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Alteration of dopamine uptake into rat striatal vesicles and synaptosomes caused by an in vitro exposure to atrazine and some of its metabolites

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Abstract

Studies have shown that both *in vivo* and *in vitro* exposure to the herbicide atrazine (ATR) results in dopaminergic neurotoxicity manifested by decreased striatal dopamine (DA) levels. However, the mechanism behind this reduction is largely unknown. A decrease in striatal DA could be due to ATR exposure affecting vesicular and/or synaptosomal uptake resulting in disrupted vesicular storage and/or cellular uptake of DA. Hence, we investigated the effects of *in vitro* ATR exposure on DA uptake into isolated rat striatal synaptosomes and synaptic vesicles. In addition to ATR, effects of its major mammalian metabolites, didethyl atrazine (DACT), desethyl atrazine (DE) and desisopropyl atrazine (DIP) were investigated. ATR (1–250 μ M) inhibited DA uptake into synaptic vesicles in a dose dependent manner. Of the three ATR metabolites tested, DACT did not affect vesicular DA uptake. DE and DIP, on the other hand, significantly decreased vesicular DA uptake with the effect of 100 μ M DE/DIP being similar to the effect of the same concentration of ATR. Kinetic analysis of vesicular DA uptake indicated that ATR significantly decreased the V_{max} while the K_m value was not affected. Contrary to the inhibitory effects on vesicular DA uptake, synaptosomal DA uptake was marginally (6–13%) increased by ATR and DE, but not by DACT and DIP, at concentrations of ≤ 100 μ M. As a result, ATR, DIP and DE increased the synaptosomal/vesicular (DAT/VMAT-2) uptake ratio. Collectively, results from this study suggest that ATR and two of its metabolites, DIP and DE, but not its major mammalian metabolite, DACT, decrease striatal DA levels, at least in part, by increasing cytosolic DA, which is prone to oxidative breakdown.

Keywords

Atrazine; dopamine uptake; synaptosomes; synaptic vesicles; neurotoxicity

1. Introduction

Genetics is not a major contributory factor to Parkinson's Disease (PD) when the onset of symptoms occurs after the age of 50 (Tanner et al., 1999). Increasing evidence indicates that the environment plays an important role in the etiology of basal ganglia pathologies, such as PD. Living in a rural area, farming, drinking well water, and occupational exposure to

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agricultural chemicals are all suspected PD risk factors (Gorell et al., 1998; Tanner et al., 1999; Priyadarshi et al., 2001; Di Monte et al., 2002; Brown et al., 2006). Among the risk factors for PD, pesticide exposure has received particular attention and it has been associated with increased incidence of PD in agricultural workers in rural environments (Semchuk et al., 1992; Schapira, 1999; Priyadarshi et al., 2000; Brown et al., 2006). For example, increased incidence of PD associated with high use of agricultural pesticides was observed in rural California (Ritz and Yu, 2000), while in the state of Washington, a trend towards increased PD incidence in pesticide applicators and crop farmers was observed (Firestone et al., 2005). Furthermore, case control studies suggest that the risk of PD is increased when the duration of exposure to pesticides exceeds a particular threshold (Seidler et al., 1996; Gorell et al., 1998). Specific pesticides that have been associated with PD-like symptomology (i.e., dopaminergic toxicity) are paraquat (Fredriksson et al., 1993; McCormack et al., 2002), maneb (Morato et al., 1989; Takahashi et al., 1989), and rotenone (Alam and Schmidt, 2002; Gao et al., 2003). In the case of paraquat and maneb, greater dopaminergic toxicity was observed in animals exposed to both of these pesticides (Thiruchelvam et al., 2000). Increased levels of organochlorine pesticides, such as dieldrin, have also been detected in the brains of PD patients (Fleming et al., 1994) and dieldrin has been demonstrated to be toxic to the basal ganglia in animal models (Richardson et al., 2006).

