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Dopaminergic toxicity of the herbicide atrazine in rat striatal slices

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Abstract

A possible link between Parkinson's disease and pesticide exposure has been suggested, and recently it was shown that the herbicide atrazine (ATR) modulates catecholamine metabolism in PC12 cells and affects basal ganglia function in vivo. Hence, the objectives of this study were to: (i) determine if ATR is capable of modulating dopamine (DA) metabolism in striatal tissue slices *in vitro* and (ii) to explore possible mechanisms of its effects. Striatal tissues from adult male Sprague Dawley rats were incubated with up to 500 μ M ATR in a metabolic shaker bath at 37 °C and an atmosphere of 95% O₂ and 5% CO₂ for 4 h. At the end of incubation, samples were collected for both tissue and media levels of DA and its metabolites (3,4-dihydroxyphenylacetic acid, DOPAC and homovanillic acid, HVA), which were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD). To gain some mechanistic insight in to the way ATR affects DA metabolism, several pharmacological manipulations were performed. Striata exposed to ATR at concentrations of 100 μ M and greater had a dose-dependent decrease of tissue levels of DA. At doses of ATR 50 μ M and greater, the DOPAC+HVA/DA ratio was dose-dependently increased. Tyrosine hydroxylase (TH, the rate-limiting enzyme in DA synthesis) protein levels and activity were not affected by ATR treatment. However, high potassium induced DA release into the medium was decreased, whereas the increase in media DA observed in the presence of the DA uptake inhibitor nomifensine was increased even further by ATR in a dose-dependent manner. All of these effects of ATR were observed at levels that were not toxic to the tissue, as LDH release into the medium (lactate dehydrogenase, an index of non-specific cytotoxicity) was not affected by ATR. Taken together, results from this study suggest that ATR decreases tissue DA levels not by affecting TH activity, but possibly by interfering with the vesicular storage and/or cellular uptake of DA.

Keywords

atrazine; pesticide neurotoxicity; dopamine; striatum; Parkinson's disease

1. Introduction

The contribution of environmental contaminants to the etiology of neurodegenerative diseases, such as Parkinson's disease (PD), is now being broadly recognized (Calne and Langston 1983; Di Monte 2003; Koller et al. 1990; Mackin 2000). Of importance, a recent twin study for PD incidence indicated that genetic factors do not play a major role in causing adult onset PD (Tanner et al. 1999). While several environmental factors have been implicated in the

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etiology of PD, the evidence for a positive relationship between PD incidence and pesticide exposure is increasing (Le Couteur et al. 1999; Lockwood 2000; Ascherio et al. 2006; Frigerio et al. 2006). In animal studies, mice exposed to the fungicide maneb, the herbicide paraquat, or their combination, exhibit PD symptomatology (McCormack et al. 2002; Thiruchelvam et al. 2000). Other pesticides, such as dieldrin (Kitazawa et al. 2003; Richardson et al. 2006) and heptachlor (Miller et al. 1999), also cause dopaminergic perturbations *in vivo* and *in vitro*.

The herbicide atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine; ATR) is a very widely used broad-spectrum pesticide and is applied on major crops such as corn, soybeans, and sugarcane (EPA 2003). For example, in 1997 in the U.S. alone, more than 33,800 metric tons of ATR were applied on crops (Gianessi 2000). Besides its widespread use, an additional concern is the fact that ATR is the most frequently detected pesticide in ground and surface waters (Dorfler et al. 1997; Koskinen and Clay 1997). In this regard, a meta-analysis of existing data not only confirmed pesticide exposure as a risk factor for PD but also found a significant association between well water consumption and PD incidence (Priyadarshi et al. 2001).

Many of the toxicological studies conducted so far have focused primarily on the effects of ATR on the endocrine and reproductive systems (i.e., Cooper et al. 2000; Stoker et al. 2000; Narotsky et al. 2001; Stoker et al. 2002). One prominent effect observed following ATR exposure is the disruption of the hypothalamic control of pituitary-ovarian function, possibly by affecting hypothalamic dopamine (DA) and norepinephrine (NE) which are regulatory molecules for pituitary prolactin and luteinizing hormone secretion, respectively (Cooper et al. 2000).

