The Endocrine Disruptor Atrazine Accounts for a Dimorphic Somatostatinergic Neuronal Expression Pattern in Mice

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INTRODUCTION

Recently several environmental chemicals that have either agricultural or industrial food packaging and consumer applications have been shown to possess endocrine-disrupting properties that pose a potential threat to wildlife and, above all, to humans (Vos et al., 2000). In this last decade, the widespread application of the environmental contaminant atrazine (2-chloro-4-ethylamino-6-isopropylamino-S-triazine) in agricultural activities has proven to have a major impact on water and soil conditions (Koskinen and Clay, 1997), both because of its perversiveness, provoked by its mobility through ecosystems, and by the slow degradative properties of the herbicide (Hayes, 1993). Indeed, low ecological doses of atrazine have been correlated with altered sexual development events of aquatic endemic vertebrates such as the frog *Xenopus laevis* (Hayes et al., 2002), and in fish it is not only responsible for altered testicular structural formation, but it is also involved in the modification of steroidogenesis and socio-sexual behaviors (Saglio and Trijasse, 1998).

In response to the many threats posed by atrazine in aquatic environments, interest has grown in determining its toxic effects on terrestrial vertebrates, especially since publications of experimental studies have demonstrated a modification of chromosomal and DNA arrangements in rodents treated with this herbicide (Surrales et al., 1995). Other investigators have reported a neurotoxic effect consisting in either myelosuppressive actions or overall nerve fiber morphological damages (Sawicki et al., 1998). In line with these effects, works have also taken into account the possibility that atrazine might influence morphological features such as body weight, bone length, and overall developmental processes through concomitant interference with growth hormone releasing hormone (GHRH) and thyroid stimulating hormone (TSH) at the hypothalamic level (Gould et al., 1997). In this context, the role of neurotransmitters becomes an important regulatory factor, especially in the case of some neuropeptides such as somatostatin which, by interacting with another major neuronal system, i.e., GABA, controls the secretion of growth hormone (GH) that may prove to be critical for inducing early cerebral damage (Stumm et al., 2004).

The neuro-tetradecapeptide somatostatin (ss), which is recognized primarily for its role in basal and stimulated GH release, is widely distributed in major brain regions. In mammals ss, originally isolated from the hypothalamus, exerts...
a major inhibitory role on GH secretion, and it is involved in the control of many neuroendocrine and cognitive functions such as body temperature, satiety, sleep, and memory (Schindler et al., 1996). At present, five different sst receptor subtypes (sst1,3,5) have been cloned and have been shown to possess the heptahelical architecture typical of the G protein–coupled receptors (Csaba and Dournaud, 2001). Of these subtypes, the widely distributed sst2,3,5 which (especially in the case of the former two) not only appear relatively early during development but also remain stable during adult life, exert a key role in the organization of the central nervous system (CNS) (Thoss et al., 1995). From these indications, plus the recognition that the pulsatile release of GH is strikingly different in male and female rats, it seems that the GH secretion profile depends on sexually dimorphic neuroanatomical network differences that are induced by steroidogenic factors (Scanlan et al., 2003). Such GH- and sst-dependent relationships might provide further insights into the types of neurotoxicological mechanisms involved in the delay of the estrous cycle or the alteration of the hypothalamic-hypophyseal-gonadal axis in animals treated with atrazine (Wetzel et al., 1994).

Because of the antiestrogenic properties of atrazine (McMullin et al., 2004), this herbicide is capable of modifying neuroendocrine functions through the interaction of both α and β estrogen receptors (Quesada et al., 2002). Recently, other xenobiotics operating via estrogen-like mechanisms have presented potential risks of affecting spine synapse formation in hippocampal regions (MacLusky et al., 2005). Moreover, the upregulating effects of estrogens on mRNA expression of sst2,3 (Djordjievic et al., 1998) and the co-localization of such sst to estrogen receptors (Arancibia et al., 1997), point to these G-coupled subtypes as a major neuromediating target of atrazine-dependent actions in the domestic mouse (Mus musculus). In this context, it was our intention to determine the type of relationship between the developmental processes of the different sst mRNAs that is explicaded by assimilation of low ecological atrazine doses. It was also interesting to establish a regional specificity of atrazine–sst2,3,5 interactions in some hypothalamic and extra-hypothalamic estrogen-enriched brain regions (McEwen, 2002). This was done with particular attention to sites within the hypothalamus, because altered sst levels have been reported for hypothalamic nuclei (Pannell et al., 2002). In addition, extra-hypothalamic areas such as the frontoparietal cortex (layer III and V) and the hippocampus have been considered, aside their elevated densities of estrogen receptors to their sst-dependent role in memory and spatial tasks, as well as sociosexual and neurovegetative functions (Csaba and Dournaud, 2001).