Atrazine (ATR) is an extensively used herbicide throughout most of the world; about 76.5 million pounds are used per year throughout the U.S. to control variety of weeds in agricultural crops, as well as on golf courses and residential lawns where its use has increased (EPA, 2003). ATR is moderately volatile and water soluble, and tends to persist in ground and surface water, which is a main reason for long-term, low environmental exposures. Occupational exposure to higher levels of ATR is of particular concern as levels of ATR indicative of substantial exposure are detected in the saliva and urine of pesticide applicators and farmers during spraying (Hines et al., 2006). A recent study reported that not only ATR applicators but also their families are at risk of high level of exposure to this pesticide (Curwin et al., 2007). They found considerably higher amounts of urinary ATR and its metabolites in applicators and their families living on the farm.

The metabolism of ATR has been studied in different species (Bakke et al., 1972; Ikonen et al., 1988; Catenacci et al., 1990; McMullin et al., 2003; Ross and Filipov, 2006). Major ATR metabolites reported in mammals and humans are desethyl atrazine (DE), desiopropyl atrazine (DIP), and didealkyl atrazine (DACT; Bakke et al., 1972; Erickson et al., 1979; Catenacci et al., 1993; McMullin et al., 2003; Ross and Filipov, 2006). DACT is the primary urinary metabolite detected in mice (Ross and Filipov, 2006) and rats (McMullin et al., 2003). While DE and DIP were two major ATR metabolites found in urine of occupationally exposed humans in earlier studies (Ikonen et al., 1988; Catenacci et al., 1990), very recent evidence, using modern analytical technology, indicates that DACT is the most frequently detected human metabolite of ATR (Barr et al., 2007).

Recently, ATR has been identified as a potential basal ganglia toxicant. Thus, two separate studies found that ATR exposure decreased striatal dopamine (DA) levels and caused a loss of tyrosine hydroxylase (TH)-positive dopaminergic neurons in both the substantia nigra pars compacta (SNpc) and the ventral tegmental area (VTA) in rats (Rodriguez et al., 2005) and mice (Coban and Filipov, 2007). In addition, our laboratory (Filipov et al., 2007) found that in vitro exposure to ATR decreased the tissue levels of DA in striatal slices while media levels of DA and its turnover [(DOPAC + HVA)/DA] ratio were increased. Earlier studies with PC12 cells demonstrated that in vitro exposure to ATR ($\geq 12.5 \mu\text{M}$) significantly decreased intracellular DA in a concentration-dependent manner (Das et al., 2000). In another study, this group (Das et al., 2001) reported that the ATR metabolites, DE, DIP, and DACT, affected PC12 cells DA homeostasis in a manner different from the effects of ATR, i.e., they increased

intracellular DA. While experimental evidence for the dopaminergic toxicity of ATR is increasing, at this time, there is no epidemiological data linking ATR exposure and PD.

Dopamine is converted to 3, 4-dihydroxyphenylacetic acid (DOPAC) intraneuronally and to homovanillic acid (HVA) extraneuronally in the synaptic cleft (Cooper et al., 2003). Upon its release, DA undergoes rapid reuptake to terminate its action and maintain DA homeostasis. Reuptake is accomplished in two ways: synaptosomal uptake through the dopamine transporter (DAT), which transports DA from the extracellular space into the cytosol, and vesicular uptake by vesicular monoamine transporter-2 (VMAT-2), which stores DA into synaptic vesicles (Cooper et al., 2003). Perturbation of either of these two uptake mechanisms will result in altered DA homeostasis. For example, the importance of both DAT and VMAT-2 was demonstrated in DAT- knockout mice (Giros et al., 1996) and VMAT-2-knockout mice (Takahashi et al., 1989; Miller et al., 1999; Fon et al., 1997; Wang et al., 1997). Thus, compared to normal mice, extracellular DA remained 300 times longer in DAT-knockouts (Giros et al., 1996), and reduced vesicular storage was observed in VMAT-2-knockout mice (Miller et al., 1999).