Nevertheless, there are studies that have investigated the neurotoxic potential of ATR (Castano et al. 1982; Podda et al. 1997). In terms of the potential of ATR being a basal ganglia toxicant, *in vitro* exposure to ATR for up to 24 h dose-dependently decreased cellular levels of DA in PC12 cells (Das et al. 2000). While ATR treatment also decreased both intra and extracellular NE levels, NE levels were less sensitive to ATR than DA (Das et al. 2000) and a longer exposure duration was required in order to observe effects (Das et al. 2003). This suggests a possible greater sensitivity of DA to ATR. More recently, two *in vivo* studies observed that ATR exposure altered striatal neurochemistry and caused dopaminergic neuron loss in the substantia nigra of rats (Rodriguez et al. 2005) and mice (Coban and Filipov 2006).

Acutely-isolated brain slices are very widely used in the field of neuroscience, as they have certain experimental advantages over *in vivo* studies and, particularly, cell lines (Somjen et al. 1987). Acutely-isolated striatal slices, in particular, contain all of the molecular machinery that allows them to maintain DA homeostasis and intact architecture of the DA terminal fields for several hours and have been used for numerous neurotoxicity studies (i.e., Bemis and Seegal 1999; Chishti et al. 1996; Filipov et al. 2005; Hirata et al. 2001; Hirata and Nagatsu 2005; Huguet et al. 1997). A significant advantage of striatal slices over PC12 cells is the fact that the monoamine transporter responsible for packaging DA into the vesicles, the vesicular monoamine transporter (VMAT), is type 1 for the PC12 cells (peripheral- type) and type 2 (VMAT-2) for the striatal slices (CNS-type); this differential distribution of VMATs is associated with marked differences in the sensitivity of rat brain and adrenal catecholamine stores to pharmacological depletion by tetrabenazine (Adamson et al. 1997).

Based on the existing data (Das et al. 2000; Rodriguez et al. 2005; Coban and Filipov 2006), our working hypothesis for the current study was that ATR exposure of rat striatal slices will cause a decrease in striatal DA levels and that the decrease will be associated with altered DA metabolism. To test this hypothesis, effects of ATR exposure on DA metabolism in striatal slices were assessed. Besides confirmation of the *in vitro* effects seen in PC12 cells (Das et al.

2000) and the *in vivo* effects observed in Rodriguez et al. (2005) and Coban and Filipov (2006), by using various pharmacological manipulations, we sought to determine potential mechanisms of the neurotoxicity of ATR.

2. Materials and methods

2.1. Chemicals

ATR (ATR; 2-chloro-4-ethylamino-6-isopropylamino-s-triazine; 98% purity; lot 301-49A) was obtained from ChemServices (West Chester, PA). A 50 mM stock was prepared in absolute ethanol and exposure solutions were made from it. All other chemicals, unless specified, were obtained from Sigma (Saint Louis, MO).

2.2 Animals, tissue collection, and exposure

Male Sprague-Dawley rats (2.5 to 4 months old; Harlan, Indianapolis, IN) were housed for 1 week of acclimatization and throughout the experiment on a 12 h light/dark cycle, with water and food available *ad libitum*. All procedures employed in this study were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of Mississippi State University.

Slices containing the striatum were collected and processed according to Bemis and Seegal (1999) and Chishti et al. (1996). Briefly, following CO₂ euthanasia (mid-morning, 9–11 AM), the brains were quickly removed, rinsed with oxygenated ice-cold Hank's-buffered saline solution (HBSS; pH 7.4) and four 350 µm thick coronal sections were collected using an oscillating tissue slicer (EMS-4000, Electron Microscopy Sciences, Hatfield, PA). Immediately following sectioning, 2-mm diameter punches of the striatal tissue were collected bi-laterally (a total of 8 punches per animal), completely randomized, and transferred into 24-well plates (Costar) containing 0.5 ml of HBSS supplemented with 1% horse serum, a concentration range of ATR, and (if necessary) other pharmacological manipulators.

