Age-Dependent Methamphetamine-Induced Alterations in Vesicular Monoamine Transporter-2 Function: Implications for Neurotoxicity

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ABSTRACT

Tens of thousands of adolescents and young adults have used illicit methamphetamine. This is of concern since its high-dose administration causes persistent dopaminergic deficits in adult animal models. The effects in adolescents are less studied. In adult rodents, toxic effects of methamphetamine may result partly from aberrant cytosolic dopamine accumulation and subsequent reactive oxygen species formation. The vesicular monoamine transporter-2 (VMAT-2) sequesters cytoplasmic dopamine into synaptic vesicles for storage and perhaps protection against dopamine-associated oxidative consequences. Accordingly, aberrant VMAT-2 function may contribute to the methamphetamine-induced persistent dopaminergic deficits. Hence, this study examined effects of methamphetamine on VMAT-2 in adolescent (postnatal day 40) and young adult (postnatal day 90) rats. Results revealed that high-dose methamphetamine treatment caused greater acute (within 1 h) decreases in vesicular dopamine uptake in postnatal day 90 versus 40 rats, as determined in a nonmembrane-associated subcellular fraction. Greater basal levels of VMAT-2 at postnatal day 90 versus 40 in this purified fraction seemed to contribute to the larger effect. Basal tissue dopamine content was also greater in postnatal day 90 versus 40 rats. In addition, postnatal day 90 rats were more susceptible to methamphetamine-induced persistent dopaminergic deficits as assessed by measuring VMAT-2 activity and dopamine content 7 days after treatment, even if drug doses were adjusted for age-related pharmacokinetic differences. Together, these data demonstrate dynamic changes in VMAT-2 susceptibility to methamphetamine as a function of development. Implications with regard to methamphetamine-induced dopaminergic deficits, as well as dopamine-associated neurodegenerative disorders such as Parkinson’s disease, are discussed.

According to the Substance Abuse and Mental Health Services Administration for 2003 (2004), 1.3% of adolescents aged 12 to 17 and 5.2% of young adults aged 18 to 25 have used illicit methamphetamine (METH) at least once in their lifetime. This is of concern because it is well established that high-dose methamphetamine administrations lead to persistent dopaminergic deficits in rodents, nonhuman primates, and humans, as determined by measuring striatal dopamine levels, tyrosine hydroxylase activity, and dopamine transporter function days to months after treatment (Koda and Gibb, 1973; Wagner et al., 1980; Bennett et al., 1998; Schmidt et al., 1985; Seiden, 1985; Wilson et al., 1996). Most studies of methamphetamine and its actions have involved adult animal models. However, given the prevalence of methamphetamine use among young people, increasing attention has focused on effects of the stimulant in adolescent animal models. For example, Cappon et al. (1997) demonstrated that multiple high-dose administrations of methamphetamine reduce striatal dopamine levels in postnatal day 60, but not postnatal day 20, rats. Similarly, this high-dose methamphetamine regimen leads to a loss of tyrosine hydroxylase-positive terminals in postnatal day 60 and 80 rats but not in postnatal day 40 and younger rats (Pu and Vorhees, 1993). In addition, Kokoshka et al. (2000) demonstrated that multiple high-dose administrations of methamphetamine lead to long-term decreases in dopamine transporter activity, tyrosine hydroxylase activity, and the binding of the dopamine transporter ligand WIN35498 in postnatal day 90 rats but not in postnatal day 40 rats. These studies suggest that the persistent dopamine deficits caused by methamphetamine are more apparent in older rats. Mecha-

ABBREVIATIONS: METH, methamphetamine; VMAT-2, vesicular monoamine transporter-2; WIN35498, 2-b-carbomethoxy-3-β-(4-fluorophenyl)tropane.
nisms underlying these age-dependent differences remain to be elucidated.