Based on our previous findings with striatal slices (Filipov et al., 2007) and on the mechanisms of the maintenance of DA homeostasis, we hypothesized that decreased tissue DA could be caused by direct effects of ATR on vesicular and/or synaptosomal uptake resulting in disruption of vesicular DA storage and/or cellular uptake. Therefore, to test this hypothesis, the present study investigated the effects of in vitro ATR exposure on DA uptake into rat striatal synaptosomes and vesicles. In addition we investigated the effects of ATR's major metabolites, DACT, DE, and DIP on vesicular and synaptosomal uptake of DA.

2. Materials and Methods

2.1. Animals

Adult Sprague-Dawley rats (2–4 months old, 260–300 g, Harlan, Madison, WI) were used as source of striatal synaptosomes and vesicles in these studies. Animals were housed in a climate controlled room (23 ± 0.5 °C and humidity at $55 \pm 5\%$) under a 12-h light/dark cycle (lights on at 06:00 h) with free access to 18% protein rodent diet (Harlan) and water. All procedures were in accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23) and were approved in advance by the Institutional Animal Care and Use Committee (IACUC) of Mississippi State University.

2.2. Chemicals

Atrazine, 2-chloro-4-(ethylamine)-6-(isopropylamine)-s-triazine (ATR; lot # 301-49A, purity: 98%), desethyl atrazine (DE; lot # 266-60A, purity: 99.5%), desiopropyl atrazine (DIP; lot # 282-112A, purity: 98%) and didealkyl atrazine (DACT; lot # 285-100A purity: 96%) were purchased from ChemService (West Chester, PA, USA). MicroScint™-20 cocktail, Top Count NXT™, Filtermate 196 and plate sealer 496 were purchased from Packard Bioscience Company (Meriden, CT, USA). HEPES was purchased from (Acros, NJ, USA) and Whatman unifilter-24™ GF/B and [³H] dopamine hydrochloride ([³H] DA; 1 mCi) were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Polyethylenimine, dopamine hydrochloride (DA), pargyline, adenosine 5-triphosphate disodium (ATP), and reserpine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.3. Exposure solutions

Stock solutions (50 mM) of ATR, DE and DIP were prepared in absolute ethanol (EtOH) and DACT and reserpine were prepared in dimethyl sulfoxide (DMSO); ATR, DE and DIP

completely dissolved in EtOH, whereas DACT and reserpine were not soluble in EtOH but dissolved completely in DMSO. Exposure solutions were prepared with incubation buffer (described below). Since 250 μ M of ATR was used in a single vesicular/synaptosomal study and 100 μ M was the highest concentration used for ATR/DE/DIP and DACT in all other studies, control, vesicles, and synaptosomes were exposed to EtOH and DMSO vehicles in volume present in 100 μ M ATR/DE/DIP and DACT, respectively. In pilot experiments, we determined that EtOH vehicle equivalent to 500 μ M ATR did not cause any alterations in synaptosomal and vesicular uptake

2.4. Preparation of synaptic vesicles

Striatal vesicles were prepared according to (Roz and Rehavi, 2003) with minor modifications. Briefly, rats were euthanized with CO₂, the striata were quickly removed, weighed and homogenized in 10 vol. of ice-cold physiological sucrose solution (0.32 M sucrose, 10 mM Tris HCl; pH 7.4) using a glass-Teflon pestle homogenizer (RZR1, Heidolph, Germany). Homogenates were centrifuged (1500 $\times$ g for 10 min at 2 °C) and the supernatants were briefly stored at 4 °C. The pellets were resuspended in the same volume of ice-cold homogenizing buffer and centrifuged at 1500 $\times$ g for 10 min at 2 °C. The two supernatants were combined and centrifuged at 20,000 $\times$ g for 20 min at 2 °C. The resulting supernatant was discarded and pellets were disrupted by osmotic shock via homogenization in 10 volumes of 5 mM Tris-HCl buffer using a glass-Teflon homogenizer followed by incubation on ice for 45 min. Each homogenate was centrifuged at 20,000 $\times$ g for 20 min and the supernatant was further centrifuged at 62,000 $\times$ g for 40 min. The resulting pellet (synaptic vesicles) was re-suspended in 0.32 M sucrose containing 5 mM Tris HCl (pH 7.4) and stored at 2 mg protein/ml at -80 °C until use. Protein concentration was measured by the method of (Bradford, 1976).