In each of the experiments, a minimum of 4 punches/experimental condition were used and each experiment was repeated at least once. Control slices received appropriate vehicle controls based on the highest concentration of ATR/drug used. In the case of ATR, 5 µl/ well (10 µl/ ml) of 100 % ethyl alcohol was added to control wells in all experiments. In preliminary experiments, we determined that this concentration of ethanol did not cause any neurochemical/ cytotoxic effects on its own. In fact, more than 25 µl/well (50 µl/ml) of ethanol was required in this experimental paradigm for any differences from media-exposed tissues to be observed (increased media DOPAC being the most sensitive indicator). The plates were placed in a Dubnoff metabolic shaker bath (Precision Scientific Inc. Chicago, IL) and shaken at 80 oscillations per min at 37 °C temperature under an atmosphere of 95% O₂ and 5% CO₂ for 4 h. At the end of incubation, tissues and media aliquots were collected and processed for HPLC analysis as described below.

2.3. Pharmacological manipulations

a. Determination of tyrosine hydroxylase activity using L-DOPA accumulation as an indicator—Striatal slices were pre-incubated for 30 min in a medium containing 20 µM of 3-hydroxy-benzylhydrazine (NSD-1015, an L-aromatic amino acid decarboxylase [AAAD] inhibitor), which was followed by the addition of a dose-range of ATR and an additional 4 h incubation. Accumulation of 3,4-dihydroxy phenylalanine (L-DOPA) in the media was used as indicator of tyrosine hydroxylase (TH) activity (Demarest and Moore 1979), as previously described (Filipov et al. 2005). As we have previously indicated (Filipov et al. 2005), L-DOPA readily diffuses into the medium where it accumulates (more than 99.9 % of the total). Thus, L-DOPA accumulation in the medium is the appropriate indicator of TH activity under our experimental conditions.

b. Determination of the effects of ATR exposure on high potassium-stimulated dopamine release—To determine whether ATR will affect high K⁺-stimulated DA release, striatal slices were exposed to a dose-range of ATR in normal medium (1% HBSS) for 4 h. The media was then aspirated and replaced with 500 µl of fresh culture medium containing high-potassium (55 mM) for an additional 5 min incubation. The media was then collected for analysis of DA levels as described below.

c. Determination of the effects of ATR exposure on media DA levels in the presence of the DA uptake blocker, nomifensine—To determine whether the increases in media DA levels caused by ATR exposure could be modulated by a pharmacological inhibition of DA uptake, striatal slices were pre-incubated for 30 min with a concentration-range of nomifensine (20, 100, and 500 µM). ATR was then added to the culture medium and the slices were incubated for additional 4 h. Slices and media samples were then collected and tissue and media levels of DA were analyzed as described below.

2.4. HPLC analysis for DA and its metabolites

DA and its metabolites, DOPAC (3,4-dihydroxyphenylacetic acid) and HVA (homovanillic acid), were analyzed by HPLC with electrochemical detection (HPLC-ECD) with chromatographic conditions slightly modified from those previously reported (Seegal et al. 1986). Briefly, at the end of incubation, 100 µl aliquots of media were mixed with 100 µl 0.4 N perchloric acid and stored at −80 °C until HPLC analysis for monoamines and their metabolites. Each slice was sonicated in 100 µl of 0.2 N perchloric acid and frozen separately until HPLC analysis. Aliquots (20 µl tissues; 50 µl media) of the supernatants were injected into HPLC (Waters Alliance) interfaced with an electrochemical detector (Waters 2465) with a glossy carbon flow-through detector cell. The analytes were separated on a C₁₈, 5µm base deactivated reverse-phase column (Supelco, Sigma). The mobile phase with final pH of 3.65 (adjusted with 5 M phosphoric acid) consisted of 84 mM of NaH₂PO₄H₂O, 1.15 mM of octyl sodium sulfate, 0.09 mM of EDTA disodium salt, 0.25 mM of triethylamine, and 17.5 % methanol as an organic modifier. The neurochemistry data were normalized on a per mg of tissue protein basis before statistical analysis. Protein content in the tissue samples was determined using the Bradford protein assay method in a 96-well plate (Bio-Rad, Hercules, CA) using bovine serum albumin (BSA) as a standard.

