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Alterations in vesicular dopamine uptake contribute to tolerance to the neurotoxic effects of methamphetamine

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Running Title – Alterations in VMAT-2 uptake contribute to METH tolerance

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ABSTRACT

Previous studies demonstrated that tolerance to the long-term neurotoxic effects of methamphetamine on dopamine neurons could be induced by pretreating with multiple injections of escalating doses of methamphetamine. The mechanism(s) underlying this tolerance phenomenon is (are) unknown. Some recent studies suggested that aberrant vesicular monoamine transporter-2 (VMAT-2) and dopamine transporter function contribute to neurotoxic effects of methamphetamine. Hence, the purpose of this study was to explore the role of the VMAT-2 and dopamine transporter in the induction of tolerance to the long-term persistent dopaminergic deficits caused by methamphetamine. A second purpose was to investigate the potential role of hyperthermia and alterations in brain methamphetamine distribution in this tolerance. Results revealed that the methamphetamine pretreatment regimen attenuated both the acute methamphetamine-induced decrease in VMAT-2 function 2 h after the methamphetamine challenge administration and its resulting persistent dopamine deficits without attenuating the acute methamphetamine-induced decreases in dopamine transporter uptake. Furthermore, pretreatment with methamphetamine prior to a high-dose methamphetamine challenge administration also attenuated the acute methamphetamine-induced redistribution of VMAT-2 immunoreactivity within the nerve terminal. This protection was not due to alterations in concentration of methamphetamine in the brain, as both the methamphetamine-pretreated and saline-pretreated rats had similar amounts of methamphetamine and amphetamine at 30 min-2 h after the last methamphetamine challenge injection. In summary, these data are the first to demonstrate an association between the prevention of acute alterations in vesicular dopamine uptake and the development of tolerance to the neurotoxic effects of methamphetamine.

In rodents, multiple high-dose administrations of methamphetamine cause long-lasting depletions of central monoamine neurotransmitters in the brain (for review, see Gibb et al., 1994). This long-term monoamine depletion is reflected by damage to dopamine nerve terminals (Lorez, 1981; Ricaurte et al., 1982), decreased tyrosine hydroxylase activity (Kogan et al., 1976; Hotchkiss and Gibb, 1980), as well as decreases in dopamine uptake sites (Wagner et al., 1980). Similar deficits have been observed in non-human primates (Seiden et al., 1976; Woolverton et al., 1989; Melega et al., 1997) and humans (Wilson et al., 1996).

Several factors contribute to the persistent dopamine deficits caused by methamphetamine. For example, studies from Bowyer et al. (1994) and Albers and Sonsalla (1995) suggest that hyperthermia contributes to the long-term neurotoxic effects of methamphetamine on striatal dopamine neurons since prevention of elevated core body temperatures attenuates this deficit. A second contributor may be alterations in the distribution of methamphetamine in the brain. Studies by Schmidt et al. (1985) and Gygi et al. (1996) suggest that pretreatment regimens can alter the distribution of methamphetamine between brain and plasma 2 h after the methamphetamine challenge administration, and possibly contribute to the protection against the neurotoxic effects of methamphetamine. Furthermore, recent studies suggested that aberrant monoamine transporter function contributes to the neurotoxic effects of methamphetamine. For example, multiple high-dose administration of methamphetamine causes a rapid and partially reversible decrease in plasmalemmal dopamine transporter (DAT) uptake activity (Fleckenstein et al., 1997), and post-treatment with DAT reuptake inhibitors such as amfonelic acid can block the neurotoxic effects of methamphetamine (Marek et al., 1990).

Similarly, multiple high-dose administrations of methamphetamine cause a rapid decrease in vesicular dopamine uptake through the vesicular monoamine transporter-2 (VMAT-2; Hogan et al., 2000; Brown et al., 2000; Brown et al., 2002). Drugs such as methlyphenidate reversed this methamphetamine-induced decrease in activity and attenuated the persistent dopamine deficits caused by methamphetamine (Sandoval et al., 2003).

In addition to factors noted above, several studies have demonstrated the dopamine deficits caused by methamphetamine can be attenuated by pretreatment with methamphetamine. For example, pretreatment with multiple low-dose injections of methamphetamine (Stephans and Yamamoto, 1996) or with multiple escalating doses of methamphetamine prior to a high-dose methamphetamine challenge administration can lead to the development of tolerance to the acute neurotoxic effects on dopamine and serotonin systems (Schmidt et al., 1985; Gygi et al., 1996), as assessed 18 h after the last challenge injection, and the long-term deficits to monoamine content, as assessed 4 and 7 days after the challenge administration (Stephans and Yamamoto, 1996; Johnson-Davis et al., 2003). Mechanisms underlying the protection afforded by methamphetamine pretreatment are unknown. Hence, the purpose of this study was to explore the ability of a tolerance-producing methamphetamine regimen to prevent acute alterations in factors linked to the long-term dopamine deficits caused by methamphetamine. Tolerance was defined as a diminished response to methamphetamine after our dosing regimen.

