Age as a Susceptibility Factor in the Striatal Dopaminergic Neurotoxicity Observed in the Mouse following Substituted Amphetamine Exposure

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ABSTRACT: A number of substituted amphetamines, including methamphetamine (METH) are considered dopaminergic neurotoxicants. METH causes long-term depletions of striatal dopamine (DA) and its metabolites (DOPAC and HVA) that are accompanied by other changes indicative of nerve terminal degeneration. These include argyrophilia as detected by silver degeneration stains and an elevation in glial fibrillary acidic protein (GFAP), a marker of reactive gliosis in response to injury, as well as a long-term decrease in tyrosine hydroxylase (TH) protein levels. The susceptibility to the dopaminergic neurotoxicity of METH and the other amphetamines can be affected by a number of factors including age, gender, stress, and environment. Many of these susceptibility factors have been extensively investigated in the rat but less so in the mouse. As the availability of genetically altered mice continues to expand, this species is increasingly selected for study. Thus, in previous work we determined that stress, gender, and the environment can significantly impact the neurotoxicity of the amphetamines. Here we determined how age affects the striatal DA depletion and GFAP elevation induced by *d*-METH in C57BL/6 mice. Age was a significant determinant of the ability of a known neurotoxic regimen of *d*-METH (10 mg/kg × 4) to produce striatal DA depletion with one-month-old C57BL/6 mice displaying minimal and nonpersistent depletion of DA or its metabolites while mice 12 months of age displayed large and persistent depletions of DA (87%), DOPAC (71%), and HVA (94%). Large elevations in striatal GFAP were induced in mice 2–23 months of age by *d*-METH, with lower dosages of *d*-METH being effective in the older mice. In contrast, the usual neurotoxic regimen of *d*-METH was minimally effective in inducing GFAP elevations (49% over control) in one-month-old mice, despite elevations in body temperature equivalent to those observed in older mice. Although increasing the dosage of *d*-METH (20 to 80 mg/kg) did increase the GFAP response (100% over control), it was still well below that usually exhibited at the usual neurotoxic dosage (300–400% over control) in fully mature mice. These data suggest maturity of striatal dopamine systems may be an essential element in the striatal damage induced by the neurotoxic amphetamines.

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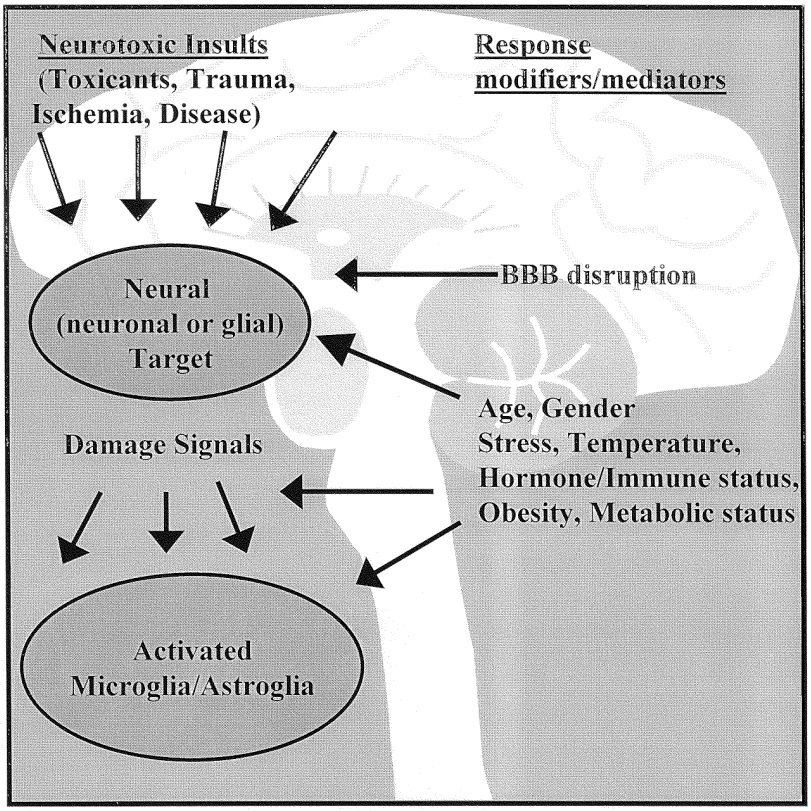

FIGURE 1. Schematic of factors that can affect the neurotoxic actions of chemicals.