2.5. Western blot analysis for tyrosine hydroxylase (TH) protein levels

At the end of the 4-hour incubation with a concentration-range of ATR, striatal slices were rinsed with HBSS, collected from the exposure wells, and placed in 100 µl of RIPA lysis buffer supplemented with protease inhibitor cocktail (Sigma). Samples were sonicated, held on ice for 30 min with occasional vortexing, and stored at −80 °C until analysis. Prior to the Western blot analysis, protein concentration in the tissue lysates was determined using the Bradford method with reagents obtained from Bio-Rad. Samples were heat-denatured for 5 min at 95 °C and then ten micrograms of total protein were loaded and separated on a 10% SDS-PAGE gel. Following electrophoresis, samples were transferred to a PVDF membrane, and the membranes were blocked in 5% milk for 1 h at RT. The membranes were then incubated overnight at 4 °C with a rabbit polyclonal antibody specific for tyrosine hydroxylase (Chemicon, Temecula, CA) at 1:3000 dilution, washed 3x, and then probed with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (Bio-Rad) at a 1:10,000 dilution. The blots were exposed to SuperSignal West Pico chemiluminescent substrate (Pierce; Rockford, IL) for up to 15 sec and then enclosed in transparent covers prior to exposure to x-ray film. Band density was analyzed using the UN-SCAN-IT software package (Silk Scientific Inc., Orem, UT). Following TH detection, certain membranes were stripped and re-probed with a polyclonal antibody against β-tubulin (1:1000, Santa Cruz Biotechnology Inc. Santa

Cruz, CA, USA). All steps after the overnight incubation of the membrane with the anti-tubulin primary antibody were identical to the procedure described above for TH.

2.6. Lactate dehydrogenase (LDH) activity determination

Activity of LDH in the medium was determined as an indicator of tissue viability using a method similar to the procedure previously described (Filipov et al. 2005), but adapted to a 96-well plate format. As LDH activity catalyzes the oxidation of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) to NADH, formation of NADH in each sample (10 μ l) was monitored kinetically for 90 sec at 340 nm wave length. Each sample was run in a duplicate and rabbit liver LDH (Sigma) was used as a standard.

2.7. Statistical analysis

Data were analyzed by analysis of variance (ANOVA). In cases where data on the figures are expressed as a percent of a vehicle control, all analyses were performed prior to the expression of the data as a percent of control, i.e., in the case of the neurochemistry data, ng/mg protein of a respective analyte was analyzed. When ANOVA *p*-values were ≤ 0.05 , mean separation was performed using the Student-Newman-Keul's (SNK) multiple comparison *post hoc* test.

3. Results

3.1. Effects of in vitro exposure to ATR on striatal DA metabolism

When rat striatal slices were exposed to a concentration range (50 – 500 μ M) of ATR for 4 h, a dose-dependent decrease in the tissue levels of DA was observed with the lowest effective concentration being 100 μ M (Figure 1). Under control exposure conditions, tissue DA levels were 316.36 ± 25.07 (mean $\pm$ SEM, ng/mg protein) and these levels were decreased to 102.82 ± 25.07 ng/mg protein by exposure to 500 μ M of ATR. Levels of DA in the media were even more sensitive to the effects of ATR, as even the lowest (50 μ M) concentration we used resulted in a significant increase of extracellular DA levels (Figure 2). Under control exposure conditions, media DA levels are low (1.14 ± 0.15 , mean $\pm$ SEM; ng/mg protein), but they increased more than five-fold (6.23 ± 0.41 , mean $\pm$ SEM; ng/mg protein) when slices were exposed to the highest (500 μ M) concentration of ATR. Effects of ATR exposure on the two main DA metabolites, DOPAC (Figure 2 insert) and HVA (data not shown), were also determined but their levels were only marginally affected. Under control exposure conditions, media DOPAC and HVA levels were 20.81 ± 1.04 and 1.95 ± 0.31 (mean $\pm$ SEM) ng/mg protein, respectively. Media HVA levels exhibited a numerical decline that did not reach statistical significance even at the 500 μ M dose of ATR ($P \geq 0.25$), whereas media DOPAC levels were modestly (21%, $P < 0.08$) decreased by the highest (500 μ M) dose of ATR (to 16.41 ± 1.04 ng/mg protein. Figure 2 insert). As a result of the dose-dependent DA decrease and the minor alterations in the DA metabolites DOPAC and HVA, the (DOPAC + HVA)/DA ratio, an index of DA metabolism, was dose-dependently increased (Figure 3).