METHODS

Materials: (±)-methamphetamine hydrochloride was provided by the National Institutes on Drug Abuse (Rockville, MD); (±)-methamphetamine, (±)-methamphetamine-d8, (±)-amphetamine, (±)-amphetamine-d5 (Cerilliant; Austin, TX); 3,4-dihydroxyphenylethyamine hydrochloride [3H]-Dopamine (54.10 Ci/mmol) (PerkinElmer Life Sciences Inc; Boston, MA); anti-rabbit immunoglobulin antibody (Biosource International; Camarillo, CA); VMAT-2 antibody (AB1767; Chemicon International; Temecula, CA). The following reagents were purchased from SIGMA-Aldrich (St. Louis, MO): 3-hydroxytyramine (dopamine), 5-hydroxytryptamine creatinine sulfate (serotonin), sodium phosphate, citric acid, methanol, sodium octyl sulfate, sodium phosphate dibasic, ethylenediaminetetraacetic acid (EDTA), adenosine triphosphate (ATP). Reagents for the methamphetamine/amphetamine extractions were purchased from Burdick & Jackson (Muskegon, MI): formic acid, acetonitrile, ammonium hydroxide, n-butyl chloride.

Animals: Male Sprague-Dawley rats (Charles River, Canada) weighing 290-310 g at the time of experiment were housed 4 per cage in hanging wire cages in a light and temperature-controlled room. Rats had free access to food and water. The Institutional Animal Care and Use Committee of the University of Utah approved the animal care and experimental procedures. These procedures were also in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Pharmacological Procedures: Our laboratory did not use our previously published protocol for these experiments (Davis et al., 2003) because we were unable to purchase rats from

the Simonsen distributor at the time of the experiments. Due to a change in vendor, to Charles Rivers, the methamphetamine pretreatment and challenge doses were reduced and the frequency of administration was altered for the present study. The evening before the experiment, rats were weighed and then re-housed in plastic cages. The tolerance-dosing regimen consisted of saline or methamphetamine pretreatments on days 1, 3, and 5. On day 1, a total of 4 injections of either saline or 2.0 mg/kg of methamphetamine were administered (s.c.) with a 4-h interval between each dose. Rats were allowed a 40-h drug-free period between each pretreatment dosing regimen. The doses for methamphetamine were increased to 4.0 mg/kg on day 3, and 6 mg/kg on day 5. Sixty-six hours after the last pretreatment regimen on day 5, rats were either challenged with 4 injections of saline or 8 mg/kg methamphetamine, s.c., with a 4-h interval between each injection. Where indicated, core body (rectal) temperatures were assessed every h during the pretreatment and challenge dosing regimens while the rats were in the cages. Rectal temperatures were measured using a BAT-12 model thermometer and a thermocoupled rectal probe (model RET-2) (Physiotemp Instruments, Clifton, NJ USA).

Monoamine Tissue Content: Rats were decapitated and the striatum was dissected from the brains 7 days after the last methamphetamine challenge injection. Tissues were quickly frozen on dry ice, then stored at -80°C. The striatum and hippocampus was sonicated (Branson Sonifier 250) in 1 ml of tissue buffer (0.05 M sodium phosphate/ 0.03 M citric acid with 15% methanol (vol/vol), pH 2.5), then centrifuged at 18,800 x g for 15 min at 4 °C to separate the supernatant

from the protein. The supernatant was centrifuged at 18,800 x g for 10 min at 4 °C, then 20 µl was injected onto a high performance liquid chromatography system (HPLC; Dynamax AI-200 Autosampler and SD-200 pump; Varian, Walnut, CA) coupled to an electrochemical detector (Eox = +0.70 V; Varian Star 9080, Walnut, CA) to quantitate the dopamine and serotonin concentrations, as described by Chapin et al. (1986). A Whatman PartiSphere C-18 column (250 x 4.6 mm, 5-µm) was used to separate the monoamines. The mobile phase consisted of MeOH (23% vol/vol), sodium octyl sulfate (0.03% wt/vol), EDTA (0.1 mM), sodium phosphate dibasic (0.05 M), and citric acid (0.03 M). The pH of the mobile phase was 2.87, and the flow rate was 1 ml/min. Protein content was determined as described by Lowry et al. (1951).