INTRODUCTION

Much data confirms the idea that toxic effects of chemicals are not solely dictated by dosage.[1] For example, factors, including gender, stress and temperature (FIG. 1), are known to impact the neurotoxicity of many agents including the substituted amphetamines. These compounds, including amphetamine (AMP), methamphetamine (METH), 3,4-methylendioxymethampetamine (MDMA), and 3,4-methylenedioxyamphetamine (MDA) are able to damage the dopaminergic innervation of the striatum as evidenced by long-lasting depletions in dopamine (DA), its metabolites DOPAC and HVA, as well as tyrosine hydroxylase (TH) activity and protein levels.[2] Accompanying this damage are elevations in a number of injury-related markers, including glial fibrillary acidic protein (GFAP), an astrocyte marker of neural injury, and cupric silver degeneration stain reflective of injury-induced argyrophilia.[2] We have previously shown that male mice are more susceptible to the striatal damage induced by the amphetamines and that both ambient and core temperature play a role in the degree of damage induced by these agents.[3–5] We have also documented that

certain stress procedures can protect against the neurotoxicity of the amphetamines through their temperature-modulating abilities.[3,6]

As the stage of maturation of the striatal dopamine system appears to play a role in many of the pharmacological actions of the amphetamines, including activation and sensitization,[7,8] age also is likely to be a determinant of their neurotoxic potential. To examine the impact of age on the striatal damage induced by METH in C57BL/6 mice, animals ranging in age from 1 to 23 months of age were treated with a repeated dosage regimen known to produce significant DA depletions and GFAP elevations in our standard mouse models of METH-induced neurotoxicity.[2,5] Our data demonstrate that age is a significant determinant in the degree of striatal damage induced by METH. Although METH was able to effect temperature increases in mice of all ages, with the exception of those 23 months of age, one-month-old mice displayed little or no striatal GFAP elevation or DA depletion in response to the standard neurotoxic dosage regimen. METH in older mice induced robust neurotoxicity as indicated by significant elevations in GFAP and decrements in DA. In fact, lower dosages of METH were able to induce neurotoxicity in older mice whereas increasing the dosage of METH to lethal levels increased neurotoxicity only minimally in immature mice. Our data suggest that maturation of the striatal dopaminergic system contributes greatly to the ability of METH to induce striatal damage.

METHODS

Materials. All reagents were analytical grade and were obtained from a variety of commercial sources. The following drugs and chemicals were obtained from the sources indicated: *d*-METH and high-performance liquid chromatography (HPLC) standards (Sigma Chemical Co., St. Louis, MO), bicinchonic acid protein assay reagent and bovine serum albumin (Pierce Chemical Co., Rockford, IL); reagents used for HPLC were of HPLC grade (Burdick and Jackson, Muskegon, MI).

Animals. Female C57BL/6J mice were received from Jackson Labs (Bar Harbor, ME) at 4-6 weeks of age and male C57BL6/N mice were obtained from the NCTR breeding colony. All mice were maintained 4-6 per cage in humidity- and temperature-controlled colonies certified by the American Association for Accreditation of Laboratory Animal Care until reaching the ages used here. Mice ranged from 1 to 23 months of age.

Dosing. Female mice were administered *d*-METH (10, 5 or 2.5 mg/kg s.c. as the base) or saline vehicle (0.9%) every 2 h beginning at 1100 h, for a total of four injections. Male mice received *d*-METH (10 mg/kg i.p. as the base) or saline vehicle every 2 h for a total of four injections.

Temperature measurement. A Bat-10 thermometer coupled to a RET-3 mouse rectal probe (Physitemp, Inc., Clinton, NJ) lubricated with mineral oil was used to record rectal temperature. Use of a fast-rise time probe and the method outlined in previous work[3] made it possible to obtain reliable measurements of rectal temperature in less than 30 s per mouse.

Tissue preparation. At 72 h following the last injection of *d*-METH, mice were killed by cervical dislocation, and striata were obtained by free-hand dissection. Stri-

ata prepared for HPLC analysis were ultrasonicated in 0.2 N perchloric acid and centrifuged at 4°C (15,000 × *g*; 7 min). One hundred and fifty microliters of the supernatant were filtered through a 0.2 μm Nylon-66 microfilter (MF-1 centrifugal filter, Bioanalytical System, W. Lafayette, IN) and 25 μl were injected directly onto the HPLC/EC system for the separation of the analytes. Striata prepared for GFAP analysis were weighed, homogenized with an ultrasonic probe (Model XL-2005, Heat Systems, Farmingdale, NY) in 10 vol hot (90–95°C) 1% sodium dodecylsulfate (SDS), and stored frozen at −70°C until analysis.

Catecholamine analysis. Concentrations of DA and its metabolites DOPAC and HVA were quantitated by a modified method of HPLC combined with electrochemical detection as described by Ali *et al.*[9]

Protein assay. Total protein in SDS homogenates was assayed by the method of Smith *et al.*[10] using bovine serum albumin as the standard.