3.2. Effects of in vitro exposure to ATR on LDH release

The observed effects on striatal neurochemistry were, apparently, not caused by non-specific cytotoxicity, as LDH levels in the culture medium remained unchanged by ATR exposure and, moreover, additional 30 min incubation with the nonionic detergent Triton X100 after the 4 h incubation with ATR/vehicle, resulted in a massive increase of media LDH that was not different between slices previously exposed to plain control medium, vehicle-containing medium, of medium containing 500 μ M ATR (Figure 4). In pilot studies, we exposed striatal slices to greater concentrations of ATR and observed that the lowest concentration of ATR that caused a significant (35%) increase in media LDH was 1 mM (data not shown).

3.3. Effects of in vitro exposure to ATR on striatal tyrosine hydroxylase protein levels and activity

To determine whether the decreases in tissue levels of DA were due to ATR-induced alterations in tyrosine hydroxylase (TH, the rate-limiting enzyme for DA synthesis) levels, the protein levels of TH in the ATR-exposed slices were analyzed by Western blotting. After 4 h of exposure, protein levels of TH were not significantly different between control and ATR-exposed striatal slices (Figure 5A). Moreover, the activity of TH (as measured by L-DOPA accumulation in the presence of the AAAD inhibitor NSD-1015) also remained unchanged by ATR exposure (Figure 5B).

3.4. Effects of in vitro exposure to ATR on high potassium-stimulated DA release

The decrease in tissue levels of DA caused by ATR (Fig. 1) was also associated with a diminished ability of the striatal slices to respond to high-potassium stimulation. In slices exposed to a high-potassium containing culture medium, the magnitude of the high K⁺-induced release was diminished by pre-exposure to ATR (Fig. 6). This effect, however, was significant only at the 250 and 500 μ M concentrations, suggesting that the decrease in tissue DA observed at doses lower than 250 μ M (Fig. 1) was not related to the ability of the slices to respond to the high K⁺ stimulus.

3.5. Effects of in vitro exposure to ATR on media DA in the presence of nomifensine

As media DA levels appeared to be particularly sensitive to the effects of ATR, it was determined whether a high-affinity DA transporter (DAT) blocker, nomifensine, could interfere with the ability of ATR to increase media DA levels. As expected, 20 μ M nomifensine itself (a concentration typical for similar studies) increased the amount of DA in the media by almost 2000% (from 1.14 ± 0.15 to 22.42 ± 1.40 , mean $\pm$ SEM ng/mg protein; Figure 7A), while not affecting the tissue DA levels (Figure 7B). However, presence of ATR in the exposure medium further increased media DA levels with the effect being dose-dependent. Similar findings were observed when the nomifensine concentration in the media was increased to 100 μ M. The effect of ATR exposure on media DA was no longer apparent only when 500 μ M nomifensine was present in the media (Figure 7A). This concentration of nomifensine however, was also associated with an almost complete depletion (4 % remaining) of the tissue levels of DA (Fig 7B).