Vesicular [^{3}H]dopamine uptake: Synaptic vesicles were prepared from rat striatum as described previously (Brown et al., 2000), 2 h after the last methamphetamine challenge injection. The 2 h time-point was selected to be consistent with the 2-h time-point evaluated for concentrations of methamphetamine in the brain. Vesicular [^{3}H]dopamine uptake was determined as described by Teng et al. (1997) with the following modifications: (1) synaptic vesicles were isolated in two ultracentrifugation steps (20,000 x g, 20 min and 100,000 x g, 45 min); (2) vesicles were incubated at 30 °C for 3 min in the presence of [3H]dopamine (final concentration, 30 nM); and (3) non-specific uptake was determined by incubating synaptic vesicles at 4 °C in the absence of adenosine triphosphate (ATP). Protein concentrations were determined using the Bradford protein assay (Bradford, 1976).

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VMAT-2 immunoreactivity: Striatal synpatosomes were prepared as described previously (Fleckenstein et al., 1997). Fresh striatal tissue was homogenized in ice-cold 0.32 M sucrose and centrifuged (800 x g,12 min; 4°C). The resulting supernatant (S1) was then centrifuged (22,000 x g, 15 min; 4°C) and the pellet (P2; whole synaptosomal fraction) was resuspended in cold distilled deionized water at a concentration of 50 mg/ml (original wet weight of tissue) and a portion was saved for western blot analysis. The remainder of the synaptosomal samples were centrifuged (22,000 x g, 20 min; 4°C) to pellet the membrane-associated fraction (P3), which was resuspended at 50 mg/ml (original wet weight of tissue) and saved for Western blot analysis. Prior to resuspension of P3, the resulting supernatant (S3; non-membrane associated fraction) was removed and saved for Western blot analysis.

Western Blot Analysis. Western blot analysis was performed as described previously by (Riddle et al., 2002). The binding of VMAT-2 antibody was performed using 50 µg of protein from the whole synaptosomal (P2), membrane-associated (P3), and non-membrane-associated fractions (S3). The bound primary VMAT-2 antibody (1:1000 dilution) was visualized with anti-rabbit immunoglobulin(Ig) antibody (1:2000 dilution). The antigen-antibody complexes were visualized by chemiluminescence. Bands on blots were quantified by densitometry measuring net intensity using Kodak 1D image analysis software (Eastman Kodak, Rochester, NY). Western blots were run on a fifteen well gel. Therefore, in order to run a blot with an n = 6 per group, only 2 groups were compared on each blot. The same samples from each group were used to run the four western blots needed to make all of the two group comparisons. Each blot was

developed on a separate film and the values from the western blot data were measured by densitometric analysis.

[^{3}H]Dopamine Uptake via Plasmalemmal Transporters: Uptake of [^{3}H]dopamine was determined in striatal synaptosomes prepared according to the method described by Fleckenstein el al. (1997). One h after the last methamphetamine challenge injection, rats were decaptitated and striatal tissues were dissected and homogenized in cold 0.32 M sucrose and centrifuged (800 g for 12 min; 4°C). The supernatant (S1) was then centrifuged (22,000 g for 15 min; 4°C) and the resulting pellet (P2) was resuspended in ice-cold modified Krebs' buffer (126nM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 16 mM sodium phosphate, 1.4 mM $MgSO_4$, 11 mM dextrose, 1mM ascorbic acid, pH 7.4) Assays were conducted in Krebs' buffer. Each assay tube contained synaptosomal tissue (the resuspended P2 from 1.5 mg of original wet weight striatal tissue) and 1 µM pargyline. Nonspecific values were determined in the presence of 10 µM cocaine. Assay tubes were preincubated for 10 min at 37°C then [^{3}H]dopamine (0.5 nM final concentration) was added to initiate the reaction. Assay tubes were incubated for 3 min at 37°C, then samples were filtered through Whatman GF/B filters (Brandel Inc., Gaitherburg, MD) soaked previously in 0.05% polyethylenimine. Filters were washed rapidly three times with 3 ml of ice-cold 0.32 M sucrose by using a Brandel filtering manifold. The radioactivity trapped in filters was counted using a liquid scintillation counter. Remaining resuspended P2 samples were assayed for protein concentrations according to the method of Lowry et al. (1951).