MATERIALS AND METHODS

Animals and treatment procedure. Domestic mice (Mus musculus) of an outbred Swiss-derived strain (CD-1) were purchased from a commercial breeder (Charles River Italia, Calco-Italy). Upon arrival, animals were kept in an air-conditioned room (temperature 21°C ± 1°C, relative humidity 60 ± 10%; lights on from 20:30 h to 8:30 h), in 33 × 13 × 14 cm Plexiglas boxes with metal tops and sawdust as bedding. Pellet food (enriched standard diet; Mucedola-Milan, Italy) and water were provided ad libitum. Adequate measures were taken to minimize pain or discomfort during all phases of animal treatment.

To establish the organizational effects of the herbicide atrazine on the sst neuroreceptor system of adult mice, we began treatment with the herbicide early, during the gestational and lactational phases of dams. It is at these biological stages that atrazine, working via activation of hypothalamic-pituitary axis, can directly or indirectly promote neuronal plasticity of the different brain regions (Bowers et al., 2004). For this purpose, dams (n = 23) were individually housed and trained (oral-administration) on gestational days (GD) 1–5 to drink 0.1 ml of corn oil during a 1–5-min period every day from a modified syringe, as previously reported (Branchi et al., 2005). This approach allows administration of compounds without the stressful handling procedure typical of administration by gavage. Subsequently, GD 14 females were divided into the following treatment groups: two groups received oral-administration from GD 14 until postnatal day 21 (PND 21) of either a low (LD, 1μg/kg per day; n = 8) or a high (HD, 100 μg/kg per day; n = 8) realistic ecological atrazine concentration compared to controls (n = 8) that received corn oil alone. The procedure was monitored to make sure that pups did not attempt to drink the solution from the syringe. Because of the necessity of adopting consistent neurotoxicological conditions of atrazine equivalent to those of field conditions, it was important to use realistic ecological concentrations as the preferred doses (Hayes, 1993). The atrazine concentrations employed for the present study, which are exceptionally lower than that of the maximum tolerated oral dose (MTD; 50 mg/kg per day) estimated for the rat (Wetzel et al., 1994), mimic those that are detectable in drinking water (3 μg/l) and soil (2 μg/l). These levels have been implicated in some forms of physiological dysfunction (Hayes et al., 2002), as well as in tumor formation (Kettles et al., 1997).

On PND 21, mothers were removed and F1 mice of both sexes (n = 6 females; n = 6 males for all treatment groups) were housed in the same Plexiglas boxes according to type of treatment received (LD, HD, or controls). At PND 60–65, the animals were sacrificed and their brains were quickly removed and stored at −40°C. During the entire experimental period, animals were treated in accordance with NIH Guide for the Care and Use of Laboratory Animals and with European Communities Council Directive of 24 November 1986 (86/609/EEC).