GFAP Immunoassay. A sandwich enzyme-linked immunosorbent assay (ELISA)[11] with the modifications specified in O'Callaghan & Miller[2] was used to assay striatal homogenates for GFAP concentration. Microtiter plate wells were coated with a rabbit polyclonal antibody to GFAP for 1 h, and nonspecific binding was then blocked with nonfat dry milk. Dilution of the SDS homogenates with sample buffer and their addition to the wells of the microtiter plates were accomplished using a Tecan robotic sample processor (model 5052, Tecan, U.S., RTP, NC), in dual-tip mode, running on RSP-Integrator/Immuno-AMI software (Tecan, U.S., RTP, NC). After several more blocking and washing steps, a mouse monoclonal antibody to GFAP was added followed by the addition of an enzyme-linked antibody directed against mouse immunoglobin G (IgG). The colored reaction product was obtained by subsequent addition of enzyme substrate and quantified by spectrometry at 405 nm using a microplate reader (UV Max running on a Soft Max program, Molecular Devices, Menlo Park, CA).

Statistics. Data analyses were conducted with the JMP, software (SAS Institute Inc., 1995). Individual variables were evaluated by analysis of variance followed by Duncan's Multiple Range Test for mean comparisons. The alpha level used to determine significance was .05.

RESULTS

Effects in C57BL/6N Male Mice

Effects of Age on Striatal Neurotoxicity of d-*METH*

Significant depletions in striatal DA were caused by treatment with *d*-METH at a standard neurotoxic dosage (10 mg/kg, i.p. × 4) (FIG. 2). It is also apparent that a much greater depletion was induced in 12-month-old mice as compared to those 1 month of age (~80% vs. 50%, respectively), suggesting this dosage regimen produced more severe neurotoxicity in the older mice. The failure of *d*-METH to produce depletions in the DA metabolites, DOPAC and HVA, in the younger mice also supports this conclusion.

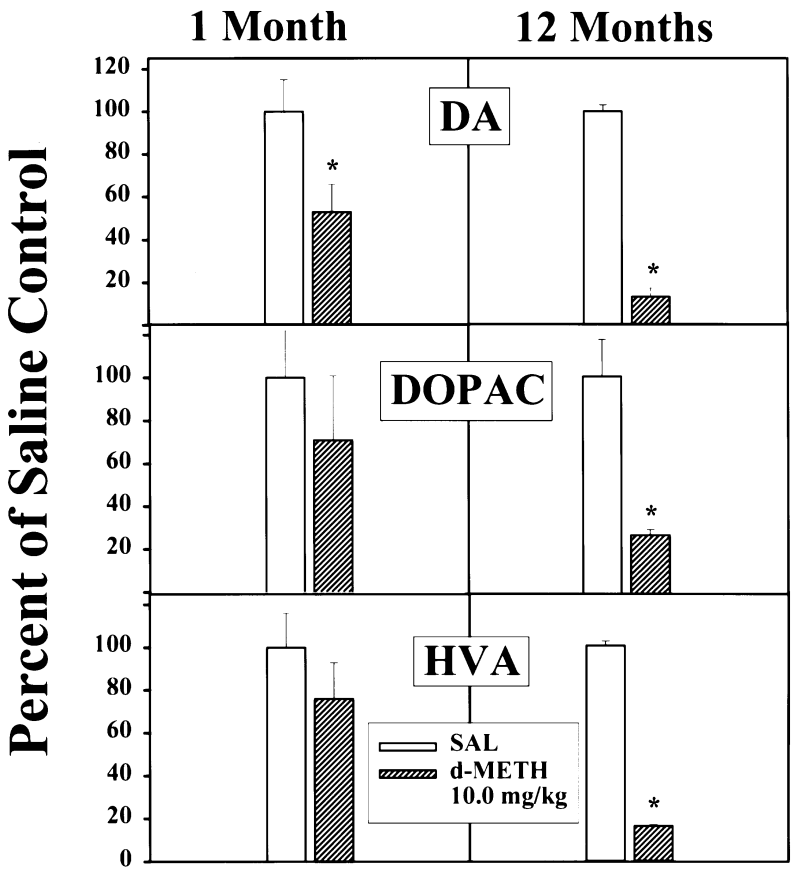

FIGURE 2. At 2 weeks following *d*-METH (10 mg/kg i.p. as the base given every 2 h × 4) DA is significantly ($p < .05$) reduced at both ages, but DOPAC and HVA concentrations are significantly reduced in the striatum of 12-month-old but not one-month-old male C57BL6/N mice. Data are presented at mean ± SEM percent of SAL for the same age mice and the $n = 5$–6 mice per group. The percent of SAL control was calculated for each animal by determining the mean value for the appropriate age SAL group and then determining the percent of that value for each SAL and *d*-METH animal. Values for DA, DOPAC, and HVA in SAL-treated mice were 433.0 (± 42.9), 93.8 (± 22.2), and 50.3 (± 6.4)ng/100 mg wet weight of tissue, respectively, for the one-month-old mice and 812.5 (± 19.6), 74.2 (± 8.5), and 438.5 (± 7.9)ng/100 mg wet weight of tissue, respectively, for the one-year-old mice. *Significantly different ($p < .05$) from SAL.

