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Title Page

Amphetamine treatment similar to that used in the treatment of adult ADHD damages dopaminergic nerve endings in the striatum of adult non-human primates

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Abbreviations: ADHD - Attention-deficit/hyperactivity disorder

DOPAC - dihydroxyphenylacetic acid

PFPA - pentafluoropropionic acid

5-HIAA - 5-hydroxyindoleacetic acid

Abstract

Pharmacotherapy with amphetamine is effective in the management of attention-deficit/ hyperactivity disorder (ADHD), now recognized in adults, as well as in children and adolescents. Here we demonstrate that amphetamine treatment, similar to that used clinically for adult ADHD, damages dopaminergic nerve endings in the striatum of adult non-human primates. Furthermore, plasma concentrations of amphetamine associated with dopaminergic neurotoxicity in non- human primates are on the order of those reported in young patients receiving amphetamine for the management of ADHD. These findings may have implications for the pathophysiology and treatment of ADHD. Further preclinical and clinical studies are needed to evaluate the dopaminergic neurotoxic potential of therapeutic doses of amphetamine, in children as well as adults.

Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a highly prevalent neuropsychiatric illness, afflicting 3-9 % of school-age children and 1-5 % of adults world-wide (Leung and Lemay, 2003; Wilens et al., 2004, Biederman and Faraone, 2004). For years, psychomotor stimulant drugs have been the mainstay of ADHD treatment (Greenhill et al., 2002; Fone and Nutt, 2005) and, in the last decade, their use has increased substantially (Olfson et al., 2003; Robison et al., 2004). Of the various stimulant drugs used in the treatment of ADHD, amphetamine is among the most often prescribed (Greenhill et al., 2002; Fone and Nutt, 2005), both in children and adults (Wilens et al, 2004; Dodson, 2005).

As use of amphetamine in the treatment of ADHD has increased, a large body of preclinical data has accrued indicating that amphetamine has the potential to damage brain dopamine-containing neurons in experimental animals. In particular, animals treated with amphetamine develop lasting reductions in striatal dopamine, its major metabolite dihydroxyphenylacetic acid (DOPAC), its rate-limiting enzyme tyrosine hydroxylase (TH), its membrane transporter (DAT), and its vesicular transporter ($VMAT_2$) (see Gibb et al., 1994; McCann and Ricaurte, 2004). Anatomic studies indicate that lasting dopaminergic deficits after amphetamine are due to damage of dopaminergic nerve endings in the striatum, with sparing of dopaminergic nerve cell bodies in the substantia nigra.

Despite these preclinical data, and growing awareness of potential long-term adverse effects of stimulant ADHD medications (see Volkow and Insel, 2003; Fone and Nutt, 2005), there has been little expressed concern over possible dopaminergic neurotoxicity in humans receiving amphetamine for the treatment of ADHD. In large measure, this appears to be due to the fact that, as noted by various authors (Vitiello, 2001a; 2001b; Greenhill et al., 2002; Fone and Nutt, 2005), doses, routes and regimens of administration used in amphetamine neurotoxicity studies in animals differ significantly from those used in the treatment of ADHD.

The purpose of the present study was to determine if amphetamine treatment, similar to that used clinically in the therapy of adult ADHD, produces long-term effects on brain dopaminergic neurons in non-human primates. Initial studies used baboons as experimental subjects because their size (20-30 kg) allows for administration of amphetamine at an absolute dose similar to that used in ADHD. To further simulate the clinical use of amphetamine, we trained baboons to self-administer amphetamine by mouth. We tested a 3:1 mixture of *dextro*- and *levo*-amphetamine, because one of the more common formulations used in the treatment of ADHD consists of a combination of 75 % dextroamphetamine and 25 % levoamphetamine. In a final set of studies, we used squirrel monkeys, to test the species generality of our findings.

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Methods

Animals: Baboons (*Papio anubis*, weighing 20-30 kg) and squirrel monkeys *(Saimiri sciureus*, weighing 0.83 – 0.95 kg) were used. All animals were drug-naive (except for health maintenance medications administered by the veterinary staff) and in good health. Baboons were 10-15 years of age and of both genders. Squirrel monkeys were feral-reared; thus, their precise ages were unknown, but all were adult males. Baboons were housed singly in standard steel cages, at an ambient temperature of 26 ± 1 ^{O}C and 20-40 % humidity, with free access to food (New World Primate Diet) and water. Squirrel monkeys were housed in pairs in standard steel cages with free access to food (New World Primate Diet) and water in a second colony room maintained at an ambient temperature of 26 ± 1 ^{O}C and 20-40 % humidity. The facilities for housing and care of the animals are accredited by the American Association for the Assessment and Accreditation of Laboratory Animal Care. Animal care and experimental manipulations were approved by the Institutional Animal Care and Use Committee at the Johns Hopkins University School of Medicine, and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Drugs and chemicals: S(+)- and R(-)-amphetamine were obtained through the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA), and their chemical authenticity was confirmed by means of gas chromatography/mass spectroscopy (GC/MS).