4. Discussion

The most significant finding of this study was that, as we had hypothesized, ATR exposure decreased striatal tissue levels of DA. The decrease was associated with increased media levels of DA and increased (DOPAC+HVA)/DA ratio, while TH levels and activity were not affected in this exposure paradigm. Thus, data from the described experiments suggest that ATR is a dopaminergic toxicant and are in agreement with previous *in vitro* studies where exposure to ATR dose-dependently decreased cellular levels of DA in PC12 cells (Das et al. 2000, 2003). Moreover, decreased striatal DA levels were also reported after *in vivo* exposure to rats (Rodriguez et al. 2005) and mice (Coban and Filipov 2006). As the link between PD and pesticide exposure (Ascherio et al. 2006; Frigerio et al. 2006; Priyadarshi et al. 2000) is now well established, collectively, these studies indicate that in addition to rotenone (Sherer et al. 2003), paraquat (Brooks et al. 1999), maneb (alone or in combination with paraquat) (Thiruchelvam et al. 2003), dieldrin (Kitazawa et al. 2003; Richardson et al. 2006) and heptachlor (Miller et al. 1999), among others, exposure to the herbicide ATR may also be a contributory factor to PD.

As the decrease in tissue levels of DA was associated with a dose-dependent increase of the DOPAC+HVA/DA ratio, DA metabolism might have been affected by ATR. Following

reuptake, DA is converted to DOPAC primarily intraneuronally, whereas HVA is produced primarily extraneuronally (Cooper et al. 2003). Thus, an inhibition of vesicular uptake caused by ATR would increase the levels of free cytosolic DA, which is converted by a monoamine oxidase (MAO) to DOPAC. The fact that DOPAC levels were only moderately decreased by the highest concentration of ATR argues that DOPAC levels were maintained due to increased substrate (cytosolic DA) availability resulting from an inhibition of vesicular uptake. In addition to possible inhibition of vesicular uptake by ATR, cellular uptake may have also been compromised by ATR exposure. Two of our findings argue that: first, media (extracellular) levels of DA were dose-dependently increased by ATR; second, HVA levels were not affected significantly by ATR. Whether ATR interacts directly with the transporters responsible for vesicular and cellular uptakes, VMAT-2 and DAT, respectively, remains unknown at present and will be the subject of future studies. In the case of DAT however, our experiments with the DAT blocker nomifensine indicate that the actions of ATR on DA uptake are not eliminated unless tissue levels of DA are almost completely depleted (Figure 7B), suggesting that either ATR does not interact with DAT directly and the increase in media DA levels in the presence of the nomifensine is due to an inhibition of vesicular uptake, or, that the mechanism of ATR's interaction with DAT is distinct from that of nomifensine.

Our data from the experiments with high-potassium stimulation also hint towards ATR-caused interference with vesicular storage of DA. It is well established that at least two different pools of DA exist intraneuronally, a storage pool and a readily-releasable pool of DA, with the later possibly being comprised of newly synthesized DA (Leviel et al. 1989; Zetterstrom et al. 1988). High-potassium stimulation releases DA primarily from the releasable pool (Zetterstrom et al. 1988). In our study, media DA levels following high-potassium challenge, albeit decreased, were less sensitive to the effects of ATR than the tissue levels of DA, perhaps suggesting that even though ATR affects both storage and releasable pools of DA, it interferes more with the storage pool of DA than it does with the releasable (newly synthesized) pool.

Another possible reason for the decrease in tissue DA levels that we observed in the present study could be an inhibition/decreased expression of the rate limiting enzyme for DA synthesis tyrosine hydroxylase (TH; Cooper et al. 2003). However, when we examined both TH activity and protein levels, no ATR-caused alterations were observed (Figure 5). Similarly, despite affecting cellular DA concentrations, the expression of TH, albeit numerically decreased, was not significantly affected by ATR in PC12 cells (Das et al. 2003). Also, TH protein levels in the striatum of mice exposed to ATR were not different among treatments at a time when DA levels were decreased (Coban and Filipov 2006). Even though ATR exposure may still have some effect on the TH phosphorylation status, which was not examined in the current study, it appears that TH is not a major target for ATR exposure and that ATR acts by regulating some other cellular mechanism, such as interfering with vesicular storage of DA as indicated above.

The fact that NE levels were also affected by ATR exposure to PC12 cells (Das et al. 2000) and that the number of TH-positive neurons was decreased by ATR not only in the substantia nigra pars compacta, but also in the ventral tegmental area, which projects to the nucleus accumbens, in both rats (Rodriguez et al. 2005) and mice (Coban and Filipov 2006), suggest that ATR does not target the nigrostriatal pathway exclusively. On the other hand, DA may be more sensitive than NE as suggested by the studies of (Das et al. 2000) and our findings with the short-term exposure of striatal slices.