Effects in C57BL/6J Female Mice

Effects of Age on Body Temperature Changes Induced by d-*METH*

The standard neurotoxic regimen of *d*-METH (10 mg/kg, s.c. × 4) clearly caused hyperthermia in mice ranging from 1 to 12 months of age (FIG. 3). Transformation of these temperature curves into areas under the curve (AUCs; FIG. 4) confirm *d*-METH can significantly elevate temperatures above those observed in saline-treated

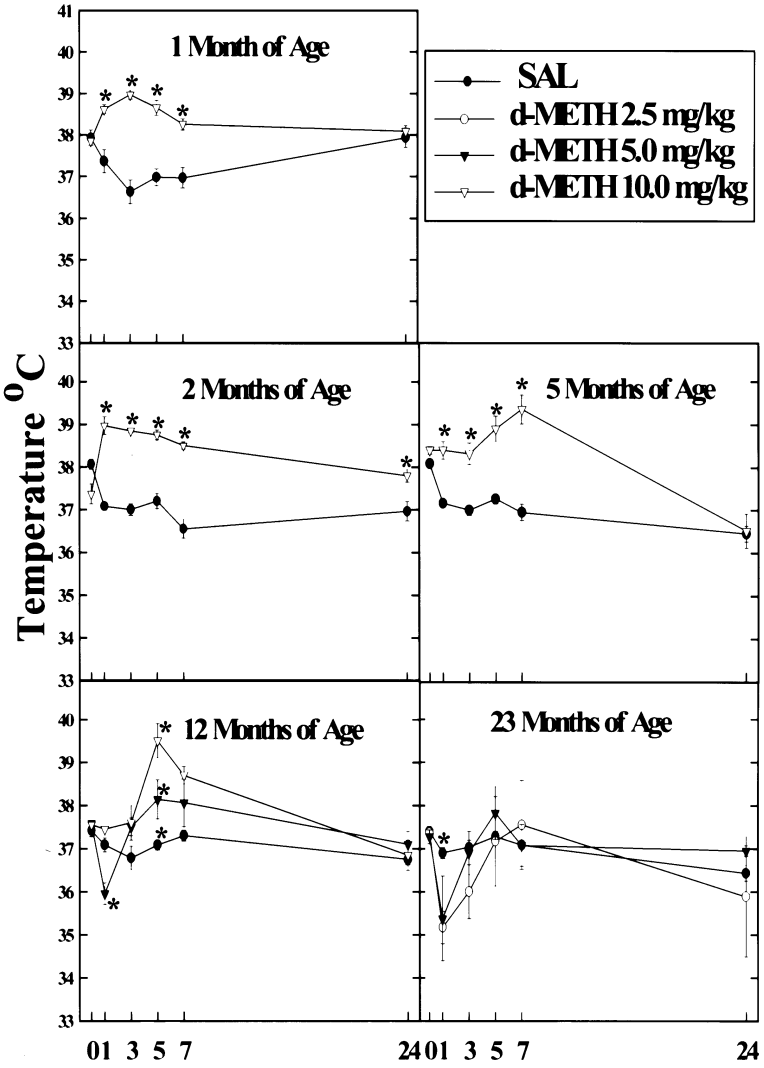

FIGURE 3. Effect of age at treatment on the alterations in rectal temperature induced by *d*-METH administered as the base s.c. every 2 h for a total of four injections. Data are presented as the mean ± SEM and the $n = 5–6$ mice per group with the exception of the 12 months of age group given 10 mg/kg. Data are presented from the three survivors. *Significantly different ($p < .05$) from SAL.

mice. In contrast, the temperature curves and AUC measure suggest that 23-month-old mice display a minor but nonsignificant decrease in response when given either 2.5 or 5.0 mg/kg. A dosage of 10 mg/kg × 4 was lethal at 23 months of age.

It is also evident that age does not alter the temperature curves or AUCs displayed in response to saline injection and handling. As we have noted in previous work han-