Doses were expressed as the base weight. For GC/MS determinations, racemic amphetamine was obtained from Lipomed (Cambridge, MA), racemic-D_8-amphetamine was purchased from Cerelliant (Round Rock, TX), and pentafluoropropionic acid (PFPA) was purchased from Fluka (St. Louis, MO). Clean Screen with Clean Thru tips solid-phase extraction columns (6 cc) were obtained from United Chemical Technologies (Bristol, PA). Other drugs and chemicals were obtained from the following sources: dopamine hydrochloride, DOPAC, 5-hydroxytryptamine (5- HT) creatinine sulphate complex, 5-hydroxyindoleacetic acid dicyclohexylammonium salt (5- HIAA), sodium octyl sulphate and ethylenediaminetetraacetic acid (EDTA), quinine hemisulfate salt (Sigma-Aldrich, St. Louis, MO, USA), [^{3}H]WIN35,428 was purchased from Perkin Elmer (Billerica, MA) and [^{3}H]DTBZ from American Radiolabeled Chemicals (St. Louis, MO).

Drug administration: The oral route of administration was used. For baboons, this was accomplished by first training the animals to self-administer the drug vehicle orally. The training procedure (Kaminski et al., 2003) involved administration of orange fruit drink (Tang, Kraft Foods, Rye Brook, NY) containing quinine, in increasing concentrations, immediately followed by administration of unadulterated orange drink (40 ml). Training lasted until the baboons were reliably drinking the most concentrated quinine solution (approximately 43 mg

quinine/liter orange drink). Subsequently, amphetamine treatment was initiated by dissolving the appropriate amounts of dextroamphetamine and levoamphetamine (as sulfate salts) in distilled water, then adding the amphetamine isomer mixture to the orange drink. A 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine, respectively, was used for all animal treatments. Dose selection was based upon published reports (Greenhill et al., 2002; Spencer et al., 2001; Wilens et al., 2004). In our initial baboon study, the amphetamine mixture was given twice daily (at 0930 and 1530 hr) for approximately 4 weeks, using doses shown in Table 1.

In a second baboon study using different animals, we measured plasma amphetamine concentrations at the end of each of four weeks of treatment and, similar to our first study, baboons were trained to self-administer the amphetamine mixture orally twice daily (at 0900hr and 1500 hr) for 4 weeks, using doses shown below. As before, a 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine, respectively, was used (Table 2).

In a subsequent study involving squirrel monkeys, oral administration of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine was accomplished by orogastric gavage, while the animals were gently restrained in a plexiglass chair. Squirrel monkeys received the amphetamine isomer mixture twice-daily (0930 and 1530 hr) for 4 weeks. In these studies, rather than increasing the dose of the amphetamine isomer mixture automatically at the end of

each week (as in the baboon study above), dose increments were made contingent upon plasma drug concentrations. In particular, when the plasma amphetamine concentration reached a level comparable to that observed clinically (between 100 and 150 ng/ml - see Discussion), dosage increments were stopped, and the animals were maintained on that dose of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine for the remainder of the 4-week treatment period, as below (Table 3).

Determination of plasma amphetamine concentrations: Samples were analyzed by solid-phase extraction and gas chromatography-mass spectrometry using a procedure from United Chemical Technologies (http://www.unitedchem.com/s/pdfs/13-Applications % 20Manual.pdf) adapted for plasma. Briefly, 0.5 mL of specimen was added to 25 µL of a 10 µg/mL aqueous solution of internal standard (d_8-AMP), followed by 125 µL 1.6 M periodic acid solution. Samples were allowed to stand at room temperature for 10 min after vortexing. Thereafter, 150 µL of 1.6 M KOH and 1 mL 0.1 M monobasic phosphate buffer (pH 6.0) were added, again followed by vortexing. The pH was adjusted to 5.0 to 7.0, if necessary. The samples were then centrifuged at 1800 rpm for 3 minutes. The supernatant was collected in 5 mL, 75 x 12 mm plastic test tubes and decanted onto CSDAU Clean Screen Extraction columns (UCT, Inc.) preconditioned with 1.5 mL of methanol followed by 1.0 mL of 0.1 M monobasic phosphate buffer (pH 6.0). Supernatant samples were added to the columns and low vacuum was applied.