Amino cupric silver staining. For the neuronal degeneration processes induced by the two concentrations of atrazine, brains of F1 male and female mice, belonging to the control (n = 6), LD (n = 6), and HD (n = 6) treatment groups, were mounted on the freezing stage of a sliding cryostat (Microm-HM505E), according to common cryostat procedures for unfixed brains (Canonaco et al., 1997). A serial set of representative coronal sections (30 μm) was selected for amino cupric silver staining procedures, according to previously published methods (de Olmos et al., 1994). The brain sections were rinsed with distilled H2O, placed into dishes containing the pre-impregnating solution [AgNO3, distilled H2O, dl-alanine, Cu(NO3)2, Cd(NO3)2, La(NO3)2, neutral red, pyridine triethanolamine, isopropanol], heated in a microwave oven (45–50°C) for 50 min, and cooled at room temperature for 3 h. The sections were then rinsed in distilled H2O and after a quick rinse in acetone, they were placed in an impregnating solution (AgNO3, distilled H2O, ethanol, acetone, LiOH, NH4OH) for 50 min, followed by a 25-min permanence in a reducer solution (formalin, citric acid monohydrate, ethanol, distilled H2O) at a temperature ranging from 32° to 35°C. These sections were left in distilled H2O overnight and the next day they were placed in a first bleaching solution (potassium ferricyanide in potassium chlorate solution, lactic acid) for 60 s at room temperature. Subsequently, they were bleached in a second solution (potassium permanganate, sulphuric acid) for 60 s and rinsed with distilled H2O. For the stabilization phase, sections were transferred in sodium thiosulfate solution and rinsed again with distilled H2O. Finally, they were immersed in a rapid fixer solution for 5 min and counterstained with 0.5% neutral red solution (Carlo Erba, Italy) for 20 min.
Total RNA was extracted from mice brain belonging to the control group (3 females and 3 males) using TRI reagent (Sigma, Italy) based on established extraction methods (Chomczynski and Sacchi, 1987) and dissolved in DEPC-water (Sigma, Italy). The reverse transcription reaction was performed using 2 methods (Chomczynski and Sacchi, 1987) and dissolved in DEPC-water males) using TRI reagent (Sigma, Italy) based on established extraction specific primer sets: sst 2 for 5'-GAGGTGGA TACGGTTGTGC; sst3 for 5'-CCTCTACTTGCCCTCCGA; sst5 for 5'-TGTGCGGCAACACGCTGGT and rev 5'-AGTTGGAATACGGTTGTGC; sst2 for 5'-TGGCATCAACCATGGTCCAC; and rev 5'-CTCTCTTGGTCCTTGGCA; sst3 for 5'-GCTCCAGTGGTCCGAGGGT and rev 5'-TCTGACAGGCCGCTTGGC. The number of PCR cycles was determined preliminarily by performing from 20–35 and 15–30 amplification reactions for subtypes, and the number was fixed at 35 cycles, consisting of denaturation at 94°C for 40 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min.

The single PCR product of all sst was separated by agarose gel (1%), purified with Wizard Kit (Promega-Italy) for sequence reactions, and synthetic oligonucleotide probes (Roche, Italy) were designed from sequencing of the above PCR fragments. To perform in situ hybridization, antisense and sense probes were labeled by 3'-tailing with digoxigenin-11-dUTP according to the DIG oligonucleotide tailing kit (Roche-Italy). The labeling reaction was handled by incubating antisense and sense probes at 37°C for 30 min and then stopped with 0.2 M EDTA pH 8.0. Probe concentration was determined by the quantification of known standards on Hybond N° filters (Amersham, Italy). For in situ hybridization, brain sections (thickness 12 μm) of female (n = 4) and male (n = 4) mice of all treatment groups were mounted on polylysine coated slides (Carlo Erba, Italy) that were stored at −40°C. Subsequently, 100 ng of antisense probe in 100 μl of hybridization solution was added to each section for overnight in situ permanence at 50°C in a humidified chamber (Kia et al., 2002). Nonspecific hybridization was obtained on slides incubated with the sense probe. For immunological detection, sections were cover slipped for 45 min with PBS buffer containing 2% normal sheep serum and 0.3% Triton X-100 (Sigma, Italy), then 1:100 anti-digoxigenin alkaline phosphatase antibody (Roche, Italy) was added for 2 h at room temperature, and alkaline phosphatase color reaction buffer (NBT/BCIP) was applied to sections and incubated for 72 h in a humidified dark chamber.

The neuronal hybridization signals were observed in a bright-field Dialux EB 20 microscope under a phase contrast objective, and transcriptional activity was evaluated with a Panasonic Telecamera (Canon Objective Lens FD 50 mm, 1:3.5) attached to a Macintosh computer-assisted image analyzer system running an image software of National Institutes of Health (Scion Image 2.0) in which an internal standard was used for the calibration of optical density (O.D.) values.