2.5. Assay for vesicular uptake of DA

Vesicular uptake of DA was determined according to (Roz and Rehavi, 2003) with slight modifications. Two sets of experiments were performed: studies with [³H] DA present in the incubation medium and kinetic studies where in addition to [³H] DA, a concentration-range of cold DA was also present. The total reaction incubation volume was 300 μ l. Frozen synaptic vesicles were thawed and diluted to a concentration of 15 μ g protein/100 μ l with assay buffer (100 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 10 mM potassium phosphate buffer; pH 7.4). In the presence of 1 mM Na₂ATP (final concentration), aliquots of 200 μ l vesicles (30 μ g protein) were incubated at 30 °C for 15 min with either vehicles (EtOH or DMSO), ATR (0.1–250 μ M), its metabolites (DE, DACT, or DIP at 1 and 100 μ M), or with reserpine (1 μ M), as a positive control. This was followed by an incubation with cold DA (0.1–100 nM, final concentration, kinetic experiments only) and [³H] DA (6.6 nM, final concentration) for 3 min. The uptake was terminated by adding 2.5 ml of ice cold 0.15 M KCl. Non-specific uptake was determined by incubating at 0 °C. Following termination of uptake, the samples were filtered under vacuum through Whatman unifilter-24™ GF/B (presoaked in 0.3% polyethylenimine) and then washed twice with 2.5 ml ice-cold 0.15 M KCl. The filters were dried overnight at room temperature and radioactivity was counted in 300 μ l MicroScint™-20 cocktail with Top count NXT™ (Packard Bioscience Company, USA). The specific [³H] DA uptake was calculated by subtracting nonspecific from total uptake. For presentation, data were expressed as a percent of appropriate control (EtOH or DMSO).

2.6. Preparation of striatal synaptosomes

Synaptosomes were prepared as previously described (Kirby et al., 1999). Briefly, following euthanasia with CO₂, striata were quickly removed, weighed and homogenized in 20 volumes of ice-cold physiological sucrose solution (0.32 M sucrose, 4.2 mM HEPES; pH 7.4) using a glass-Teflon pestle homogenizer (RZR1). The homogenate was centrifuged at 1500 $\times$ g for 15

min at 2 °C. The supernatants were collected and recentrifuged at $10,000 \times g$ for 15 min at 2 °C. The resulting synaptosomal pellets were gently suspended (one striatum/ml) in ice-cold incubation buffer (0.02% L-ascorbic acid, 50 μ M pargyline, 50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM sucrose, pH 7.4) and held on ice until use.

2.7. Assay for synaptosomal uptake of DA

Synaptosomal DA uptake was measured as previously described (Kirby et al., 1999) with some modifications. The total volume of reaction was 250 μ l. Aliquots of 100 μ l synaptosomal membranes were incubated for 15 min at 37 °C with vehicles (EtOH or DMSO), ATR (1–250 μ M), its metabolites (DE, DACT, and DIP at 100 μ M), or the DA uptake blocker nomifensine (10 μ M) followed by incubated with cold DA (1 nM–1 μ M, final concentration, kinetic experiments only) and [^3H] DA (50 nM, final concentration) for 5 min. Uptake was terminated by addition of ice-cold wash buffer (50 mM Tris-HCl, 125 mM NaCl, 5 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM sucrose, pH 7.4) and rapid vacuum filtration over GF/B filter (Whatman unifilter-24TM). Filters were washed once more with ice-cold wash buffer, allowed to air dry, and then radioactivity was counted in scintillation liquid. The specific [^3H] DA uptake was determined as described above for vesicular uptake. In the studies with ATR and its metabolites where no cold DA was used, data were expressed as a percent of appropriate control (EtOH or DMSO).