Columns were washed sequentially with 0.5 mL aliquots of 1.0 M acetic acid and 1.5 mL of methanol. The columns were dried thoroughly at high vacuum for 5 min. The analytes were eluted with 1.5 mL of a mixture of methylene chloride, 2-propanol, and ammonium hydroxide (80:20:2 by volume). The eluates were collected in conical glass centrifuge tubes. To the collected eluates, 15 µL acidified methanol was added. Samples were evaporated under a continuous nitrogen stream until completely dry. After samples were evaporated to dryness, 50 µL of the derivatizing agent, pentafluoropropionic anhydride (PFPA), was added and centrifuge tubes were capped, vortex-mixed, and placed on a heat block for 30 minutes at 50^{o}C. After derivatization, the samples were dried under a gentle stream of nitrogen. Samples were then reconstituted with 50 µL ethyl acetate and transferred to autosampler vials. Standards were prepared as follows: A 10 µg/mL amphetamine standard solution was prepared by diluting 0.1 mL of a 1.0 mg/mL ampoule to 10 mL of methanol. The contents of 100 µg/ml ampoule of D_8-Amphetamine internal standard solution were diluted to 10 mL with methanol to prepare a 10 µg/mL D_8-Amphetamine internal standard solution. Final concentrations of standards used in the analysis of plasma samples were 5, 20, 100 and 500 ng/mL. Analysis was performed using a Agilent Technologies Model 6890N gas chromatograph equipped with a 30 m x 0.25 mm (i.d.) HP-5ms (5 % phenyl)-methylpolysiloxane capillary column (0.25 µm film thickness) interfaced with an Agilent Technologies Model 5973 inert electron impact mass spectrometer. Helium was

used as the carrier gas (flow rate, 1.3 mL/min) for 1 µL splitless sample injections. The initial oven temperature was 90^{o}C with a 1-min hold followed by ramps at 20 ^{o}C/min to 180 ^{o}C with a 2-min final hold. Total run time was 7.5 min. Specimens were analyzed in selected ion monitoring mode for the following ions (where q is the quantitative ion): D_8-AMP [pentafluoropropionic (PFP) derivative], m/z 193 (q), 126, and 96; AMP (PFP derivative), m/z 190 (q), 118, and 91. Amphetamine peak areas were integrated using the Agilent ChemStation Software® (Rev. D.01.00), and the ratio of the area of the calibrator and its internal standard were used for calculations.

Brain dissection: Two weeks after drug treatment, animals were sacrificed under deep sodium pentobarbital anesthesia (60 mg/kg; i.p.), and the brain was removed from the skull in a cold room (4^{o}C). Regional dissection of the brain was performed using the Emmers and Akert (1963) atlas as a guide for the squirrel monkey and the Riche et al. (1988) atlas for the baboon. Brain regions of interest were isolated from coronally cut sections (approximately 4-5mm thick) by means of free dissection. Tissue from each brain region was wrapped in aluminum foil, then stored in liquid nitrogen until assay.

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Determination of regional brain monoamine concentrations: Regional brain concentrations of DA and 5-HT were determined by high performance liquid chromatography with electrochemical detection (HPLC-EC), as described previously (Yuan et al., 2002).

Quantitative DAT autoradiography: Frozen brains were sectioned using a Microm HM505E cryostat at -20 ^{o}C. Half-hemisphere coronal sections (20 µm) were thaw-mounted onto gelatin-coated microscope slides and the DAT was labeled with 50 pM [^{125}I] RTI-121, using the method of Staley et al. (1995). Autoradiographs were digitized with a Dage CCD 72 camera and MCID Elite 6.0 image analysis system (Amersham Biosciences Niagara Inc, St. Catherines, Ontario, Canada).

DAT binding; [^{3}H]WIN-35,428-labeled DATs were measured using the method of Madras and colleagues (1989), with minor modification. Briefly, frozen striatal tissue was weighed, homogenized for 15 sec in 20 volumes (w/v) of a 0.32 M sucrose phosphate buffer (pH=7.4) at 0-4 ^{o}C, and centrifuged in a Sorvall RC2B centrifuge at approximately 45000 g for 15 min at 0-4 ^{o}C. The supernatant was discarded and the pellet was resuspended in 20 volumes of sucrose phosphate buffer, then centrifuged once again at approximately 45000 g for 15 min at 0-4 ^{o}C. The resulting pellet was suspended in buffer for a final tissue concentration of 10

mg/ml wet weight. [^{3}H]WIN 35,428 was used at a predetermined saturating concentration of 30 nM. Cocaine, at a final concentration of 30 μM, was used to displace specific [^{3}H]WIN 35,428 binding, to estimate nonspecific binding. Tubes were incubated in sextuplicate for 60 min, at 0-4 ^{o}C. The incubation was terminated by rapid filtration, using a 48-well cell harvester (Brandell, Gaithersburg, MD) and Whatman GFB filters soaked with 0.05 % PEI. Filters were washed three times using ice-cold sucrose phosphate buffer. Radioactivity was measured with a Packard- 1500 Tricarb Liquid Scintillation Analyzer. Specific [^{3}H]WIN 35,428 binding was calculated by subtracting the average value of the six tubes containing excess cocaine from the average of the six tubes without cocaine. Specific [^{3}H]WIN 35,428 binding was expressed in DPM/mg original wet weight tissue.