**Statistical analysis.** Data of in situ hybridization that were evaluated as means of O.D. (± SE) were performed in at least three independent experiments carried out on different brain slices. Statistically significant differences between experimental groups were determined by one-way analysis of variance (ANOVA) followed by an unpaired t-test.

**RESULTS**

**Amino Cupric Silver Staining**

The use of the silver impregnation procedures for the study of degenerative changes in the nervous system has recently demonstrated to be an excellent histological tool for the evaluation of damaged neuronal elements in specific brain areas after exposure to neurotoxins, hypoxia, and other forms of trauma (de Olmos et al., 1994). Indeed, from the necessity of identifying not only the nature but also the specific dose linked to the neuronal damage to the different brain regions of Mus musculus, it was possible, with amino cupric silver staining methods, to show a HD atrazine-dependent dimorphic neurodegeneration pattern. In particular, a consistently evident argyrophilic reaction of axonal processes and perikarya was demonstrated to be an excellent histological tool for the study of degenerative changes in the nervous system.
neuronal cells accompanied by significant perikaryal swelling plus well-developed astrocytic formations in the female. These dimorphic morphological features were also typical of the hypothalamus, as demonstrated by significantly darker axonal processes in the female (Fig. 1c) arcuate hypothalamic nucleus (Arc) than in males (Fig. 1d), with respect to their corresponding controls (Fig. 1e, f), neither of which displayed any type of staining abnormality and hence no degenerative phenomena. Conversely, in other extra-hypothalamic areas the effects of this herbicide did not prove to be of a dimorphic nature, as shown by mostly shrunken and folded perikarya occurring in a similar manner for the pyramidal neurons of the striatal and cortical areas of both sexes (Fig. 2).

**RT-PCR Detection of sst2,3,5 and In Situ Hybridization**

Before any correlations between the above atrazine-induced structural deformations and altered ssergic neuro-signaling events could be handled, it was necessary to initially supply the nucleotidic sequential arrangement of the major ss subtypes (sst2,3,5) in the two sexes. For this part, reverse-transcriptase PCR (RT-PCR) investigations yielded identical amplified bp products in the brains of both male and female mice (Fig. 3) as illustrated by 743 bp (sst2), 786 bp (sst3), and 566 bp (sst5) fragments exhibiting a high degree of nucleotide homology (>85%) with respect to the same subtypes of the rat. The subsequent application of antisense probes developed for in situ hybridization analyses did in fact provide a heterogeneous distribution pattern of the different subtype mRNAs. On the one hand, the predominance of neurons expressing sst2 mRNA was detected in the oriens pyramidal layer (Or-Py) and stratum radiatum (Rad) of the hippocampus plus the medial preoptic area (MPOA) of the hypothalamus (Fig. 4). On the other hand, high levels of neurons expressing sst3 mRNA were preferentially found in other extra-hypothalamic areas, namely, the cortical layer III (Cor III), and some amygdalar areas such as lateral amygdaloid nucleus (Lat). As far as sst5 is concerned, this subtype, even though it was widely located in most areas of the telencephalon, was of lesser significance than the other two subtypes.

It is worthwhile to note that a mixed distribution pattern of these subtypes was, instead, obtained when the mice were treated with both HD and LD atrazine concentrations. Indeed for the latter concentration, it was sst2 that responded in a dimorphic nature, as shown by mostly shrunken and folded perikarya occurring in a similar manner for the pyramidal neurons of the striatal and cortical areas of both sexes (Fig. 2).

**FIG. 2.** Representative sections showing no dimorphic amino cupric pattern in female \((n = 6, \text{A})\) and male \((n = 6, \text{B})\) mice treated with HD of atrazine. The effects of this herbicide (arrows) were similar in the striatum \((a, b)\) and in the cortex \((c, d)\) of both animal sexes. Scale bar = 1.6 mm \((\text{A, B})\), 100 μm \((\text{a, b, c, d})\).