2.8. Statistical analysis

Kinetic parameters (K_m and V_{max}) of DA uptake were determined by nonlinear regression and represented on Eadie-Hofstee plots (Prism, Graphpad software, San Diego, CA) and expressed in nM and pmol/mg protein/min, respectively. Statistical analysis of the data was carried out using analysis of variance (ANOVA) followed by Fisher's *post hoc* multiple comparison test (SigmaStat). Data are expressed as mean $\pm$ SEM. All analyses were performed prior to the expression of data as a percent control. Differences were considered to be statistically significant with $p < 0.05$.

3. Results

3.1. Effects of ATR on vesicular uptake of DA

When rat striatal vesicles were exposed to a concentration range (0.1–250 μ M) of ATR for 15 min, a dose-dependent decrease in the vesicular DA uptake was observed with the lowest effective concentration being 1 μ M (Fig. 1). Of the three ATR metabolites tested, DACT (up to 100 μ M) did not affect vesicular DA uptake whereas both DE and DIP significantly (about 35%) decreased the vesicular DA uptake with the effect of 100 μ M DE or DIP being similar to the effect 100 μ M ATR (67% of control or 33% decrease). At the 1 μ M concentration, ATR was more potent than DE and DIP in inhibiting vesicular uptake of DA (Fig. 1). As previously reported (Roz and Rehavi, 2003; Beckstead et al., 2004) reserpine was demonstrated to be a potent inhibitor of DA uptake into synaptic vesicles, i.e., 1 μ M of reserpine decreased vesicular DA uptake by more than 90% (Fig. 1). The effects of ATR exposure on the kinetics of vesicular DA uptake are represented on Fig. 2 with kinetic parameters (V_{max} and K_m) shown in the table inset. Kinetic analysis of vesicular DA uptake demonstrated that ATR significantly and dose-dependently decreased V_{max} of vesicular uptake (90.6% and 71.8% of control for 10 and 100 μ M of ATR, respectively). The K_m value was not affected ($p = 0.93$; Fig. 2, table inset). The degree of inhibition of vesicular DA uptake by ATR decreased when concentration of cold DA was increased to 100 nM, but the effect was still significant (Fig. 3).

3.2. Effects of ATR on synaptosomal uptake of DA

As shown in Fig. 4, little or no change was observed in synaptosomal DA uptake when striatal synaptosomes were exposed to ATR and its metabolites, DACT, DE and DIP for 15 min. The synaptosomal DA uptake was moderately (6–13%) increased by ATR at concentrations of 100 μ M or less; however, 6% reduction was observed with 250 μ M ATR (Fig. 4). In contrast to the results with ATR, both DACT and DIP did not affect the synaptosomal DA uptake whereas 100 μ M DE marginally (9%) increased synaptosomal DA uptake (Fig. 4). As previously reported (Compton and Johnson, 1985; L'Hirondel et al., 1995), nomifensine is a potent DAT inhibitor and blocks DA uptake into synaptosomes. We used it as a positive control and found that 10 μ M of nomifensine decreased synaptosomal DA uptake by more than 95 % (Fig. 4). Kinetic analysis of synaptosomal DA uptake showed that ATR did not have a statistically significant effect on $V_{\max}$ or K_m values (data not shown).

3.3. Effects of ATR on synaptosomal/vesicular (DAT/VMAT-2) uptake ratio

As shown in Fig. 5, ATR increased the ratio of synaptosomal/vesicular (DAT/VMAT-2) uptake in a dose-dependent manner. Of the three ATR metabolites tested, DACT (100 μ M) did not affect synaptosomal/vesicular uptake ratio whereas both DE and DIP increased the ratio (1.6 fold) to an extent similar to 100 μ M ATR.

4. Discussion

Dopamine is one of the major neurotransmitters in the CNS and its marked decrease in the striatum is a hallmark feature of PD. The risk of PD or PD-like pathology in animal models associated with exposure to pesticides has been reported in multiple studies (i.e., Morato et al., 1989; Takahashi et al., 1989; Fredriksson et al., 1993; Alam and Schmidt, 2002; McCormack et al., 2002; Gao et al., 2003). With respect to ATR, several studies indicate that both in vivo (Rodriguez et al., 2005; Coban and Filipov, 2007) and in vitro (Das et al., 2000; Das et al., 2003; Filipov et al., 2007) exposure to this herbicide causes dopaminergic neurotoxicity manifested with decreased DA. In order to clarify the mechanism underlying this reduction, we investigated the effects of in vitro exposure to ATR and its major metabolites, DACT, DE, and DIP on DA uptake into rat striatal synaptosomes and vesicles.