VMAT binding: [^{3}H]DTBZ, used to label type 2 vesicular monoamine transporter (VMAT) sites. [^{3}H]DTBZ binding was measured using the method of Vander Borght et al. (1996), with minor modifications. Briefly, tissue samples were homogenized for 15 seconds in 20 volumes (w/v) sodium phosphate buffer, (25 mM, pH 7.7), then centrifuged in a Sorvall RC2B centrifuge at approximately 45000 g for 15 minutes at 0-4 ^{o}C. The resulting pellet was resuspended in 20 volumes (w/v) sodium phosphate buffer, then homogenized again for 15

seconds, and recentrifuged at approximately 45000 g for 15 minutes at 0-4 ^{o}C. The supernatant was discarded and the resulting pellet was resuspended in buffer at a final concentration of 10 mg of original wet weight tissue per ml. Membrane preparations were incubated with a predetermined saturating concentration of [^{3}H]DTBZ (15 nM) in 25 mM sodium phosphate buffer, pH 7.7, for 90 minutes at 30 ^{o}C in a shaking water bath. Each sample was run in sextuplicate, such that 3 tubes were used to define total binding, and 3 tubes were used to determine nonspecific binding. Nonspecific binding was determined in the presence of 1 μM tetrabenazine, and represented approximately 8-10 % of total binding. The incubation was terminated by rapid filtration, as above. Filters were washed three times with 10 ml sodium phosphate buffer and residual radioactivity was measured using a Packard-1500 Tricarb Liquid Scintillation Analyzer. Specific binding, calculated by subtracting nonspecific binding from total binding, was expressed as DPM/mg original wet weight tissue.

Western blot analysis: A rat anti-DAT monoclonal antibody (Chemicon, CA) was used as primary antibody (1:1000), and goat anti-rat Ig horseradish peroxidase linked antibody (Amersham Bioscience, NJ) was used as secondary antibody (1:1000). An ECL Western blotting detection system (Amersham Biosciences, NJ) was used to visualize the signals produced. Actin polyclonal antibody (Santa Cruz, CA) was used to correct the loading protein

amount, and the NIH image program (Image J) was used to semi-quantitatively analyze the band intensity (http://rsb.info.nih.gov/ij/), as described previously (Xie et al., 2004). Tissue used for these studies was from the same animals used for the other neurochemical studies herein reported.

Statistics: Data were analyzed by ANOVA and, where appropriate, by independent samples, two-tailed students t-test. Results were considered significant when $p<0.05$. Data analysis was performed using the Statistical Program for the Social Sciences (SPSS for Windows, Release 10.5).

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Results

Two to four weeks after cessation of treatment, the first group of baboons (n=3) that had self-administered escalating doses of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine twice daily for approximately 4 weeks showed significant reductions in striatal dopamine concentration, the density of [^{3}H]WIN35,428-labeled DAT sites, the amount of DAT protein and the number of [^{3}H]DTBZ-labeled $VMAT_2$ sites; quantitative autoradiographic studies showed that the regional density of [^{125}I]RTI-121-labeled DAT sites was comparably reduced (*Fig. 1*). A closer examination of regional monoamine data revealed lasting dopaminergic deficits in the caudate nucleus and putamen of comparable magnitude (44 - 47 % depletions), while smaller, but significant, deficits (approximately 30 %) were also evident in the nucleus accumbens (*Fig. 2a*). Analysis of regional brain serotonergic neuronal markers in the same animals revealed no significant differences (*Fig. 2b*).

To ensure that the dose and dosing parameters used in the above study accurately approximated those used clinically, we trained another group of baboons (n=3) to self-administer escalating oral doses of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine (see Methods) and measured plasma concentrations of amphetamine at the end of each week of treatment. As the dose of the amphetamine isomer mixture was increased, plasma levels

of amphetamine rose (*Fig. 3a*). The mean (± s.e.m.) plasma amphetamine concentration at the end of the 4-week treatment period was 168 ± 25 ng/ml. Similar to the baboons in our first study, this group of baboons had significant reductions in brain dopaminergic neuronal markers when examined two weeks after cessation of amphetamine treatment (*Fig. 3b-d*).

To determine the species generality of our findings, we carried out an additional study using squirrel monkeys. However, in this study, once the plasma concentration of amphetamine reached a level comparable to that reported clinically (100 to 150 ng/ml - see Discussion), dose increments were stopped. As in baboon studies, plasma levels of amphetamine rose when the dose of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine was increased from 0.25 to 0.5 mg (*Fig. 4a*). Interestingly, despite maintaining the amphetamine dose constant at 0.5 mg for the last two weeks of treatment, plasma amphetamine concentrations decreased modestly, but significantly, to a mean (± s.e.m.) plasma concentration of 125 ± 14 ng/ml at the end of the 4-week treatment period (*Fig. 4a*).