Methamphetamine and amphetamine concentrations: The striatum was dissected from the brain of the saline/methamphetamine and methamphetamine/methamphetamine groups, 66 hr after the last 6 mg/kg pretreatment injection (t = 0, corresponding to the time for the first injection of the methamphetamine challenge administration), then 30 min, 1 hr, and 2hr after the last methamphetamine challenge injection (8 mg/kg) and quickly frozen on dry ice, then stored at -80°C. On the day of extraction, brain tissues were thawed on ice. Brain samples were weighed and sonicated in 1 ml of doubled deionized water (ddH_2O) using a Branson Sonifier 250. When samples were equilibrated to room temperature, 500 µl of each sample were transferred into 16 x 100-mm silanized glass test tubes with screw caps. Two hundred and fifty nanograms of deuterated methamphetamine and deuterated amphetamine were added as an internal standard to 500 µl of homogenate brain samples. Samples were vortexed for 5 s then the pH was made alkaline (pH > 12) with 100 µl of concentrated ammonium hydroxide. Five hundred microliter aliquots of brain homogenates were extracted with 5 ml of n-butyl chloride: acetonitrile (4:1 vol/vol) at room temperature for 30 min with gentle rocking. Samples were centrifuged for 20 min at 1,200g then the organic layer containing methamphetamine and amphetamine were transferred to silanized glass test tubes (13 x 100-mm) with screw caps. Samples were evaporated half-way to dryness at 20 °C, then 50 µl of 1% HCl in methanol was added to stabilize the analytes before samples were completely dried down. When samples were completely evaporated to dryness, samples were reconstituted in 100 µl of 95% formic acid/5% acetonitrile solution and transferred into vials prior to analysis by liquid chromatography/mass spectrometry/mass spectrometry. Methamphetamine and amphetamine concentrations in brain were determined with a ThermoFinnigan TSQ7000 tandem mass spectrometer operating in

atmospheric pressure chemical ionization mode. Chromatographic separation was achieved on a Metasil Basic 3µ, 100 x 3.0 mm column (MetaChem Technologies Inc, Torrance, CA) with a mobile phase consisting of 95% formic acid (0.1%) and 5% acetonitrile. Selected reaction monitoring (SRM) of ions was performed and transitions monitored as follows: *m/z* 150.1 → *m/z* 91 (methamphetamine), *m/z* 158 → *m/z* 92 (methamphetamine-d8), *m/z* 136 → *m/z* 91 (amphetamine) and *m/z* 141.1 → *m/z* 92 (amphetamine-d5). Accuracy was within 20% of the fortified non-deuterated methamphetamine and amphetamine target samples from brain homogenate quality control samples. The limit of quantitation for this assay was 5 ng/ml.

Statistical Analysis: Dopamine content, VMAT-2 uptake, and DAT uptake were analyzed with one-way ANOVA with followed by least significant difference (LSD) post hoc comparision (SPSS version 11.5). Methamphetamine and amphetamine concentrations, and VMAT-2 immunoreactivity were analyzed by using Student's *t*-test. Analysis of data for core rectal temperatures was first attempted using ANOVA with repeated measures, however the underlying statistical assumptions for this test (compound symmetry and homogeneity of variance) were not met. Therefore, a one-way ANOVA followed by post-hoc testing with Student's *t*-test was performed at each individual time-point *when the assumption of homogeneity of variance was met*. The fixed factor was "treatment"; the.dependent variable was temperature**.** When the assumption of homogeneity of variance was not met for a particular time point, data was analyzed using Kruskal Wallis Analysis of Variance by Ranks, followed by post-hoc testing with

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Mann Whitney U. Statistical significance for all comparisons was set at $p \leq 0.05$. Data represent means $\pm$ standard error of the mean (S.E.M.).

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Results

Results presented in figure 1 demonstrate that a multiple high-dose challenge administration of methamphetamine (4 x 8 mg/kg/injection, 4-h interval, s.c.) significantly decreased striatal dopamine tissue content 69%, when compared to the value for the saline control group, seven days after treatment ($p \leq 0.05$). Pretreatment with multiple injections of escalating doses of methamphetamine prior to the high-dose challenge attenuated the methamphetamine-induced depletion of striatal dopamine 36%, when compared to the value for the saline control; ($p < 0.05$). Dopamine content in the saline/methamphetamine group was decreased 51%, when compared to the value for the methamphetamine/methamphetamine group ($p < 0.05$). The pretreatment regimen (methamphetamine/saline group), by itself, decreased dopamine concentrations 27%, when compared to the value for the saline control ($p < 0.05$).

On each day of the pretreatment regimen, rats that received methamphetamine had greater core body temperatures than those that received saline. Figure 2 demonstrates that likewise, on the day of the challenge, the high-dose methamphetamine administration elevated core body temperatures in both the saline/methamphetamine and methamphetamine/methamphetamine groups, which differed from the saline/saline-treated rats at each time-point ($p \leq 0.05$). Importantly there was a general trend that the methamphetamine/methamphetamine group had lower body temperatures than the saline/methamphetamine group throughout the experiment, but it was only significant at 3 time-points.