The vesicular monoamine transporter-2 (VMAT-2) is the main transporter protein involved in the transport of cytoplasmic dopamine into vesicles for storage and presumably protection from oxidative consequences. It has been suggested that methamphetamine causes persistent dopaminergic deficits by preventing vesicular dopamine uptake through VMAT-2, resulting in cytoplasmic dopamine accumulation, an effect that can lead to dopamine autooxidation, free radical formation, and nerve terminal damage (Fumagalli et al., 1999; Fleckenstein and Hanson, 2003). Consistent with this hypothesis, Brown et al. (2000) demonstrated that multiple administrations of methamphetamine rapidly (within 1 h) decrease vesicular dopamine uptake in adult rats, as assessed in purified striatal vesicles prepared from treated rats. In addition, Hogan et al. (2000) demonstrated reduced binding of the VMAT-2 ligand dihydrodextrabenzene in isolated synaptic vesicle preparations obtained from mice 24 h after a neurotoxic regimen of methamphetamine. The mechanism underlying methamphetamine-induced alteration of VMAT-2 is unknown.

As noted above, methamphetamine causes age-dependent differential persistent deficits in dopaminergic neuronal function. The possibility of an age-dependent difference in the response of VMAT-2 to methamphetamine has not been investigated. Hence, the purpose of this study was to examine the effect of multiple administrations of methamphetamine on vesicular dopamine uptake in adolescent and young adult rats. The results demonstrate age-related differential susceptibility of VMAT-2 to methamphetamine administration. Implications with regard to methamphetamine-induced dopaminergic deficits, as well as dopamine-associated neurodegenerative disorders such as Parkinson’s disease, are discussed.

Materials and Methods

Animals. Neuroendocrine studies described by Ojeda et al. (1980) suggested that postnatal day 40 and 90 rats reflect adolescence and young adulthood, respectively; hence, these age groups were employed for this study. Postnatal day 40 and 90 male Sprague-Dawley rats (Charles River Laboratories, Raleigh, NC) were maintained under controlled temperature and lighting, with food and water provided ad libitum. The day before the experiment, animals were rehoused according to body mass (cage size approximately 12 × 14 × 6.5 inches; animals housed six postnatal day 40 rats per cage or three postnatal day 90 rats per cage) to permit equivalent hyperthermic responses to methamphetamine treatment and allowed to acclimate overnight. Where indicated, core body (rectal) temperatures were measured using a digital thermometer (Physiotemp Instruments, Clifton, NJ) every 1 h beginning 30 min before the first drug administration and continuing until 30 min after the final drug administration. Rats were treated concurrently and under identical environmental conditions to preclude the possibility that environmental factors (e.g., ambient temperature and sounds in the animal colony) might differentially affect core temperatures. All rats were killed by decapitation. All experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Drugs and Chemicals. [7,8-3H]dopamine (54.1 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences. Methamphetamine hydrochloride was furnished by the National Institute of Drug Abuse, National Institutes of Health (Bethesda, MD), and doses were calculated as the free base. Methamphetamine was administered as indicated in the figure legends. Reagents for the methamphetamine extractions were purchased from Burdick and Jackson (Muskegon, MI).

Vesicular Dopamine Uptake. Striatal synaptic vesicles were prepared according to a modification of a previously described method (Dubna and Standaert, 2001). Briefly, striatal tissues were dissected and homogenized in ice-cold 0.32 M sucrose and centrifuged (100,000 × g for 10 min at 4°C) to remove nuclei and large debris. The supernatant was centrifuged (10,000 × g for 15 min at 4°C), and the pellet was retained as the whole synaptosomal fraction. This fraction was subsequently lysed hypo-osmotically with water and centrifuged (25,000 × g for 20 min at 4°C) to pellet a membrane-associated fraction. The remaining supernatant was centrifuged (165,000 × g for 45 min at 4°C), and the pellet was retained as the nonmembrane-associated (cytoplasmic or vesicle-enriched) fraction. Vesicular [3H]dopamine uptake was determined in the nonmembrane-associated fraction as described previously (Brown et al., 2002). Because variability in assay components or duration of the assay can affect vesicular [3H]dopamine uptake, all samples within a given experiment were assayed concurrently. Protein concentrations were determined using the Bradford protein assay.