In addition to determining plasma amphetamine concentrations at the end of each week of treatment, we measured the concentration-time profile of amphetamine in the same 4 squirrel monkeys the day after completing the 4-week treatment period. Results of these studies showed that plasma amphetamine concentrations peaked 1 to 2 hr after oral amphetamine administration, and that the mean (± s.e.m.) peak concentration of amphetamine was 136 ± 21 ng/ml (*Fig. 4b*).

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When these squirrel monkeys were examined approximately two weeks after the final dose of the 3:1 mixture of *dextro* [S(+)] and *levo* [R(-)] amphetamine, they had reductions in striatal dopaminergic markers, although the reduction in [^{3}H]WIN35,428-labeled DAT did not achieve statistical significance (*Fig. 4c-e*).

Discussion

To our knowledge, this is the first study in which oral self-administration of amphetamine has been used to evaluate the neurotoxic potential of amphetamine in non-human primates and to demonstrate that plasma levels of amphetamine that produce brain dopaminergic neurotoxic changes in the primate CNS are on the order of those reported in some patients with ADHD treated with amphetamine (see below). In particular, the results of the present study indicate that an oral regimen of amphetamine, modeled after dosing regimens used in patients with ADHD, engenders plasma amphetamine concentrations that result in toxicity to brain dopaminergic axon terminals in baboons and squirrel monkeys. These results may have implications for the pathophysiology and treatment of ADHD, and raise the question of whether or not plasma monitoring might be indicated in ADHD patients receiving higher, chronic doses of amphetamine.

Doses of amphetamine that are used clinically range from 5 to 60 mg (Greenhill et al., 2002; Wilens et al., 2004) and, except for slow-release, longer-acting formulations that have recently become available (Greenhill et al., 2003; McGough et al., 2003), are typically prescribed for twice-daily use. Plasma concentrations of amphetamine that develop after chronic amphetamine treatment have rarely been reported. Indeed, to our knowledge, there are only two reports that provide such information. The first, an early study by Borcherding and colleagues

(1989), indicates that hyperactive children given escalating oral, twice-daily (0900 and 1300 hr) doses of dextroamphetamine over a 3-week period develop plasma amphetamine concentrations of approximately 120 to 140 ng/ml. The second, a more recent study by McGough and colleagues (2003), shows that patients with ADHD given a single daily (morning) dose of an extended release formulation of mixed amphetamine salts for a six-week period develop dose-related plasma amphetamine concentrations, with a total (*d*- plus *l*- isomers) plasma amphetamine concentration of approximately 120 ng/ml after the highest dose examined (30 mg). Thus, plasma concentrations of amphetamine achieved clinically in patients receiving chronic amphetamine for the treatment of ADHD appear to be on the order of those shown here to produce dopaminergic neurotoxic effects in adult baboons and squirrel monkeys. Importantly, the moderate magnitude of the reduction of dopaminergic axonal markers in these non-human primates suggests that plasma levels achieved approach the minimal plasma concentration "threshold" required to produce dopaminergic neurotoxic changes.

In contrast to the paucity of clinical reports on plasma amphetamine concentrations after chronic treatment, there are numerous reports on the pharmacokinetics of single, oral doses of amphetamine in humans (Brown et al., 1979; Angrist et al., 1987; McGough et al., 2003). Collectively, these studies indicate that, as the dose of amphetamine is increased, there is a corresponding increase in plasma amphetamine concentration. Furthermore, when frequency of

dosing is increased from once- to twice-daily, plasma concentrations of amphetamine increase accordingly (Greenhill et al., 2003), probably due to the fact that the elimination half-life of amphetamine in humans is on the order of 6 to 9 hrs (see Cho and Kumagai, 1994).

Although the present preclinical observations may have clinical implications, it would be premature to extrapolate them to humans receiving amphetamine treatment for ADHD for several reasons. First, the dopaminergic neurotoxicity may only occur in the context of doses of amphetamine that result in plasma concentrations comparable to those found in these experiments; lower dosage regimens that engender lower plasma amphetamine concentrations may not be associated with toxic effects on central dopaminergic neurons. Second, the mechanisms of amphetamine-induced dopaminergic neurotoxicity are not known, and, theoretically, could be operant in non-human primates (and rodents) but not in humans. Third, aspects of amphetamine metabolism in non-human primates may differ from those in humans and such differences could, potentially, result in neurotoxicity in non-human primates, but not in humans. Fourth, the relative sensitivity of brain dopaminergic neurons to amphetamine toxicity in non-human primates and humans is unknown. Fifth, it is possible that the effects observed in normal primates with amphetamine may not be observed in ADHD patients because such patients presumably have abnormal neurotransmitter function and such abnormalities may influence the expression of amphetamine neurotoxicity. Finally, it is important to note that