**FIG. 3.** Reverse-transcriptase polymerase chain reaction (RT-PCR) amplifications yielded identical bp products in the brain of both male \((n = 3)\) and female \((n = 3)\) mice. Line 1: marker; 2: (sst2, 743 bp); 3: (sst3, 786 bp) and 4: (sst5, 566 bp).
mRNA expressing neurons of the female (Fig. 6), which appeared to be the preferred sst target of atrazine in females, whereas the effects in males were low or absent (Fig. 6a). As a matter of fact, very strong (\(p < 0.001\)) and strong (\(p < 0.01\)) upregulatory effects of sst2 were reported for the female hypothalamic areas in the SCh (\(+110\%\)) and the ventromedial hypothalamic nucleus (VMN \(= +65\%\)), respectively, when compared with sst5, which was not densely concentrated in the different brain regions. A HD atrazine concentration was not only responsible for upregulatory effects of sst2 mRNA levels in the female, because a very strong and moderate downregulating activity appeared to characterize the striatum (STR \(= -96\%\)) and the Ce \((-45\%)\), respectively, of the female animals. Conversely, sst3 neurons appeared to be the major target in males after exposure to such an HD atrazine concentration (Fig. 7), as displayed by a very great number of sst3 mRNA expressing neurons that were featured in the hypothalamic areas such as Arc \((+120\%)\) and amygdalar regions and the basolateral amygdaloid nucleus (Bl \(= +78\%)\). Moreover, a very strong downregulation of this subtype (Fig. 7) was obtained in the cortical (Cor III \(= -88\%\)) and hippocampal areas (DG \(= -105\%)\) of the male, whereas in the female only the former brain area provided moderate downregulatory activity, although to a lesser extent (Cor III \(= -45\%)\). In the case of the other subtype (sst5), this herbicide was not responsible for notable changes aside from a moderate downregulation of the mRNA levels in male cortical areas (Cor III \(= -40\%)\) and female amygdalar areas (Ce \(= -50\%)\), as shown in both Figure 6 and Figure 7.

**FIG. 5.** The effects of both LD \((n = 4; \text{gray bar})\) and HD \((n = 4; \text{white bar})\) concentrations of atrazine on (a) sst2- and (b) sst3-expressing neurons in male and female mice with respect to their controls \((n = 4, \text{vertical line bar})\) were expressed as mean O.D. mRNA levels ± SE as described in Materials and Methods. The levels were compared using one-way ANOVA followed by unpaired \(t\)-test. \(x = p < 0.05\}; \(y = p < 0.01\}; \(z = p < 0.001\). For abbreviations, see Figure 4.

**DISCUSSION**

The distinct neurotoxicological effects of low ecological atrazine doses have demonstrated, for the first time, that this environmental contaminant is able to interfere with encephalic neurotransmitter functions via a preferential dimorphic interaction of the major ss subtypes. The involvement of this neuroreceptor system on similar dimorphic toxicological activities should not be surprising because several studies have begun to show that differentiated GH secretion may be due to gender-related differences in the transduction of ssergic mechanisms in brain and/or pituitary targets (Zhang et al., 1999). Just recently, results deriving from neurochemical and behavioral models have pointed to neurotoxicological threats of the broadly applied organophosphorus insecticides, which evoke encephalic damages from the embryonic stage throughout the postnatal developmental phases in both sexes (Reeves et al., 2003). In the present work, a first observation that strongly supports the dimorphic effects of atrazine is provided by amino cupric silver staining results, a specific method capable of providing immediate evidence of the infiltrative
properties of this herbicide in female mesencephalic and telencephalic regions, which are involved in the modulation of motor activities and sociosexual behaviors (Steiniger and Kretschmer, 2004). In line with such structural alterations, this staining method has proven to be a valuable histological tool for the easy and quick detection of not only structural neuronal damage of cerebral tissues under stressful conditions but also neuronal damage caused by a vast number of chemical agents, including similar pesticides (Poirier et al., 2000). Indeed, application of amino cupric silver methods has, above all, established that neurotoxicologic effects of atrazine did not occur in a dimorphic fashion for all brain areas of Mus musculus. This characteristic is further supported by similar morphological alterations, plus a numerical reduction of neurons, in hypothalamic sites of both sexes, findings that seem to corroborate the lack of dimorphic pesticide influences on some neuroendocrine structural and functional features of rodents (Cooper et al., 2000).