VMAT-2 Immunoreactivity. Striatal synaptosomes were prepared as described above. The synaptosomes were subsequently lysed hypo-osmotically with water, and a portion was reserved as the whole synaptosomal fraction. The remaining lysed synaptosomes were centrifuged (25,000 × g for 20 min at 4°C) to pellet a membrane-associated fraction, and the supernatant was retained as the nonmembrane-associated fraction. Western blot analysis was performed on all fractions as described previously (Riddle et al., 2002).

The binding of VMAT-2 antibody was performed using 50 μg of protein from whole synaptosomal fractions, 30 μg of protein from membrane-associated fractions, and 20 μg of protein from nonmembrane-associated fractions. The primary VMAT-2 antibody (AB1767; 1:1000 dilution) was purchased from Chemicon (Temecula, CA) (Riddle et al., 2002; Sandoval et al., 2003). Bound antibody was visualized with anti-rabbit immunoglobulin antibody (1:2000) purchased from BioSource International (Camarillo, CA). Antigen-antibody complexes were visualized by chemiluminescence. Bands on blots were quantified by densitometry using Kodak 1D image-analysis software (Kodak Biomax MR; Eastman Kodak, Rochester, NY).

Methamphetamine Concentrations. Striatal tissue was homogenized in 500 μl of distilled water, and the whole sample was used for analysis. Brain tissue was homogenized in an equal volume of water, and 500 μl of the homogenate was used for analysis. On the day of the assay, these homogenates were equilibrated to room temperature. Deuterated methamphetamine (250 ng; METH-d8; Radian Corporation, Austin, TX) was added as internal standard to 500 μl of homogenate. Samples were vortexed for 5 s and then made strongly basic by adding alkaline (pH >12) with 100 μl of concentrated ammonium hydroxide. Homogenates were extracted into 6 ml of n-butyl chloride/chloroform (4:1 v/v) for 30 min with gentle shaking. Samples were centrifuged for 10 min at 1200g. The organic phase containing the analyte of interest was transferred to a 16 × 100-mm silanized glass screw-capped test tube and evaporated to dryness at 20°C. Residues were reconstituted with 100 μl of 95:5 formic acid (0.1%) and 5% acetonitrile (v/v) prior to analysis by liquid chromatography/tandem mass spectrometry. A multipoint calibration curve ranging from 1 to 1500 ng/ml homogenate was prepared with drug-free rat plasma and extracted as described above. Analytical intra-assay accuracy and the lack of matrix effect were verified by concurrent analysis of quality control samples that were prepared in drug-free rat brain homogenate.

Concentrations of methamphetamine were determined with a ThermoQuest Quantum liquid chromatography tandem mass spectrometer (Thermo Electron Corporation, Waltham, MA) operating in an atmospheric pressure chemical ionization mode. Separation was achieved with a MetaChem MetaSil Basic 3-μ, 100 × 3.0-mm column.
an analysis of variance followed by Fisher's protected least significant differences.

and quantitation of dopamine levels by using the method of Chapin et al. (1986).

5% acetonitrile. Transitions monitored were m/z 150.1 → 91.1 (methamphetamine) and m/z 158.1 → 92.1 (METH-d8). Accuracy was within 10% of the fortified methamphetamine concentrations from brain homogenate quality control samples. The limit of quantitation in these experiments was 1 ng/ml.

**Vesicular Dopamine Content.** Purified striatal synaptic vesicles were prepared as described above. The resulting vesicular pellet was sonicated for approximately 10 s in tissue buffer [0.05 M sodium phosphate/0.03 M citric acid buffer with 25% methanol (v/v), pH 2.5] at a concentration of 50 mg/ml (original wet weight of tissue). A portion of sample was retained, and the protein was determined according to the method of Lowry et al. (1951). The supernatant (20 μl) was injected onto a high-performance liquid chromatograph system coupled to an electrochemical detector (+0.73 V) for separation and quantitation of dopamine levels by using the method of Chapin et al. (1986).