To determine whether alterations in brain methamphetamine concentrations contributed to the development of tolerance, striatal methamphetamine and amphetamine concentrations were measured. Neither methamphetamine nor amphetamine were detected 66 h after the last methamphetamine pretreatment injection (i.e., t = 0 in Fig. 3 corresponding to the time immediately prior to the first methamphetamine challenge injection). Results presented in Fig. 3 demonstrate that methamphetamine and amphetamine levels were elevated similarly at 0.5, 1, and 2 h after the methamphetamine challenge injection, with a statistical significant difference (i.e. greater methamphetamine levels in the methamphetamine/methamphetamine vs. saline/methamphetamine) only observed at 0.5 h.

Since the pretreatment regimen altered methamphetamine-induced hyperthermia during the challenge administration (Fig 2) it was important to determine whether this decrease in core body temperatures afforded neuroprotection by attenuating the acute methamphetamine-induced decrease in VMAT-2 function. Hence, vesicular dopamine uptake was measured in rats that were treated with methamphetamine at room temperature and or in a 6°C environment to allow or prevent methamphetamine-induced hyperthermia, respectively. Results presented in Fig. 4A demonstrate that multiple injections of methamphetamine (4 x 8 mg/kg; 2-h interval; s.c.) elevated core body temperatures and that methamphetamine-induced hyperthermia was blocked in rats that were treated with methamphetamine at 6°C. Fig 4B shows that at room temperature (24°C), methamphetamine decreased vesicular dopamine uptake 72%, when compared to the value for the saline-treated rats (24°C) ($p \leq 0.05$). The prevention of methamphetamine-induced hyperthermia did not attenuate this decrease in vesicular dopamine uptake. At 6°C, methamphetamine decreased vesicular dopamine uptake 75%, when compared to the value for

the saline treated rats ($p \leq 0.05$). Vesicular dopamine uptake was not altered in rats that were treated with saline at 6°C.

Fig. 5 demonstrates that the high-dose methamphetamine administration decreased plasmalemmal DAT uptake 72%, when compared to the value for the saline/saline group ($p \leq 0.05$), 1 h after the last methamphetamine injection on the day of the challenge administration. In rats that were pretreated with methamphetamine prior to the high-dose challenge administration, dopamine transporter uptake was decreased 58%, when compared to the value for the saline/saline group ($p \leq 0.05$). Dopamine transporter uptake was not statistically different between the methamphetamine/methamphetamine and the saline/methamphetamine groups. The pretreatment regimen per se did not significantly alter DAT uptake when compared with the saline/saline control.

Fig. 6 demonstrates that the high-dose methamphetamine administration decreased vesicular dopamine uptake 57%, when compared to the value for the saline/saline control ($p \leq 0.05$), 2 h after the last methamphetamine injection on the day of the challenge administration. In rats that were pretreated with methamphetamine prior to the high-dose challenge administration, the decrease in vesicular dopamine uptake was attenuated, when compared to the value for the saline/methamphetamine group ($p < 0.05$). The pretreatment regimen alone did not significantly alter vesicular dopamine uptake when compared to the saline/saline control. Vesicular dopamine uptake was significantly different between the saline/methamphetamine and methamphetamine/methamphetamine groups. Vesicular uptake in the saline/methamphetamine group was decreased 39%, when compared to the value for the methamphetamine/methamphetamine group ($p \leq 0.05$).

To determine whether the attenuation of the methamphetamine-induced decrease in VMAT-2 function was due to alterations in VMAT-2 protein levels, western blot analysis was performed on the three tissue fractions: non-membrane-associated (S3; vesicular enriched fraction), membrane-associated fraction (P3; membrane-bound vesicles), and whole synaptosomal fraction (P2; non-membrane-associated plus the membrane-associated fractions), 2-h after the last injection of the methamphetamine challenge administration. In the non-membrane-associate fraction (S3), results shown in Fig 7 panel A illustrates that the pretreatment regimen alone (methamphetamine/saline group) does not alter VMAT-2 immunoreactivity when compared to the saline/saline control ($p > 0.05$). Panel B demonstrates that the challenge methamphetamine administration alone decreased VMAT-2 immunoreactivity, when compared to saline/saline control. In panel C, there was no difference in VMAT-2 immunoreactivity between the methamphetamine/saline and methamphetamine/methamphetamine groups. In panel D, there was a significant difference in VMAT-2 immunoreactivity between the saline/methamphetamine and methamphetamine/methamphetamine groups ($p \leq 0.05$).