Human exposure to ATR is fairly common. In fact, it was estimated that more than half of the population in the United States is exposed to ATR (Birnbaum and Fenton 2003). Moreover, documented exposures of over 200,000 people to ATR levels above the acute Reference Dose (RfD) have been reported (EPA 2003). Of note, the acute RfD was derived from a NOAEL of 10 mg/kg/day and a LOAEL of 70 mg/kg/day (EPA 2003). The dose-range of ATR that we

used in this study, taking into account the shorter exposure duration, is comparable with the amount of ATR (up to 200 μ M for up to 24 h) used in the studies with PC12 cells (Das et al. 2000, 2003) and in line with other neurotoxicity studies with less frequently used/banned pesticides (Kitazawa et al. 2001, 2003; Peng et al. 2004; Uversky et al. 2001; Yang and Tiffany-Castiglioni 2005). Das et al. (2000) have provided justification for the relevancy of this dose-regimen to *in vivo* toxicity studies.

It is difficult to compare *in vitro* and *in vivo* results and, ultimately, pharmacokinetic data with actual levels of ATR in the brain would be the best reference for an *in vitro* dose-regimen consideration. Even though we have an ongoing project where levels of ATR in the brains of mice will be determined using our recently published LC-MS method (Ross and Filipov 2006), such data are lacking at present. Nevertheless, an earlier study with rats (Bakke et al. 1972) indicated that a relatively low dose of 14 C-labeled ATR (approximately 2.5 mg/kg) resulted in tissue residues of total 14 C-labeled ATR equivalents of approximately 7.4 μ M two days after ATR administration. Importantly, eight days after ATR administration, almost 70% of the radioactivity that was detected at two days, was still present. Considering that ATR is fairly rapidly metabolized in the periphery, these data suggests that (i) ATR and its metabolites reach greater brain levels than the levels determined two days after administration and that (ii) their elimination from the brain is not rapid.

As we have indicated in detail (Coban and Filipov 2006), data from this and the other studies (Das et al. 2000; Rodriguez et al. 2005) are potentially relevant to high levels of exposure to this pesticide as they have occurred occupationally as reported by the Agricultural Health Study (AHS, Storm 2004). As indicated in the ongoing AHS study, segments of society, such as farmers, pesticide applicators, and other individuals in the vicinity of ongoing pesticide application, including children, are intermittently exposed to much higher levels of ATR and these levels are high enough for a substantial proportion of the farmers and commercial applicators enrolled in the AHS that were exposed to ATR to experience health problems (Storm 2004). Another indicator for high occupational exposures in certain segments of the society are the facts that in ongoing metabolism studies we have been able to detect the urinary metabolite of ATR, ATR-mercapturate, only in mice acutely exposed to the 125 and 250 mg/kg doses (Ross and Filipov 2006), whereas ATR-mercapturate is readily detectable in the urine of pesticide applicators (Lucas et al. 1993), thus suggesting high occupational exposure. The finding that ATR was readily detected in the saliva of pesticide applicators further substantiates significant exposure levels during spraying with this pesticide (Hines et al. 2006). Moreover, a recent study indicates that (i) the urinary metabolite of ATR was substantially higher in fathers, mothers and children living on a farm compared with non-farm households, regardless of pesticide application, and that (ii) recent ATR application by fathers resulted in increased urinary atrazine in their children (Curwin et al., 2006). Thus, besides farmers and pesticide applicators that are exposed to ATR occupationally, farm households and rural communities are also at risk of high levels of exposure to this pesticide.

In summary, our data further support the notion that, in cases of high occupational exposure, ATR can be considered as a dopaminergic toxicant and, potentially, as a contributory risk factor for PD and other neurological disorders where DA levels are affected. Modulation of DA metabolism by possible inhibition of vesicular and/or cellular uptakes appears to be involved in the mechanism of the toxicity of ATR.

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Figure 1.