The major finding of the present study is that ATR, DE and DIP, but not DACT, inhibit DA uptake into striatal synaptic vesicles. Contrary to the inhibitory effects on vesicular uptake, synaptosomal DA uptake was marginally (6–13%) increased by ATR and DE but not by DACT and DIP at concentrations of 100 μ M or less. Such inhibition could decrease vesicular DA and increase cytosolic DA levels (Miller et al., 1999; Cooper et al., 2003). Increased cytosolic DA is in turn easily oxidized and reactive oxygen species (ROS), including DA quinones, are produced (LaVoie and Hastings, 1999). Such increase in cytosolic DA will therefore result in decreased DA in the vesicles and ROS-mediated toxicity to DA neurons (Chiueh et al., 2000). Of the 3 metabolites examined, only DACT was found to be ineffective in altering synaptosomal or vesicular uptake of DA. Kinetic studies indicated that ATR significantly decreased $V_{\max}$ of vesicular uptake while the K_m value was not affected. Thus, our data suggest that ATR causes dopaminergic neurotoxicity by affecting primarily vesicular DA uptake. These data are in accord with and provide an explanation for the dose-dependent decrease of cellular levels of DA in PC12 cells (Das et al., 2000; Das et al., 2003) and in striatal slices (Filipov et al., 2007). Decreased striatal DA levels were also observed after both acute and chronic in vivo exposure of ATR to rats (Rodriguez et al., 2005) and mice (Coban and Filipov, 2007). In the present study, at the higher concentrations, DE and DIP were similar to ATR in their ability to decrease vesicular uptake. Considering the fact that in PC 12 cells (Das et al., 2001) and in striatal slices we observed that these metabolites of ATR are not as potent and/

or had differential effects with ATR (Filipov et al., 2004), it is conceivable that ATR has effects that are in addition to its ability to decrease the vesicular uptake of DA.

The membrane carrier dopamine transporter (DAT) and the vesicular monoamine transporter (VMAT-2) are essential for maintaining normal dopaminergic function in the brain. Under normal circumstances, neuronal activation promotes vesicular release of DA into the synaptic cleft. DAT transports DA from the synaptic cleft to the presynaptic nerve terminal, and VMAT-2 transports cytosolic DA into vesicles for storage and protection from oxidation (Cooper et al., 2003). We found that ATR and two of its metabolites, DE and DIP, decreased vesicular uptake of DA. Such inhibition of vesicular uptake causes excessive accumulation of free DA in the cytosol which in turn generates oxidative stress by producing free radicals and reactive metabolites (Cubells et al., 1994; Miller et al., 1999) and consequently neuronal death as observed in in vivo studies with ATR (Rodriguez et al., 2005; Coban and Filipov, 2007). In addition, high concentrations of cytosolic DA inhibit mitochondrial respiration which also can cause cytotoxicity (Berman and Hastings, 1999). When the effects of ATR and its metabolites on synaptosomal DA uptake were examined, ATR, DE, and DIP slightly increased it. Thus, overall, the net result was a dose-dependent increase of the synaptosomal/vesicular (DAT/VMAT-2) uptake ratio. The ratio of DAT to VMAT-2 is an indication of neuronal sensitivity to DA toxicants and a high DAT/VMAT-2 ratio is proposed to increase the susceptibility of individual neurons to toxicants while a low DAT/VMAT-2 ratio decreases their susceptibility (Miller et al., 1999). This suggests that the inhibition of VMAT-2 that we observed in our present study would lead/contribute to DA neurons damage. Considering that VMAT-2 is not unique to DA neurons (Cooper et al., 2003), ATR, DE and DIP are expected to have similar effects on noradrenergic and serotonin neurons as well.