amphetamine neurotoxicity data from the present studies were obtained in adult non-human primates; as such, while they may have implications for adults receiving amphetamine for the treatment of ADHD, their implications for children are less clear because studies assessing the influence of age on the ontogeny of amphetamine neurotoxicity suggest younger animals are less susceptible to the neurotoxic effects of amphetamine (Cappon et al., 1997; Miller et al., 2000). Future studies in young adolescent primates are needed.

It is reasonable to wonder why, if clinically relevant doses of amphetamine produce toxic effects on brain dopaminergic neurons in non-human primates, evidence of dopaminergic neurotoxicity has not been revealed in clinical or neuroimaging studies of patients with ADHD. With regard to clinical studies, it is important to recognize that parkinsonism may not become manifest until central dopaminergic function is reduced by approximately 80-90 % (Koller et al., 1991), a degree of dopamine reduction that is approximately two-fold greater than that observed in the current study. Abnormalities in cognitive function, another potential functional consequence of dopaminergic loss (Robbins, 2003) may be difficult to distinguish from the underlying symptoms of ADHD for which amphetamine is being prescribed. Indeed, given the fact that abnormalities in dopaminergic neural function are believed, in part, to underlie symptoms of ADHD (Fone and Nutt, 2005), any indication of abnormal dopaminergic function in amphetamine-treated patients might be attributed to underlying disease, rather than

amphetamine neurotoxicity. With respect to neuroimaging studies, it is important to recognize that most subjects with ADHD who have been included in PET/SPECT studies of the DAT have been medication-naïve individuals (Krause et al., 2000; 2003; Jucaite et al., 2005) and that in those isolated instances in which ADHD patients treated with stimulants have been included, those treated with amphetamine (rather than methylphenidate) were either excluded from the analysis (Krause et al., 2003), or were not analyzed separately (Dougherty et al., 1999; Krause et al., 2002). Notably, results of these imaging studies have not been entirely consistent, with both increases (Dougherty et al., 1999; Krause et al., 2000) and no change (van Dyck, 2002) in DAT density reported in the striatum of ADHD patients. Thus, for a variety of reasons, the absence of previous clear clinical or PET/SPECT data showing evidence of DAT changes consistent with dopaminergic neurotoxicity in patients with ADHD previously treated with amphetamine should not be construed as evidence that it does not occur.

In summary, the present results indicate that amphetamine treatment similar to that used clinically for the management of adult ADHD produces brain dopaminergic neurotoxicity in adult non-human primates, and engenders plasma concentrations of amphetamine that are on the order of those reported in some ADHD patients. Although it would be premature to extrapolate the present findings to ADHD patients treated with amphetamine, they provide an impetus to conduct controlled studies aimed at determining the potential for amphetamine to produce

dopaminergic neurotoxicity in ADHD cohorts (adult, adolescent and childhood forms). The present results also suggest that neurotoxic effects of amphetamine could potentially occur in other disorders that require chronic amphetamine treatment (e.g., narcolepsy), and that it might be prudent to monitor plasma amphetamine concentrations in patients considered at risk (e.g., those receiving higher amphetamine doses for extended periods). Finally, the present findings may also have implications for the drug abuse field, because plasma amphetamine concentrations in some abusers substantially exceed those here shown to produce dopaminergic neurotoxicity in non-human primates (Peters et al., 2003; Nakashima et al., 2003).

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Footnotes

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Legends for Figures

Fig. 1: Effect of chronic oral amphetamine treatment on dopaminergic neuronal markers in the striatum of baboons (n=3) examined two to four weeks after amphetamine treatment. Shown are (A) dopamine, (B) [^{3}H]WIN35,428-labeled DAT, (C) [^{3}H]DTBZ-labeled $VMAT_2$, (D) Western blots of striatal DAT and (E) [^{125}I]RTI-121-labeled DAT autoradiogram. Values represent the mean ± s.e.m. from 3 independent subjects. *Designates $p < 0.05$, two-tailed t-test.

Fig. 2. Regional concentrations of (A) dopamine and (B) serotonin in baboons (n=3) two to four weeks after amphetamine treatment. Cd = caudate; Pu t= putamen; OT = olfactory tubercle; NAc = nucleus accumbens, FC = frontal cortex; PC = parietal cortex; TC = temporal cortex. Values shown represent the mean ± s.e.m. from 3 independent subjects. *Designates $p < 0.05$, two- tailed t-test..