Results from the whole synaptosomal fraction (P2 fraction) resembled the data shown in the non-membrane-associate fraction (S3). Only the saline/methamphetamine group was significantly less than the other groups ($p \leq 0.05$), however none of the remaining groups differed from one another (data not shown). Furthermore, there was no difference in VMAT-2 immunoreactivity among the P3 fractions (data not shown).

Discussion

The purpose of this study was to explore the roles of the VMAT-2 and DAT function, in addition to investigating the roles of hyperthermia and alterations in brain methamphetamine concentration, in the induction of tolerance to the long-term persistent dopaminergic deficits caused by methamphetamine. Studies have shown that pretreatment with multiple low-dose injections of methamphetamine or with multiple escalating doses of methamphetamine prior to a high-dose methamphetamine challenge administration can lead to the development of tolerance to the acute neurotoxic effects on dopamine and serotonin systems (Schmidt et al., 1985; Gygi et al., 1996; Stephans and Yamamoto, 1996; Johnson-Davis et al., in press). Although using a different dosing paradigm, our data confirms previous findings by illustrating that methamphetamine pretreatment can attenuate the long-term depletion of striatal dopamine content, 7 days after the challenge administration.

Several laboratories have shown that hyperthermia plays an important role in mediating the long-term neurotoxic effects of methamphetamine on striatal dopamine neurons (Bowyer et al., 1994; Albers and Sonsalla, 1995). For example, lowering the environmental temperature to 4°C while administering a neurotoxic regimen of methamphetamine, as well as administering pharmacological drugs that produce hypothermia in combination with a neurotoxic regimen of methamphetamine, attenuates the methamphetamine-induced depletion of striatal dopamine (Bowyer et al., 1992; Ali et al., 1996). Collectively, these experiments demonstrate a link between hyperthermia and methamphetamine-induced neurotoxicity, suggesting that the attenuation of methamphetamine-induced hyperthermia could play a role in tolerance to the neurotoxic effects of the stimulant. Thus, it was important to determine whether the

development of tolerance to the neurotoxic effects of methamphetamine was due to the attenuation of methamphetamine-induced hyperthermia during the challenge administration. Results from this present experiment demonstrate that methamphetamine pretreatment can attenuate methamphetamine-induced hyperthermia at a few time points during the challenge administration, when using this 4-h tolerance-dosing regimen. This suggests that hyperthermia plays a small role in the tolerance phenomenon. However, other factors clearly contribute to the protection from the neurotoxic effects of methamphetamine as well. Findings that other mechanisms contribute to the tolerance are consistent with our previous study which we reported that, when dosing with a 2-h interval, both the pretreated and non-treated rats had similar core body temperatures on the day of the challenge administration (Johnson-Davis et al., 2003).

Since the pretreatment regimen altered methamphetamine-induced hyperthermia during the challenge administration, it was important to determine whether this decrease in core body temperatures afforded neuroprotection by attenuating the acute methamphetamine-induced decrease in VMAT-2 function. This was particularly important given the report by Ugarte et al. that hyperthermia contributed to the decrease in VMAT-2 function in mice caused by methamphetamine-induced toxicity (Ugarte et al., 2003). Hence, vesicular dopamine uptake was measured in rats that were treated with methamphetamine at room temperature and/or in a 6°C environment to allow or prevent methamphetamine-induced hyperthermia, respectively. Our results are consistent with previous findings from Brown et al. (2002) that the decrease in VMAT-2 function, after a single methamphetamine injection to rats, is not temperature

dependent. Despite the blockade of methamphetamine-induced hyperthermia, exposure to methamphetamine decreased VMAT-2 uptake similarly at both 24 and 6°C. Therefore, these data suggest that the attenuation in methamphetamine-induced hyperthermia, during the methamphetamine challenge administration, did not afford any protection against the methamphetamine-induced decrease in VMAT-2 function.

In addition to the role of hyperthermia, some studies suggest that alterations in methamphetamine redistribution could contribute to the tolerance of the neurotoxic effects of methamphetamine. Schmidt et al. (1985) and Gygi et al. (1996) reported, using different dosing paradigms, that methamphetamine pretreatment altered the distribution of methamphetamine between the brain and plasma following a challenge methamphetamine administration. Specifically, methamphetamine-pretreated rats had half the amount of methamphetamine in the brain and twice the amount of methamphetamine in plasma, when compared to non-pretreated rats. These studies suggested that this reduction in brain methamphetamine concentration could lead to a decrease in the amount of neurotoxicity to dopamine neurons, and therefore, induce tolerance. In contrast to these findings, the present data indicate that methamphetamine pretreated rats have similar methamphetamine concentrations in the brain than the non-pretreated rats with our current dosing paradigm. Methamphetamine concentrations in the brains of the pretreated rats were significantly higher at the 30 min time-point, but were similar to the non-pretreated rats at the 1 h and 2 h time-points. Thus, using our tolerance-inducing dosing regimen, this phenomenon cannot be attributed to alterations in brain methamphetamine concentrations between the pretreated and non-pretreated rats.

