoordination after 1–2 h of treatment. These signs were reversible approximately at 6 h after dose. After the last dose of amitraz, NE, DA and 5-HT and its metabolites levels were determined in the brain regions hypothalamus, midbrain, prefrontal cortex, striatum and hippocampus by HPLC. Amitraz caused changes in the NE, DA and 5-HT and their metabolite levels in a brain regional-, dose- and age-related manner. In the brain regions studied, amitraz induced a statistically significant increase in 5-HT, NE and DA content with age interaction, but the NE increases in prefrontal cortex and hippocampus was without age interaction. Moreover, in the brain regions studied, amitraz induced a statistically significant decrease in the metabolite 5-HIAA, MHPG, DOPAC and HVA levels displaying an age interaction, excepting the 5-HIAA decrease in midbrain and the DOPAC decrease in hypothalamus and striatum which were without age interaction. Furthermore, amitraz evoked a statistically significant decrease in 5-HT, NE and DA turnover in the brain regions studied. The present findings indicate that amitraz significantly altered CNS monoaminergic neurotransmitters in a brain regional-, dose- and age-related manner.

1. Introduction

Formamidine pesticides were developed in the late 1950s and early 1960s to combat mites and other types of insects that had developed resistance to conventional insecticides. Amitraz, one of the most important members of the formamidine class of acaricide/insecticides, is widely used in veterinary medicine for the control of external parasites (Müller, 1983; Radakovic et al., 2013), and in agriculture for the protection of fruits, vegetables and cotton against phytophagous (Harrison et al., 1973; Hollingworth, 1976). It also was used in humans to treat pediculosis or scabies (Kalyoncu et al., 2002). 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; Moser and MacPhail, 1989). Since neuronal MAO participates in metabolic inactivation of biogenic monoamines, including the neurotransmitters serotonin, norepinephrine, and dopamine, a monoaminergic mechanism of action of amitraz was quickly postulated. Recently it was demonstrated that maternal exposure to amitraz affected the development of the monoaminergic systems (Del Pino et al., 2011). In addition, amitraz alters prostaglandin synthesis (Yim et al., 1978), exerts local anesthetic effects (Chinn et al., 1977; Pfister and Yim, 1977) and has α_2 receptor agonist properties (Altobelli et al., 2001; Costa et al., 1988; Costa and Murphy, 1987). Other biological activity of amitraz has been recently reported; amitraz induced hepatic 17β-estradiol (E2) and testosterone (T) metabolism in female and male rats (Chou et al., 2008).

Amitraz is able to cross the blood–brain barrier (FAO, 1980). Amitraz exposure induce clinical signs such as sedation, loss of righting reflex, motor incoordination and coma between others in dogs (Folz et al., 1984; Hsu and Schaffer, 1988) and humans (Jorens et al., 1997; Proudfoot, 2003; Ulukaya et al., 2001; Varma et al., 2012) as well as behavioral, physiological and neurochemical effects in laboratory rodents (Boyes and Moser, 1987; Florio et al., 1993; Moser and MacPhail, 1989; Moser et al., 1987; Palermo-Neto et al., 1994, 1997).

The mechanisms by which amitraz induce its neurotoxic effects are not clear. In rats, low to intermediate doses of amitraz

Abbreviations: TH, tyrosine hydroxylase; TRH, tryptophan hydroxylase; L-TYR, L-tryptophan; L-DOPA, L-3,4-dihydroxyphenylalanine; 5-HIA, 5-hydroxyindolaldehyde; 5-HTP, 5-hydroxytryptophan; AAAD, aromatic L-amino acid decarboxylase; DHMA, 3,4-dihydroxymandeliv acid; NM, normetanephrine; VMA, vanillylmandelic acid; DBH, dopamine-β-hydroxylase; DHPG, 3,4-dihydroxyphenilglycol; AR, aldehyde reductase; COMT, catechol-O-metyltransferase; AD, aldehyde dehydrogenase.

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(6.25–25 mg/kg bw) showed a decrease in motor activity and a change in rates and patterns of operant response (Moser et al., 1987). High doses of amitraz (50–100 mg/kg bw) affected the amplitude of evoked potentials and decreased the body weight and body temperature of the treated rats (Boyes and Moser, 1987). In addition, only doses ≥ 100 mg/kg bw produced a inhibition of MAO. Thus, MAO inhibition is not the primary mechanism involved in motor activity effects induced by amitraz. According to Moser and MacPhail (1986, 1989), motor effects might be caused by the action of amitraz on the α_2-adrenergic receptor, because of the dose range over which it produces MAO inhibition is much higher than that which suppressed motor activity. However, Florio et al. (1993) described that amitraz effects on motor activity were a consequence of its MAO inhibition effects within the CNS, an action that could be responsible for the enhancement of catecholamine levels, although these effects were not observed in a dose-dependent way.