**Statistical Analysis.** Statistical analyses were performed using an analysis of variance followed by Fisher’s protected least significant difference post hoc comparison. Analyses between two groups were conducted using a Student’s t test. Differences among groups were considered significant if the probability of error was less than 5%.

**Results**

The results presented in Fig. 1 demonstrate greater levels of VMAT-2 immunoreactivity in postnatal day 90 compared with postnatal day 40 rats, as assessed in a nonmembrane-associated vesicular fraction prepared from rat striata. No significant differences in VMAT-2 immunoreactivity were observed as a function of age in corresponding membrane-associated or whole synaptosomal fractions. Consistent with this finding, the results presented in Fig. 2 demonstrate that vesicular dopamine uptake was greater in postnatal day 90 versus postnatal day 40 rats, as assessed in nonmembrane-associated vesicles prepared 1 h after saline treatment.

Multiple administrations of methamphetamine (4 × 5 mg/kg/injection, 2-h intervals) caused a 65% decrease in vesicular dopamine uptake, as assessed 1 h after treatment in nonmembrane-associated vesicular fractions prepared from saline- and methamphetamine-treated postnatal day 90 rats. This regimen produced brain methamphetamine concentrations of 4.3 ± 0.5 ng/mg, as assessed 1 h after treatment. The administration of 4 × 10 mg/kg/injection (2-h intervals) of methamphetamine to postnatal day 40 rats produced comparable brain methamphetamine concentrations (5.0 ± 0.4 ng/mg; $P \leq 0.3119$) as assessed 1 h after treatment but caused a lesser decrease in vesicular dopamine uptake (i.e., 45%). The administration of a higher dose of methamphetamine (4 × 15 mg/kg/injection, 2-h intervals) to postnatal day 40 rats, a regimen that produced greater brain methamphetamine concentrations (10.7 ± 1.1 ng/mg tissue), did not further decrease vesicular dopamine uptake.

The greater methamphetamine-induced decreases in vesicular dopamine uptake in postnatal day 90 rats presented in Fig. 2 were not the result of differences in body temperature, because rats experienced comparable hyperthermic responses throughout the methamphetamine treatments (Fig. 3). Core temperatures of postnatal day 40 and 90 saline-treated rats decreased slightly over the course of the experiment.

To examine the persistent consequences of methamphetamine, postnatal day 40 and 90 rats received 4 × 10 mg/kg injection (2-h intervals) and 4 × 5 mg/kg/injection (2-h intervals) of methamphetamine, respectively (i.e., doses that produced equivalent brain methamphetamine concentrations 1 h after treatment [Fig. 2]). For comparison, another group of postnatal day 90 rats were given 4 × 10 mg/kg injection methamphetamine (2-h intervals). Rats were decapitated 7 days later. The results revealed that basal striatal dopamine content was greater in postnatal day 90 versus 40 rats (Fig. 4). Both methamphetamine regimens decreased striatal dopamine content and vesicular dopamine uptake (Figs. 4 and 5, respectively) in postnatal day 90 rats. Methamphetamine was without effect on either parameter in postnatal day 40 rats.

**Discussion**

Considerable recent attention has focused on the neurochemical events contributing to the transition from adolescence to adulthood and to the impact of these changes on a variety of parameters such as behavior (Spear, 2000) and susceptibility to neurotoxic insults (Katoh et al., 1990; Bossi et al., 1993; Imam and Ali, 2001). Several factors likely...
controls (six rats). noreactivity and there was only a small nonstatistically related differences in membrane-associated VMAT-2 immunoreactivity in postnatal day 90 versus 40 rats. The latter increase might be due to the increase in nonmembrane-associated (i.e., cytoplasmic) VMAT-2 but did not reach statistical significance, because the total quantity of VMAT-2 protein in the whole synaptosomal fraction under study.