Fig. 3: Plasma amphetamine concentrations and striatal dopaminergic markers in the second group of baboons (n=3) that had previously self-administered gradually escalating doses of amphetamine for 4 weeks. Shown are (A) plasma concentrations of amphetamine determined at the end of each week of treatment (1 hr after the second daily dose), (B) dopamine concentrations (C) [^{3}H]WIN35,428-labeled DAT sites and (D) [^{3}H]DTBZ-labeled $VMAT_2$

sites two weeks after treatment. [1]Different from week 1; [2]Different from week 2; [3]Different from week 3; [4]Different from week 4 (one-way ANOVA); *Designates $p < 0.05$, one-way ANOVA two-tailed t-test. Values represent the mean ± s.e.m. from 3 independent subjects.

Fig. 4: Plasma amphetamine concentrations and striatal dopaminergic markers in squirrel monkeys (n=4) previously treated with oral doses of amphetamine for 4 weeks. Shown are (A) amphetamine concentrations, determined 1hr after the second daily dose at the end of each week of treatment; (B) amphetamine concentration-time profile in the same 4 monkeys administered a 0.5 mg dose of amphetamine the day after they had completed the 4-week treatment period; (C) dopamine concentrations; (D) [^{3}H]WIN35,428-labeled DAT sites and (E) [^{3}H]DTBZ-labeled $VMAT_2$ sites two weeks after amphetamine treatment. [1]Different from week 1; [2]Different from week 2; [3]Different from week 3; [4]Different from week 4 (one-way ANOVA); *Designates $p < 0.05$, two-tailed t-test. Values represent the mean ± s.e.m. from 4 independent amphetamine- treated animals and 4-5 controls.

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Table 1

	Day 1	Days 2-5	Days 6-13	Days 14-27
Amphetamine Dose	2.5 mg	5.0 mg	10 mg	20 mg
Dose in mg/kg:				
Baboon #1 (21 kg)	0.12 mg/kg	0.24 mg/kg	0.48 mg/kg	0.95 mg/kg
Baboon #2 (20 kg)	0.13 mg/kg	0.25 mg/kg	0.50 mg/kg	1.00 mg/kg
Baboon #3 (20 kg)	0.13 mg/kg	0.25 mg/kg	0.50 mg/kg	1.00 mg/kg

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Table 2

	Day 1-7	Days 8-14	Days 15-21	Days 22-28
Amphetamine Dose	5 mg	10 mg	15 mg	20 mg
Dose in mg/kg:				
Baboon #1 (30kg)	0.17 mg/kg	0.33 mg/kg	0.50 mg/kg	0.67 mg/kg
Baboon #2 (30 kg)	0.17 mg/kg	0.33 mg/kg	0.50 mg/kg	0.67 mg/kg
Baboon #3 (20 kg)	0.25 mg/kg	0.50 mg/kg	0.75 mg/kg	1.00 mg/kg

Table 3

	Day 1-7	Days 8-14	Days 15-21	Days 22-28
Amphetamine Dose	0.25 mg	0.50 mg	0.50 mg	0.50mg
Dose in mg/kg:				
Monkey #1 (0.83 kg)	0.30 mg/kg	0.64 mg/kg	0.68 mg/kg	0.65 mg/kg
Monkey #2 (0.88 kg)	0.28 mg/kg	0.63 mg/kg	0.65 mg/kg	0.63 mg/kg
Monkey #3 (0.95 kg)	0.26 mg/kg	0.58 mg/kg	0.65 mg/kg	0.64 mg/kg
Monkey #4 (0.84 kg)	0.30 mg/kg	0.64 mg/kg	0.68 mg/kg	0.68 mg/kg