Studies with PC 12 cells have shown that ATR decreased cellular DA content (Das et al., 2000). However, the rate limiting enzyme, tyrosine hydroxylase (TH), was not significantly affected by ATR (Das et al., 2003). Similarly, we ((Filipov et al., 2007) reported that in striatal slices exposed to ATR for 4hrs, TH protein expression and activity was not affected while tissue levels of DA were decreased. This suggests that TH is not a major ATR target. Other possible targets that could be involved in ATR neurotoxicity are aromatic amino acid decarboxylase (AAAD), voltage sensitive-calcium channels (VSCCs), and perturbed mitochondrial function. High potassium stimulates the VGCCs and increases Ca^{++} entry through the channels which results in increased DA release. It has been reported that ATR decreased high potassium-induced DA release in striatum of freely moving rats (Rodriguez et al., 2005). Several recent studies indicated that mitochondrial dysfunction is key factor of dopaminergic degeneration of in PD (Beal, 2000; Fiskum et al., 2003; Greenamyre and Hastings, 2004) and pesticides such as rotenone directly inhibit mitochondrial function (Sherer et al., 2003; Testa et al., 2005). At present, there are no data on the effects of atrazine on brain mitochondria. However, a recent study found that exposure to ATR results in apoptosis of ZC 7901 cells which is associated with a disruption of the mitochondrial membrane potential (Liu et al., 2006). Thus, direct mitochondrial effects of ATR, but not if its metabolites, especially at higher exposure levels, may provide one possible explanation for the greater effects of ATR than DIP and DE on tissue/cellular DA levels (Das et al., 2000; Das et al., 2001; Filipov et al., 2004; Filipov et al., 2007).

It has been reported that more than 200,000 people in the United States are exposed to ATR over the acute reference dose (EPA, 2003). The doses (0.1–250 μ M) used in this study are lower or in the range to those used in previous studies (Das et al., 2000; Das et al., 2003; Filipov et al., 2007). In vivo studies reported that DACT is the major urinary metabolite of ATR in mice (Ross and Filipov, 2006), rats (McMullin et al., 2003) and, apparently, humans (Barr et al., 2007). In our present study, ATR and its metabolites DE and DIP affected vesicular DA uptake, whereas DACT had no effect. Thus, it appears that, at least in terms of dopaminergic

effects, DACT, although a major metabolite is not an important contributor to the dopaminergic toxicity of ATR in vivo. Even though DACT is the major mammalian metabolite of ATR, animals and humans exposed to ATR also produce substantial amounts of DE and DIP (Ikonen et al., 1988; Catenacci et al., 1990; Ross and Filipov, 2006; Barr et al., 2007). For example, in mice exposed to a single oral dose of ATR, maximal plasma levels of ATR, DE, DIP, and DACT ranged 0.2–1.2, 0.2–6.7, 0.3–7.0, and 10.5–92.5 μM , for mice exposed to 5 or 250 mg/kg ATR, respectively (Ross and Filipov, 2006). Of note, exposure of rats to 6 or 12 mg/kg of ATR, doses lower than the 70 mg/kg/day used for calculation of the Lowest Observed Adverse Effect Level (LOAEL; EPA, 2003), resulted in brain levels of ATR and DE of up to 2.7 ppm. Importantly, in humans, the higher the ATR exposure level is, the lower the % of DACT and the higher the % of DE/DIP/ATR in the urine is. This suggests that in cases of high occupational exposures, more ATR, DE, and DIP will reach the brain. Collectively, these findings may provide insight into which metabolites, besides ATR, i.e., DE and DIP, are most likely to be involved in the dopaminergic neurotoxicity of ATR previously reported in vivo (Rodriguez et al., 2005; Coban and Filipov, 2007).

The proposed mechanism of action of ATR and some of its metabolites on DA neurons is shown in Fig. 6. Our data indicate that ATR, DIP and DE, but not its major mammalian metabolite, DACT, decrease striatal DA levels by inhibiting VMAT-2 while moderately activating the membrane DA transporter (DAT). As TH was not affected by ATR (Das et al., 2003; Filipov et al., 2007) other possible ATR targets such as aromatic amino acid decarboxylase (AAAD), monoamine oxidase (MAO), and mitochondrial function, in particular, remain to be investigated in future studies.