The results from this study demonstrate greater basal VMAT-2 immunoreactivity and vesicular dopamine uptake in postnatal day 90 versus 40 rats, as assessed in a purified nonmembrane-associated (presumably cytoplasmic) vesicular subcellular fraction. These data suggest the emergence of additional cytoplasmic VMAT-2, and presumably associated vesicles, during the transition from adolescence to adulthood in the rat. Whether the additional VMAT-2 proteins reflect additional VMAT-2 per nerve terminal and/or vesicle or the development of new terminals containing VMAT-2 cannot be ascertained from these findings. Notably, there were no age-related differences in membrane-associated VMAT-2 immunoreactivity and there was only a small nonstatistically

Fig. 3. Multiple administrations of methamphetamine caused equivalent increases in core body temperature of postnatal day 40 and 90 rats. Animals received METH (four administrations; 5, 10, or 15 mg/kg/injection subcutaneously; 2-h intervals) or saline (Sal) (four injections; 1 ml/kg/injection subcutaneously; 2-h intervals), as indicated by arrows. Values represent means and vertical lines ± 1 S.E.M. of determination in six rats. *, values significantly different from age-matched saline-treated controls (P ≤ 0.05).

Fig. 4. METH decreased striatal dopamine (DA) tissue content in postnatal day 90 but not in postnatal day 40 rats 7 days after administration. Postnatal day 40 rats received either methamphetamine (four injections; 5 or 10 mg/kg/injection subcutaneously; 2-h intervals) or saline (Sal) (four injections; 1 ml/kg/injection subcutaneously; 2-h intervals). Postnatal day 90 rats received methamphetamine (four injections; 5 or 10 mg/kg/injection subcutaneously; 2-h intervals) or saline (four injections; 1 ml/kg/injection subcutaneously; 2-h intervals). All animals were sacrificed 1 h after the last administration. Data represent the means and vertical lines ± 1 S.E.M. of determinations in six rats. *, values significantly different from age-matched saline-treated controls (P ≤ 0.05). #, value significantly different from saline-treated postnatal day 40 rats (P ≤ 0.05). Values presented above bars represent mean brain methamphetamine concentrations (nanograms per milligram of tissue) ± 1 S.E.M. of determinations in six rats.

significant increase in whole synaptosomal VMAT-2 immunoreactivity in postnatal day 90 versus 40 rats. The latter increase might be due to the increase in nonmembrane-associated (i.e., cytoplasmic) VMAT-2, but did not reach statistical significance, because the total quantity of VMAT-2 protein in the nonmembrane-associated fraction is much smaller than the quantity of VMAT-2 protein in the whole synaptosomal fraction under study.

The function of the additional VMAT-2 that emerges during development from postnatal day 40 to 90 is unknown; however, one function might be to sequester the larger dopamine content found in young adult versus adolescent rats (Nomura et al., 1976; Giorgi et al., 1987; Fig. 4) and thereby prevent its autooxidation, an effect that has been linked to the formation of reactive oxygen species (Cadet et al., 1994; Cubells et al., 1994; Yamamoto and Zhu, 1998). If so, the loss of this sequestration capacity would predictably make dopamine neurons in postnatal day 90 rats more susceptible to the persistent neurotoxic effects of methamphetamine. Con-

contributing to one or more of these transitional changes, including drug pharmacokinetics (i.e., metabolism, excretion, etc.; Spear, 2000), stress-induced gonadarche (Spear, 2000), and/or antioxidant systems (Kim et al., 2002). Changes in dopaminergic systems have also been reported (Araki et al., 1997; Cappon et al., 1997; Kokoshka et al., 2000), although this study is the first to evaluate the impact of methamphetamine on VMAT-2 function during this developmental period.

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sistent with this premise, the results reveal that high-dose methamphetamine treatment administered at doses producing equivalent brain methamphetamine concentrations 1 h after treatment (5 and 10 mg/kg/injection to postnatal day 90 and 40 rats, respectively) caused a greater percentage decrease in vesicular dopamine uptake both 1 h and 7 days after treatment in postnatal day 90 versus 40 rats.