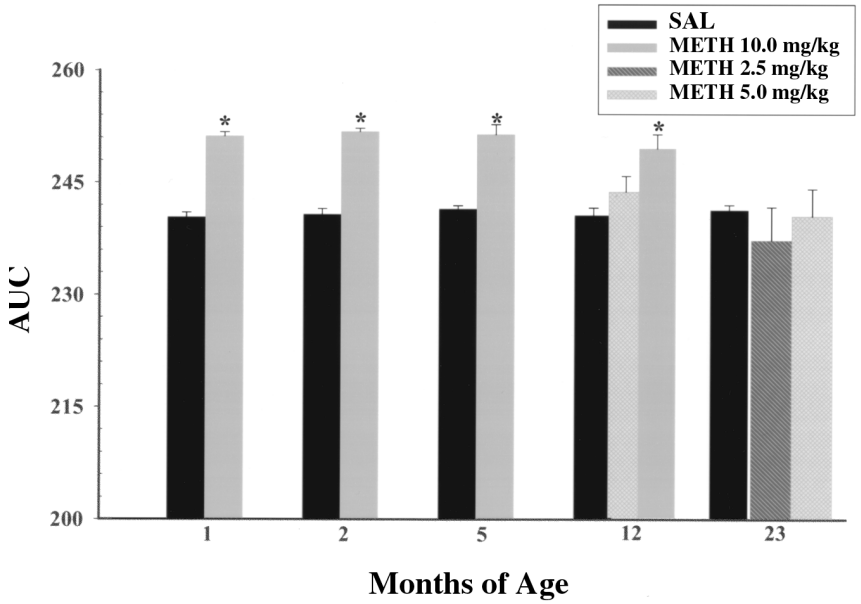

FIGURE 4. *d*-METH increases core body temperature in female C57BL/6J mice as reflected by significant ($p < 0.05$) increases in the area under the temperature curve (AUC) induced in *d*-METH treated mice relative to those receiving SAL at 1, 2, 5, 12, but not 23, months of age. Note that mice received 10 mg/kg of *d*-METH at 1, 2, 5, and 12 months of age, but only 2.5 or 5.0 mg/kg at 23 months of age. *d*-METH was administered as the base s.c. every 2 h for a total of four injections. Temperature curves were transformed to an AUC utilizing the Trapezoidal Rule using the rectal temperatures obtained at 0.5, 1, 3, 5, and 7, but not 24, h after the first injection of *d*-METH. Data are presented as the mean ± SEM and the $n = 5$–6 mice per group with the exception of the 12 months of age group given 10.0 mg/kg *d*-METH. Data are presented from the three survivors. *Significantly different ($p < .05$) from SAL.

dling results in an elevation in temperature at the beginning of the dosing session that gradually habituates,[3] all ages of mice display this handling induced hyperthermia and habituation with repeated handling.

Effects of Age on Striatal Neurotoxicity of d-*METH*

Due to a freezer failure, samples for the assessment of DA and its metabolites were lost. Therefore only GFAP data are available from these animals. *d*-METH given at the standard neurotoxic dosage (10 mg/kg, s.c. × 4) clearly resulted in significant elevations in GFAP, an indication of astrogliosis, in mice aged 2, 5, 12, or 23 months of age (FIG. 5). In contrast, mice at one month of age displayed a limited nonsignificant elevation in GFAP in response to 10 mg/kg of *d*-METH. They showed an increase of 49% relative to the 300% or greater increases displayed by the older mice. Further, mice at 12 months of age displayed a large GFAP increase (over 300%) to a lower dosage regimen of *d*-METH (5.0 mg/kg × 4), suggesting increased susceptibility to *d*-METH neurotoxicity at this age. Mice at 23 months of age received lower dosages (5.0 or 2.5 mg/kg, s.c. × 4) but still displayed elevations in