In conclusion, results from this study suggest that ATR and two of its metabolites, DE and DIP, but not its major mammalian metabolite DACT, decrease striatal DA levels, at least in part, by increasing cytosolic DA, thereby promoting its breakdown. Inhibition of vesicular uptake and storage induced by ATR and the resulting increase in levels of free cytosolic DA could lead to the generation of oxidative stress, which may be partly responsible for the previously documented loss of DA neurons in the SNpc and VTA.

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Fig. 1.

Effects of ATR and its metabolites on vesicular uptake of dopamine (DA). Synaptic vesicles from striata of Sprague-Dawley rats were incubated for 15 min at 30 °C with vehicles (EtOH, DMSO), ATR (0.1–250 μM) and its metabolites, DE, DACT, and DIP (1 and 100 μM), or with reserpine (Res., 1 μM, a positive control), and then incubated with [³H] DA (6.6 nM) for 3 min. Data are presented as mean percent of control ± SEM (n = 9–19 for EtOH, DMSO, and ATR; n = 6 for DACT, DE, and DIP; n = 3 for reserpine). Bars with different letters are significantly different from one another and from bars without any letters (*p* < 0.05). Mean % of control is indicated in the ovals embedded in each treatment bar. For further information see the text.

Fig. 2. Effects of ATR on the kinetics of DA uptake into synaptic vesicles isolated from striata of Sprague-Dawley rats are represented on an Eadie-Hofstee plot. The vesicles were incubated for 15 min at 30 °C with ATR (0–100 μM) or with reserpine (1 μM) as a positive control, and then incubated with cold DA (0.1–100 nM) and [³H] DA (6.6 nM) for 3 min. Each data point indicates the average of at least two separate experiments assayed in triplicate. The table inset summarizes the kinetic values of vesicular DA uptake parameters ($V_{\max}$ and K_m) which were determined by non linear isotherm regression (Prism, Graphpad).

Fig. 3.

Effects of ATR on DA uptake into synaptic vesicles in the presence of cold DA isolated from striata of Sprague-Dawley rats. Vesicles were incubated for 15 min at 30 °C with an EtOH vehicle or ATR (10 and 100 µM), and then incubated with cold DA (0.1–100 nM) and [^3H] DA (6.6 nM) for 3 min. Data are presented as mean percent of control $\pm$ SEM ($n = 6-9$). Within a particular cold DA concentration, bars with different letters are significantly different from one another and from bars without any letters ($p < 0.05$). Mean % of control is indicated in the ovals embedded in each treatment bar. For further information see the text.

Fig. 4.

Effects of ATR and its metabolites on synaptosomal uptake of DA. Striatal synaptosomes from Sprague-Dawley rats were incubated for 15 min at 37 °C with vehicles (EtOH, DMSO), ATR (1–250 μM) and its metabolites, DE, DACT, DIP (100 μM), or with nomifensine (Nom., 10 μM) as a positive control, and then incubated with [³H] DA (50 nM) for 5 min. Data are presented as mean percent of control ± SEM (n = 6 EtOH, ATR, and DACT; n = 3 for DE, DIP, and nomifensine). Bar with different letters indicate significant different from one another and from bars without any letters (p < 0.05). Mean % of control is indicated in the ovals embedded in each treatment bar. For further information see the text.

Fig. 5. Effects of ATR and its metabolites on synaptosomal/vesicular uptake (i.e. DAT/VMAT-2 function) ratio. Uptake ratios were calculated by dividing synaptic uptake % control to vesicular uptake % control in the absence of cold DA. Data are presented as mean $\pm$ SEM. Each bar presents $n \geq 6$ from a minimum of two independent experiments. Bars with different letters are significantly different from one another and from bars without any letters ($p < 0.05$).

catechol-*O*-methyltransferase (COMT), dopamine transporter (DAT) and vesicular monoamine transporter-2 (VMAT-2).