Amitraz is an insecticide and acaricide of relatively recent discovery and commercialization; hence it is not surprising that there are few studies in the scientific literature of its neurotoxicity in mammals. Due to the numerous cases of human poisoning by amitraz that have been and are still being described worldwide (Aging et al., 2004; Demirel et al., 2006; Kalyoncu et al., 2002; Shitole et al., 2010; Veale et al., 2011; Yaramis et al., 2000; Yilmaz and Yildas, 2003), it is important to have more information on its neurotoxic effects and underlying mechanisms involved. The present study evaluated in rats the effects of amitraz on norepinephrine, serotonin and dopamine and metabolites levels, as well as the neurotransmitter rate (turnover), a measure of presynaptic neuronal activity (Dam et al., 1999; Seider and Slotkin, 1990) in five brain regions (hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex), major areas of monoaminergic systems involved in cognitive performance, learning and memory, and motor activity, which could be targets for amitraz. On the basis of current reports suggesting that innervation of monoaminergic neurons in the brain progresses with increasing age as well as developmental changes in catecholamine biosynthetic enzymes (Nomura et al., 1976; Rinaman, 2001), the present study was carried out using male rats at 30 days of age (immature or pubertal rats) and at 60 days of age (adult rats). The aim of our work is to determine amitraz effects on CNS monoamine levels in a brain regional-, dose- and age-related manner.

2. Materials and methods

2.1. Chemicals

Amitraz (1,5 di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triaza-penta-1,4-diene), purity 99% (w/w) was provided by Virbac Laboratoires, 06511 Carros Cedex, France. All other chemicals were of the highest quality grade and obtained from commercial sources.

2.2. Animals and experimental design

All experiments using live animals were undertaken in accordance with the ethics requirements and authorized by the official ethical committee of our university. Male Wistar rats, at 30 and 60 days old each weighting 100–110 and 200–210 g, respectively (Charles River Inc., Margate, Kent, UK) were used. The animals were individually housed in polycarbonate cages with sawdust bedding and 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. Twenty-four male rats at 30 days of age were assigned randomly to four groups of 6 animals each, a control group and three amitraz treated groups. Moreover, twenty-four male rats at 60 days of age were assigned randomly to others four groups of 6 animals each, a control group and three amitraz treated groups. Both 30 and 60 days old animal treated groups received amitraz orally at the dose of 20, 50 and 80 mg/kg bw [equivalent to 1/30, 1/12 and 1/7.5 of the LD_{50} (mean LD_{50} was previously calculated, data not shown)] for 5 consecutive days. The amitraz treated group rats were deprived of food for 6 h before the oral administration of amitraz, but were allowed water ad libitum. Amitraz was dissolved in corn oil (8 mg amitraz/ml) and was administered orally by gavage in a maximum volume of 2.1 ml/rat depending of animal weight and dose. Control animals received the vehicle (corn oil) on the same schedules.

The animal body weights were measured during the study and food and water consumption of each animal was also assessed. The animals received the treatment at the same time each day, specifically between 10.0 h and 11.0 h am. Three hours after the last dose, the animals were sacrificed by decapitation. The brain was removed quickly and the hypothalamus, midbrain, hippocampus, striatum and prefrontal cortex tissues rapidly dissected out at 4 °C (Glowinski and Iversen, 1966). Tissues were rapidly weighed and stored at −80 °C until analysis.

2.3. Determination of monoamine levels

The five brain regions analyzed in the present study were hypothalamus, midbrain, 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 (1 min) by sonication (Labsonic-U-Braun) before neurochemical evaluation was performed. The homogenates were centrifuged for 15 min at 20,000 × *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 (Del Pino et al., 2011; Martínez-Larrañaga et al., 2003). 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; Del Pino et al., 2011). 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 reverse phase HPLC system.

For the analysis of catecholamines NA, 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-9 A, a 5 m 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. The HPLC system consisted of a Shimadzu liquid chromatograph, model LC-10AS, a 25 m 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). Excitation and emission wavelengths of the detector were 275 and 315 nm, respectively.

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 Statgraphics software, version Plus 4.1 for Windows. Results are presented as percentage change from control (%) and expressed as mean ± S.D. of 6 animals per group. One-way ANOVA was carried out for each age to determine significant dose-dependent effect of amitraz on 5-HT, NA, DA and metabolite levels and the corresponding turnover values in the brain regions, followed, where appropriate, by Duncan post hoc test. Two-way analysis of variance (ANOVA) was employed to determine significant interaction between amitraz treatment groups and animal age on 5-HT, NA, DA and metabolite levels. The results were considered significant at $P < 0.05$.