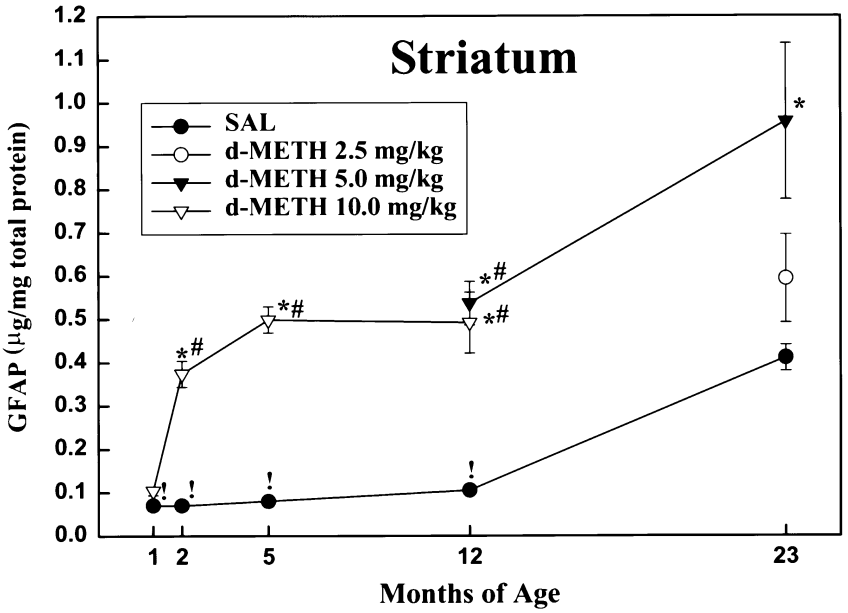

FIGURE 5. *d*-METH significantly ($p < 0.05$) increases striatal GFAP levels. All mice received an injection s.c. every 2 h beginning at 10 AM for a total of four injections, but the dosage varied by age. Mice aged 1, 2, 5, and 12 months received 10 mg/kg. An additional group of mice aged 12 months received 5 mg/kg as did mice aged 23 months. Another group of mice aged 23 months received 2.5 mg/kg. Note that mice aged one month exhibited a minimal GFAP response to a dosage of 10 mg/kg, whereas mice 2 months of age exhibited a pronounced increase as did 23-month-old mice receiving 5.0 mg/kg. Also note that control mice exhibit an age-related increase in striatal GFAP levels as we have previously noted.[26,27] Data are presented as the mean ± SEM with an $n = 5–6$ mice per group with the exception of the group of 12-month- old mice given 10.0 mg/kg *d*-METH. Data are presented from the three survivors. *Significantly different ($p < .05$) from SAL.

GFAP. However, these data are complicated by the age-related increase in the basal concentration in GFAP that is apparent in the 12-month-old mice and significantly elevated by 23 months (~ threefold increase). Thus, when *d*-METH increases in GFAP are expressed as a percent of the basal level, increases of only 45 and 132% (2.5 and 5.0 mg/kg groups, respectively) are found.

Effect of Increasing Dosage of d-*METH on Temperature and Striatal Neurotoxicity in One-Month-Old Mice*

All dosages of *d*-METH, except 80 mg/kg, s.c. × 4, caused an elevation in rectal temperature relative to saline-treated mice as indicated by the temperature curves (FIG. 6) and AUCs (FIG. 7). Note that the 10.0 mg/kg data in these figures were redrawn from FIGURES 3 and 4 to aid in comparison. A dosage of 80 mg/kg × 4 produced no elevations in temperature and also produced about 50% lethality.

Dosages of *d*-METH above 10 mg/kg × 4, the standard neurotoxic regimen, produced significant astrogliosis, as indicated by elevations in GFAP (~ 100%), but

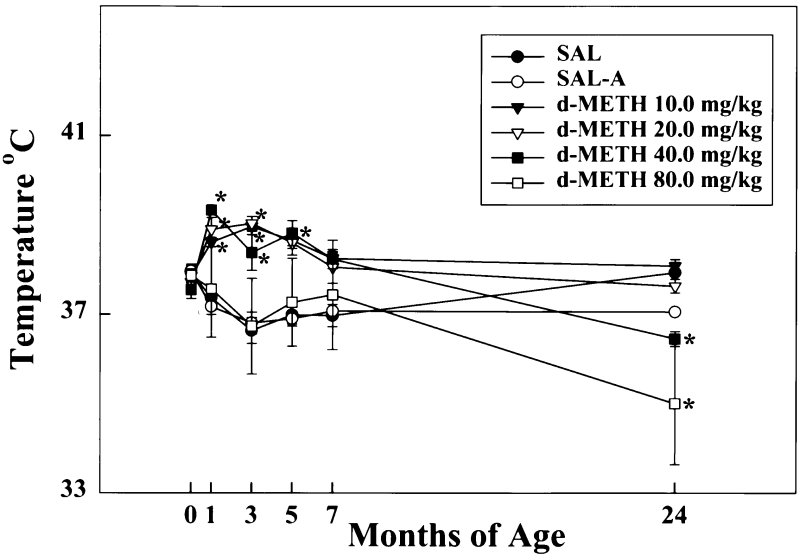

FIGURE 6. Effect of dosage of *d*-METH on rectal temperature in one-month old C57BL/6J female mice. *d*-METH was administered as the base s.c. every 2 h for a total of four injections at a dosage of 0.0, 20.0, 40.0 or 80.0 mg/kg. Data from mice one-month-of-age given 10 mg/kg *d*-METH and presented in FIGURE 3 are replotted here. Data are presented as the mean ± SEM.