Lastly, recent studies suggest that aberrant monoamine transporter function contributes to the neurotoxic effects of methamphetamine. For example, a study by Fumagalli et al. (1999) showed that impairment of the function of VMAT-2 (i.e. as demonstrated in VMAT-2 knockout mice) potentiates methamphetamine-induced neurotoxicity to dopaminergic neurons, in vivo. Likewise, Fumagalli et al. (1998) illustrated that DAT (-/-) knock-out mice were protected against neurotoxicity to dopamine neurons when exposed to methamphetamine. Clearly the role of monoamine transporter function is complex. The current theory for methamphetamine-induced neurotoxicity suggests that the decrease in VMAT-2 function, by methamphetamine, results in the disruption of vesicular storage of dopamine. This disruption can lead to the accumulation of interneuronal dopamine, which oxidizes into reactive oxygen species (Graham et al., 1978; Hastings et al., 1995), and damage the nerve terminals of dopamine neurons (Cubells et al., 1994; Liu and Edwards, 1997). Hence, for this study, it was important to explore the roles of the VMAT-2 and DAT in the induction of tolerance to the long-term persistent dopaminergic deficits caused by methamphetamine.

Results from this experiment demonstrates that acutely (i.e. 1 h after the last methamphetamine challenge injection) methamphetamine impaired DAT uptake similarly in both the pretreated and non-pretreated rats. This suggests that tolerance was not caused by an acute prevention of the methamphetamine-induced decrease in plasmalemmal dopamine uptake. In contrast, 2 h after the last methamphetamine challenge injection, there was an attenuation in the methamphetamine-induced decrease in VMAT-2 function in the methamphetamine/methamphetamine rats. The decrease in vesicular uptake was not due to residual methamphetamine because isolation of the vesicles removes any residual methamphetamine from the preparation to levels of <1 nM (Fleckenstein et al., 1997), a

concentration that is below the necessary concentration to alter dopamine uptake in our preparations. Concurrent with this decrease in vesicular dopamine uptake, western blot analysis demonstrated that exposure to methamphetamine caused a significant decrease in VMAT-2 immunoreactivity in the non-membrane-associated fraction (S3). This finding is consistent with data from Riddle et al. (2002) which showed that exposure to a high-dose methamphetamine administration redistributes VMAT-2 protein from the nerve terminal and could possibly explain the mechanism underlying the methamphetamine-induced decrease in VMAT-2 uptake as shown by Hogan et al. (2000), Brown et al. (2000), Brown et al. (2002). In addition, data from our experiment showed the rats that were pretreated with methamphetamine prior to the challenge administration had greater VMAT-2 immunoreactivity in the non-membrane-associated fraction (S3), when compared to the saline/methamphetamine rats. This finding could explain the attenuation in the methamphetamine-induced decrease in VMAT-2 function, and suggest that the pretreatment regimen attenuates the ability of the high-dose methamphetamine challenge administration to traffick vesicles out of the nerve terminal, therefore providing neuroprotection to striatal dopamine function.

One possible mechanism for the apparent redistribution of VMAT-2, and presumably associated synaptic vesicles, is that the methamphetamine pretreatment regimen causes dopamine to preferentially act on the D_2 dopamine autoreceptor. Since previous data suggests that D2 agonists are more potent at D_2 autoreceptors than postsynaptic D_2 receptors (Drukarch and Stoof, 1990). The methamphetamine pretreatment regimen could possibly alter the function of proteins involved in synaptic vesicle trafficking. For example, D_2 receptors are coupled to the inhibitory G-protein to decrease adenylyl cyclase activity and thus decrease cAMP formation (Stoof and Kebabian, 1981; Vallar and Meldolesi, 1989). Therefore, activation of the D_2

autoreceptor would lead to the decrease in PKA activity and could possibly prevent the phosphorylation of synapsin. Synapsins are proteins that are associated with synaptic vesicle membranes in the nerve terminal (DeCamilli et al., 1983a; DeCamilli et al., 1983b; Huttner et al., 1983) and functions to cluster synaptic vesicles in the cytoplasm (Bahler and Greengard, 1987) and regulate neurotransmitter release from the nerve terminal, when phosphorylated (Llinas et al., 1985; Bahler and Greengard, 1987). Consequently, activation of D2 receptors and associated decrease in PKA activity, during the methamphetamine pretreatment regimen, could prevent phosphorylation of synapsin and potentially inhibit synaptic vesicles from trafficking, thus allowing more vesicles to cluster in the non-membrane-associated fraction. This mechanism may explain why the methamphetamine pretreated rats had more VMAT-2 immunoreactivity in the non-membrane associated fraction (S3) than the non-pretreated rats, and provided neuroprotection against the high-dose methamphetamine challenge administration by allowing more VMAT-2 to sequester dopamine. Of course, results from our present study do not preclude the involvement of other possible mechanisms through which tolerance could occur, such as a potential up-regulation of anti-oxidant activity.