Effects of atrazine (ATR) exposure on tissue levels of dopamine (DA). Striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, tissues were processed and striatal levels of DA quantified by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. ^{a, b, c} The presence of different letters on top of bars indicates significant differences due to ATR exposure with the different letters being different from one another as well as from bars without any letters (control bars and bars representing ATR concentrations that are not different from the controls; $P < 0.05$).

Figure 2.

Effects of atrazine (ATR) exposure on media levels of dopamine (DA, main figure) and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC, figure insert)). Striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, culture medium was collected and processed for quantification of DA and DOPAC levels by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. a, b, c, d, e The presence of different letters on top of bars indicate differences due to ATR exposure with the different letters being different from one another as well as from bars without any letters (control bars and bars representing ATR concentrations that are not different from the controls; $P < 0.05$). ^ indicates $P < 0.08$ compared to control for media DOPAC.

Figure 3.

Effects of atrazine (ATR) exposure on (DOPAC + HVA)/DA ratios. Striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, tissues and culture medium were collected and processed for quantification of DA and its metabolites DOPAC and HVA by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. a, b, c, d, e, f The presence of different letters on top of bars indicate differences due to ATR exposure with the different letters being different from one another as well as from bars without any letters (control bars and bars representing ATR concentrations that are not different from the controls; $P < 0.05$).

Figure 4.

Effects of atrazine (ATR) exposure on lactate dehydrogenase (LDH) release in the culture medium. Striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, culture medium was collected and processed for quantification of LDH activity as described in detail in the Material and Methods section. To subset of tissues that was exposed for 4 h to plain culture medium, medium containing 5 μ l/well 100% ethyl alcohol vehicle, or 500 μ M ATR, exposure media was collected and replaced with a fresh medium containing 0.5% Triton X100. Following an additional 30 min of exposure, LDH activity in the media was quantified as described in the Materials and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. Lack of different letters on top of bars indicates lack of differences

due to ATR exposure ($P > 0.3$). * Presence of asterisks indicates significant effect of Triton X100 ($P < 0.001$).

Figure 5.

Effects of atrazine (ATR) exposure on tyrosine hydroxylase (TH) protein levels (A) and activity (B). Striatal levels of TH were determined after 4 h of exposure to ATR by a Western blot analysis as described in the Materials and Methods section. For determination of TH activity, following 30 min pre-incubation with the AAAD inhibitor NSD-1015, striatal tissues were exposed to several different concentrations of ATR for 4 h. At the end of exposure, total levels of L-DOPA (a DA precursor, used as an index for TH activity) were quantified by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. Lack of different letters on top of bars indicates lack of differences due to ATR exposure ($P > 0.5$).

Figure 6.

Effects of atrazine (ATR) exposure on high-potassium stimulated dopamine (DA) release. Striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, culture medium was replaced with high-potassium (55 mM) medium and slices exposed to it for additional 5 min. Media was collected and levels of DA in it were quantified by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. ^{a, b} Presence of different letters on top of bars indicate significant differences due to ATR exposure with the different letters being different from one another as well as from bars without any letters (control bars and bars representing ATR concentrations that are not different from the controls; $P < 0.05$).

Figure 7. Effects of atrazine (ATR) exposure on media (top) and tissue levels of dopamine (DA) in the presence of several different concentrations of nomifensine, a DA reuptake inhibitor. Following 30 min of pre-exposure to nomifensine, striatal tissues from male Sprague Dawley rats were exposed to a concentration-range of ATR for 4 h. At the end of exposure, DA levels in the culture medium and the tissue were quantified by HPLC-ECD as described in detail in the Material and Methods section. Data are presented as mean $\pm$ SEM. Each bar represents $n \geq 8$ from a minimum of two independent experiments. *, **, *** Presence and number of asterisks indicate effect of nomifensine ($P < 0.05$). a, b, c Presence of different letters on top of bars indicate significant differences due to ATR exposure with the different letters being different from one another as well as from bars without any letters (control bars and bars representing ATR concentrations that are not different from the controls) within a particular nomifensine concentration ($P < 0.05$).