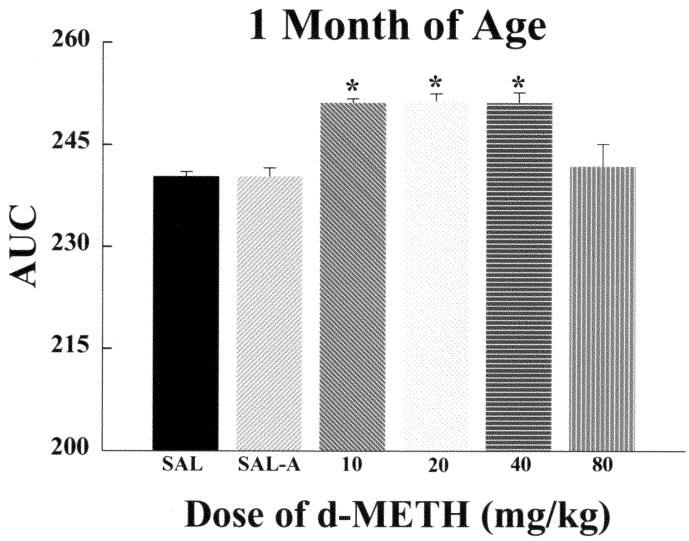

FIGURE 7. *d*-METH increases core body temperature in female C57BL/6J female mice at 10.0, 20.0, 40.0, but not at 80.0, mg/kg as reflected by a significant ($p < .05$) increase in the area under the temperature curve (AUC). *d*-METH was given as the base s.c. every 2 h for a total of four injections. Data from mice given SAL or 10 mg/kg *d*-METH are replotted from FIGURE 3 for comparison.

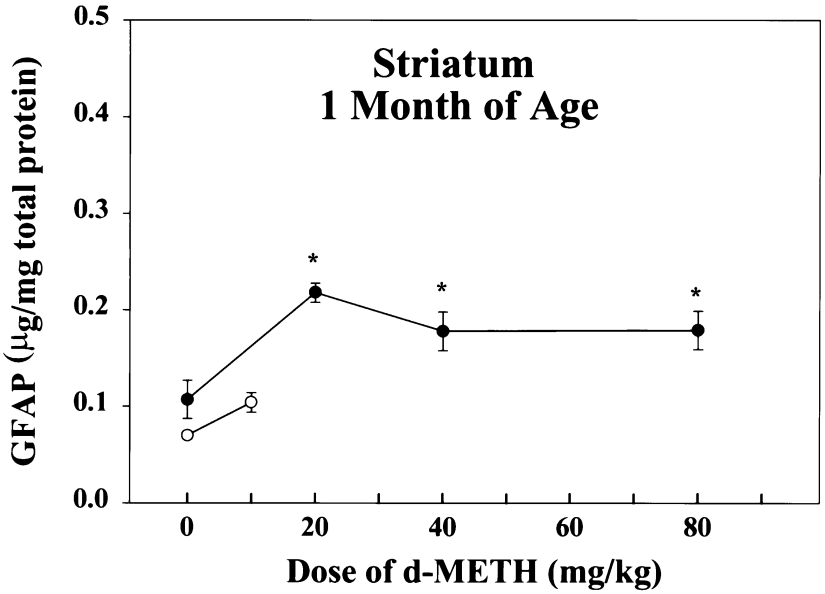

FIGURE 8. *d*-METH (s.c. as the base every 2 h for four injections) at one month of age in C57BL/6J female mice significantly ($p < .05$) increases striatal GFAP levels at 20, 40 or 80 mg/kg relative to controls given 0.9% NaCl but not in a dose-related fashion. Data from mice one month of age given SAL or 10 mg/kg *d*-METH are replotted from FIGURE 2 for comparison. Note that the 10.0 mg/kg × 4 regimen does not significantly increase GFAP and that there is no difference between the GFAP values for the two saline control groups.

these elevations were still well below those displayed by older mice given the standard regimen (~ 300– 400%). Further, increasing dosages of *d*-METH did not result in dose-related increases in the GFAP response. All dosages above the standard neurotoxicity regimen produced increases between 65 and 100%.

DISCUSSION

Our data clearly indicate that the striatal neurotoxicity associated with repeated exposure to *d*-METH is age-related. *d*-METH-induced striatal neurotoxicity was minimal in one-month-old mice as only small depletions of DA and its metabolites or elevations in GFAP were observed. Further, increasing the dosage of *d*-METH up to lethal dosages did not increase the GFAP elevation in these immature mice and suggests that the striatal dopamine system or some other crucial aspect of the toxicity (e.g., distribution, metabolism, etc.) is not sufficiently mature to allow *d*-METH to exert its full neurotoxic action. As such, our data adds to the body of literature suggesting that many of the actions of the amphetamines depend on sufficient development of the striatal dopamine system. Thus, their neurotoxic actions are age-related as are other effects of the amphetamines including sensitization, aggregation-enhanced toxicity, and their neurotransmitter-depleting abilities.[7,8,12–15]