It is worth noting that cerebral damage detected in some hypothalamic and extrahypothalamic areas appeared to coincide with the marked sex-linked ssergic subtype expression differences in these brain areas. However, to be able to point out the values of such atrazine-dependent neurotoxicological differences, it was necessary to show that no dimorphic expression pattern characterized the major ss subtypes (sst2,3,5) in all brain regions of our mice. Abundant sst2-expressing neurons were indeed featured in extrahypothalamic and hypothalamic regions such as the hippocampus and the MPOA of both sexes, whereas high sst3 mRNA levels were found mainly in cortical and amygdalar areas, which are in agreement with a similar pattern found in the amygdalar areas of both male and female rats (Thoss et al., 1995). In addition, even the distribution pattern of sst5 mRNA levels in our rodent model confirms the very low non-dimorphic differences of this mRNA sst in most rat brain areas, as reported Thoss et al. (1995).

The precise molecular mechanism by which atrazine interferes with the different neuronal transmitting systems has not yet been extensively investigated. Here we demonstrate that HD of this chlorotriazine herbicide exerts its neurotoxicological effects in a dimorphic regional specific fashion through the variations of distinct sst expressing neurons. A first major effect regards the elevated number of female sst2 neurons of the hypothalamic and amygdalar areas with respect to the same areas in the male, which is in line with both specific subtype and brain areas serving as preferential targets of bisphenol A (another important endocrine disruptor) effects in the female rat (Facciolo et al., 2002). Such a relationship not only appears to be in good agreement with the very evident rising trend of sst2 neurons detected in some female hypothalamic areas such as VMN and SCh, but also tends to further strengthen, in the case of former nucleus, the critical sst-estrogen receptor linked secretory role played by this hypothalamic nucleus during the perinatal developmental stages of females (Djordjijevic et al., 1998). Now an upregulatory pattern of sst2 mRNA expressing neurons was not the only direction induced by atrazine because a downregulation trend was obtained in female striatal areas and these differences might be responsible for the impairment of motor coordination and exploratory behaviors that have been reported for other rodents (Allen et al., 2003).
As far as the other major subtype sst3 is concerned, it appeared to be the main male target of atrazine effects despite a few brain areas that displayed a significantly low number of neurons expressing sst3 mRNA in the female. The male Arc proved to be a first important target site as shown by a marked upregulatory activity of sst3 expressing neurons in this hypothalamic station that is known for its control of feeding, energy expenditure and body weight (Bouret et al., 2004), via an ultradian feed-back mechanism of this subtype and GHRH neurons (Zhang et al., 1999). In a similar fashion for sst2 even in the male, atrazine accounted for a downregulation of sst3 activity in some extrahypothalamic areas such as the motor and learning cortical and hippocampal sites. This specific dimorphic activity appears to be in good agreement with the lack of sustained serergic activities being responsible for early impairments of motor coordination and sensory cognition activities in males (Zeyda et al., 2001). Moreover, a recent demonstration of the substantial loss of neuronal functions in cortical areas of mostly male rodents has also been correlated to atrazine-dependent motor difficulties in this same animal sex (Rodriguez et al., 2005). On the other hand, because microgram atrazine doses, which are equivalent to maximal tolerable quantities in adverse effects of environmentally relevant concentrations of microgram quantities of the herbicide, chlorotriazine that was not only responsible for modified pulsatile release of hypothalamic luteinizing hormone via its interaction with the other ss neureceptor system component, GABA (Shafer et al., 1999) but also linked to risk of breast cancer in humans (Kettles et al., 1997). It is obvious that we are only at the beginning and additional studies dealing with neurotoxin-receptor interactions in discrete brain areas are required to better understand the molecular mechanisms underlying the effects of atrazine. However our results strengthen public concern regarding the adverse effects of environmentally relevant concentrations of this herbicide for human health.

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