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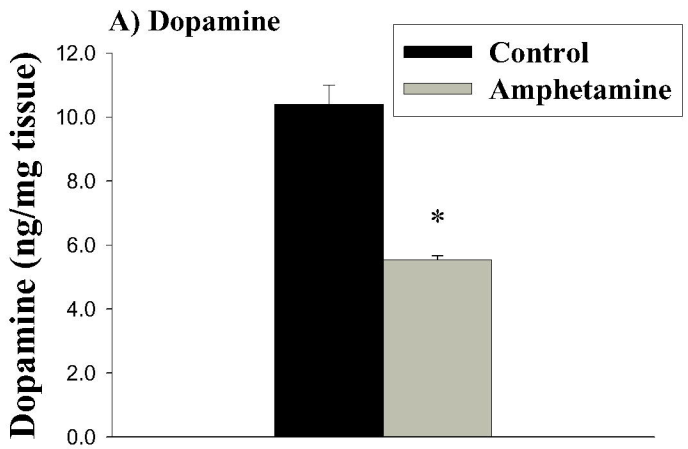
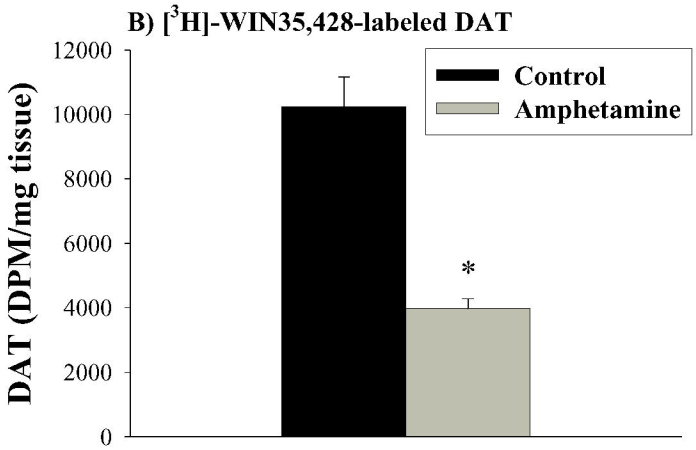
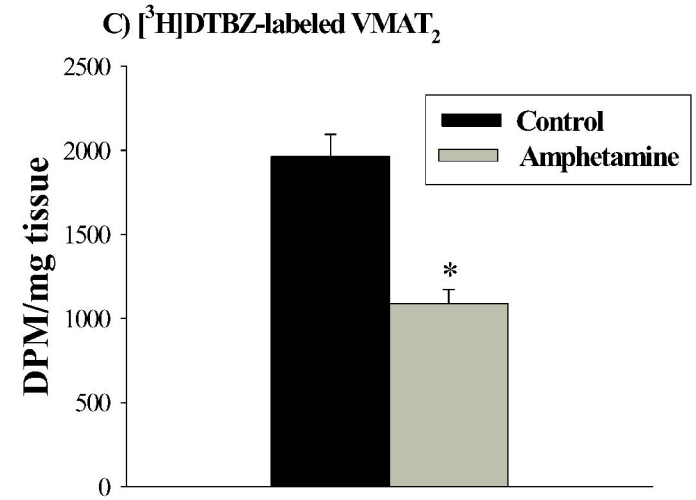
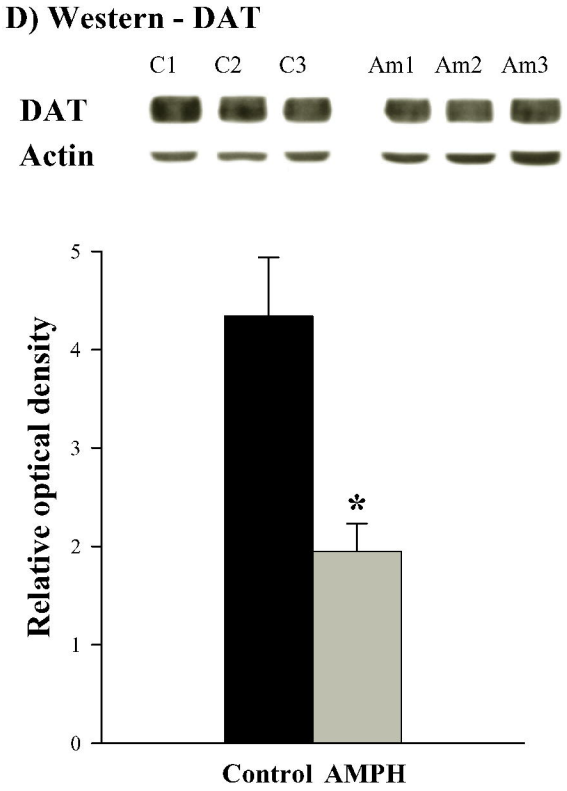
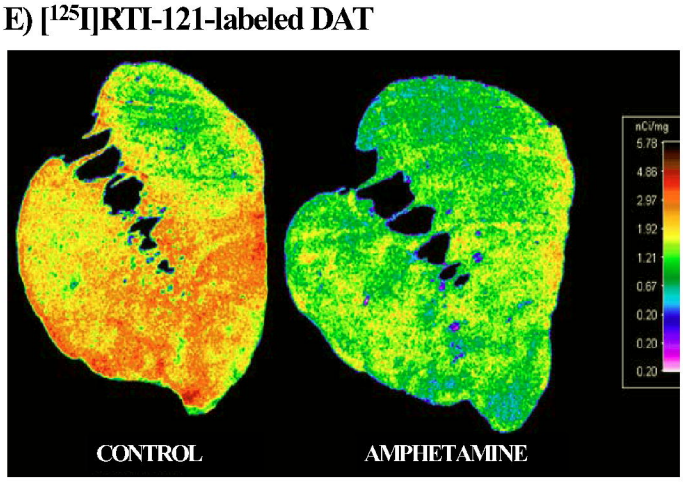
Fig 1 (JPET#87916 Page 40)











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