An increasing awareness exists of the complexity of the role of temperature in the striatal damage induced by the substituted amphetamines. *d*-METH, as well as the other neurotoxic amphetamines elevate body temperatures, sometimes to lethal levels, in both rats and mice, although the lethal elevations appear more necessary for producing striatal damage in the rat than in the mouse.[3,16–18] In our experiments, *d*-METH was able to increase body temperature, albeit by moderate increases of 1–2°C, irrespective of dosage at all ages with the exception of the mice that were 23 months of age. In the extremely old mice, body temperature was initially lowered relative to controls and never exceeded the temperature of the control mice during dosing. Obviously, mice, unlike rats (see ref. 19 a review of the role of temperature in METH neurotoxicity in the rat), can display elevations in markers associated with striatal damage in circumstances where only moderate and nonlethal elevations occur. It is also clear that manipulations capable of lowering body temperature during the period of dosing can reduce or prevent the striatal damage associated with exposure.[3,20] What is not clear is exactly what role body temperature plays in amphetamine neurotoxicity in the mouse. The data from this study do not aid in clarifying the role, but do indicate GFAP elevations in response to *d*-METH treatment can occur with no elevation (23-month-old mice) or can be minimal although an elevation in temperature in temperature occurs (one-month-old mice).

The meager GFAP elevation in the one-month-old mice in response to the standard neurotoxic dosage regimen of *d*-METH could signify an inability of immature brain to respond to injury with astrocytic hypertrophy. This is unlikely in that chemically induced injury to much younger brain results in a robust GFAP response.[21] Also the DA and metabolite deletions accompanying *d*-METH treatment in the one-month-old mice were minimal relative to older animals and would suggest this regimen fails to damage the immature striatum rather than an inability of immature and mature brain to respond to injury in the same fashion. That exposure of the one-month-old animals to higher dosages of *d*-METH results in increased striatal GFAP concentrations lends credence to this idea. However, it is also obvious from the dose-response data that in terms of a percentage increase over saline-treated mice one-month- old mice never display as great a GFAP increase as do mature mice (2–12 months of age) despite increases in the dosage to lethal levels. Without accompanying data indicating the degree of damage induced by these higher dosages (e.g., neurotransmitter levels or TH protein content), it cannot be known whether the lack of a robust response in the one-month-old mice is due to an inability to respond or to a reduced injury signal. It is likely not due to an inability to respond because our previous work and that of others indicate immature rodents can produce a robust GFAP response to injury whether measured immunocytochemically or by immunoassay.[21–24] Given that many other aspects of amphetamine actions are not fully developed by one month, it is more likely that increasing dosages were not able to cause increasing injury because of immaturity of the dopamine system.[8]

Gliosis was apparent in the oldest mice relative to all other ages of mice examined. The 23-month-old mice displayed marked elevations in the basal levels of striatal GFAP that reached greater than 400% of the levels in 1–5-month-old mice. This robust age-associated gliosis replicates our previous work as well as that of others although there is still little understanding of the cause of the increase in GFAP message and protein level with age.[25–27] Age-related gliosis may reflect activation due

to continuous neuronal degeneration (e.g., injury) that is speculated to occur with aging, or, alternately, it is due to upregulation caused by factors other than injury.

It is also apparent that the oldest mice given *d*-METH display the greatest concentrations of striatal GFAP. One interpretation is that substantial damage to the striatum has occurred even though dosages of one-half or less of the standard neurotoxic dosage were used to avoid lethality. Although no independent assessment of terminal degeneration is available, the DA depletions seen in the 12-month-old mice were substantially greater than that of the one- month-old mice. There would be little reason to suspect that *d*-METH would produce only minimal damage in the oldest mice.

However, the interpretation of this robust GFAP response is made somewhat difficult because the oldest mice also display the highest control levels of GFAP. Consequently, an expression of the data as percentage of control indicates the *d*-METH-induced increase was much less than that observed in 12-month-old mice given the same dose. In fact, the percentage of control increase in GFAP is not much different when the oldest and youngest mice are compared. Athough astrocytic response is recognized as a dominant response to injury in the central nervous system, there has been little examination of how age may affect this response; some work, however, suggests deficient astrocytic hypertrophy when damage is extreme (e.g., extensive deafferentation) (see ref. 28 for a discussion). Although we lack an independent assessment of the degree of injury induced in mice evaluated for striatal GFAP protein level following *d*-METH exposure, our data could be considered supportive of the notion that the astrocytic response to extreme damage in very aged animals is compromised or retarded in time course relative to young animals. In contrast, other studies utilizing different deafferentation models (e.g., 6-OHDA or transection) or stab wounds suggest that aged animals are capable of mounting a more robust astrocyte response to injury, at least as assessed immunohistochemically or in terms of GFAP RNA.[28–30] Our data could also be considered supportive of this idea. Resolution of questions concerning the exact interaction between age and astrocyte response to injury, including chemically induced damage such as that seen with *d*-METH, will require direct comparisons of various measures of astrocyte reactivity in animals of different ages subjected to varying amounts of neural damage.

In summary, our work extends to neurotoxicity the idea that various aspects of substituted amphetamine action are lessened in immature animals. Whether this is related to immaturity of the nigrostriatal system or to some qualitative or quantitative differences in the response to amphetamine-induced injury in the immature animal requires further investigation.

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