The age-dependent differences in magnitude of the acute VMAT-2 response were not attributed to differences in body temperature, an important contributor to the persistent effects of methamphetamine (Bowyer et al., 1994; Ali et al., 1996; Cappon et al., 1997). Instead, the greater magnitude of methamphetamine-induced decrease seems to correlate with greater basal VMAT-2 activity in postnatal day 90 versus 40 rats. This allows the speculation that the acute decrease in vesicular dopamine uptake observed in postnatal day 90 rats likely involved the population of VMAT-2 that emerged as a function of age.

We reported previously that methamphetamine decreases vesicular dopamine uptake in the nonmembrane-associated fraction and causes redistribution of VMAT-2 proteins within nerve terminals of adult rats, as determined 1 h after treatment (Riddle et al., 2002; Sandoval et al., 2003). Present findings confirm that methamphetamine decreases vesicular dopamine uptake in the nonmembrane-associated fraction of postnatal day 90 rats 1 h after treatment. One possible explanation for the methamphetamine-induced decrease in VMAT-2 activity in postnatal day 90 rats 1 h after treatment is that this treatment causes a redistribution (or “trafficking”) of VMAT-2 within nerve terminals (Riddle et al., 2002; Sandoval et al., 2003). This contrasts with the persistent dopaminergic decreases observed after 7 days, which, although controversial (Wilson et al., 1996), likely reflects the loss of nerve terminals (Kogan et al., 1976; Ricurte et al., 1982; see also the persistent loss of total striatal dopamine content presented in Fig. 4). It is important to note, however, that the presently reported VMAT-2 findings do not necessarily demonstrate terminal loss because it involves nonmembrane-associated (versus total content of) VMAT-2. Interestingly, the methamphetamine-related acute decrease in VMAT-2 activity observed in the postnatal day 40 rats was short-term because it was no longer observed 7 days after treatment. The role of trafficking in this phenomenon is presently unknown. However, in both age groups, there seems to be a population of VMAT-2 that was resistant to both the acute and persistent effects of methamphetamine at the doses selected for study (higher doses were not administered because of increased mortality, particularly in postnatal day 90 rats). These data suggest the existence of at least three populations of VMAT-2: 1) a population resistant to the acute and persistent effects of methamphetamine found in both postnatal day 40 and 90 rats; 2) a population found in postnatal day 40 rats that responds acutely to methamphetamine but is associated with neurons resistant to its persistent effects; and 3) a population found in postnatal day 90 rats that responds to both the acute and persistent effects of methamphetamine. Further studies are required to establish whether these VMAT-2 populations are associated with distinct nerve terminals, intra-neuronal VMAT-2 populations within nerve terminals, or a functional state of the related neurons.

In summary, the present data are the first to report age-dependent effects of methamphetamine on VMAT-2 protein and its function. From these data, one can speculate that multiple populations of VMAT-2, vesicles, and/or associated neurons that are differentially susceptible to methamphetamine effects may exist. In addition, the findings presented here demonstrate that the methamphetamine-induced persistent effects observed in postnatal day 90 rats, but not in postnatal day 40 rats, is associated with long-term decrease in VMAT-2 activity, although other factors such as differences in antioxidant levels may also contribute. This lack of methamphetamine-induced persistent effects in adolescent animals may result from the fact that the dopamine systems vulnerable to the long-term effects of methamphetamine do not develop until after postnatal day 40. Future studies will elucidate further mechanisms contributing to both the susceptibility and resistance to the acute and toxic effects of the stimulant, as well as the developmental changes that allow emergence of the “methamphetamine-susceptible neurons.” Because aberrant VMAT-2 regulation may be involved in dopaminergic neuronal degeneration in Parkinson’s disease (Harrington et al., 1996; Lotharius and Brundin, 2002; Fleckenstein and Hanson, 2003), identifying and understanding the mechanistic differences underlying VMAT-2 regulation may provide important insight into the cause and treatment of this degenerative disorder.

References


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