In summary, pretreatment with methamphetamine prior to a high-dose methamphetamine challenge administration attenuated the acute methamphetamine-induced decrease in VMAT-2 uptake and redistribution of VMAT-2 immunoreactivity within the nerve terminal. This attenuation in VMAT-2 uptake was not due to blocking methamphetamine-induced hyperthermia nor was the protection due to alterations in the concentration of methamphetamine in the brain, as both the methamphetamine-pretreated and saline-pretreated rats had similar amounts of methamphetamine and amphetamine at 30 min- 2h after the last methamphetamine challenge injection. These data are the first to demonstrate an association between the prevention of acute

alterations in vesicular dopamine uptake and the development of tolerance to the neurotoxic effects of methamphetamine.

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Footnotes

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Figure Legends

Fig. 1. Quantitative analysis of dopamine concentration in striatum 7 days following the tolerance-dosing regimen with methamphetamine (METH). Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of methamphetamine (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Data are expressed as mean values $\pm$ S.E.M. *Value significantly different from SALINE/SALINE control ($p \leq 0.05$). +Value significantly different from SALINE/METH group ($p \leq 0.05$). Values in parenthesis represent the number of rats per group.

Fig. 2. The effect of the tolerance-dosing regimen with METH on body temperature. Core body temperatures were recorded every 1 h after each methamphetamine or saline injection. Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of METH (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Arrows indicate the time of METH injection. Data are expressed as mean values $\pm$ S.E.M. *SAL/METH and METH/METH groups have values that were significantly different from SALINE/SALINE and METH/SALINE groups ($p \leq 0.05$). +METH/METH group

is significantly different from the SAL/METH group ($p \leq 0.05$). Values in parenthesis represent the number of rats per group.

Fig. 3. METH and amphetamine (AMP) concentrations in striatum. METH and AMP concentrations were measured 66 h after the last 6.0 mg/kg injection (t = 0), then 0.5, 1, and 2 h after the last 8.0 mg/kg injection on the day of the challenge administration. Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of METH (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Data are expressed as mean values $\pm$ S.E.M. *METH/METH is significantly different from SALINE/METH ($p \leq 0.05$). Number of rats per group; n = 7.

Fig. 4A. The effect of environmental temperature on METH-induced hyperthermia. Core body temperatures were recorded every 1 h after each METH or saline injection. Sprague-Dawley rats from the Charles River-Canada laboratory received a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 2-h intervals) or saline vehicle at either 24 or 6°C. Arrows indicate the time of injection. Data are expressed as mean values $\pm$ S.E.M. *Values are significantly different from saline-treated controls ($p \leq 0.05$). +METH 24°C group is significantly different from the METH 6°C group. Number of rats per group, n = 6.

Fig. 4B. Vesicular DA uptake at 24 and 6°C. Sprague-Dawley rats from the Charles River-Canada laboratory received a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 2-h intervals) or saline vehicle at either 24 or 6°C. Vesicular DA uptake was assessed 1 h after the last injection. Data are expressed as mean values $\pm$ S.E.M. *Values are significantly different from saline-treated controls ($p \leq 0.05$). Number of rats per group, n = 6.

Fig. 5. Plasmalemmal dopamine transporter uptake. Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of METH (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Dopamine transporter uptake was assessed 1 h after the last challenge injection. Data are expressed as mean values $\pm$ S.E.M. *Values are significantly different from SALINE/SALINE controls ($p \leq 0.05$). Number of rats per group, n = 6.

Fig. 6. Vesicular Dopamine uptake. Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of METH (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Vesicular uptake was assessed 2 h

after the last challenge injection. Data are expressed as mean values ± S.E.M. *Values are significantly different from SALINE/SALINE controls ($p \leq 0.05$). +Values are significantly different from SALINE/METH group ($p \leq 0.05$). Number of rats per group, n = 6.

Fig. 7. VMAT-2 immunoreactivity in the non-membrane-associated fraction (S3). Sprague-Dawley rats from the Charles River-Canada laboratory were pretreated with 4 injections (s.c.) of escalating doses of METH (ranging from 2.0 – 6.0 mg/kg/injection; 4-h intervals between each injection) or saline vehicle (1 ml/kg/injection), with 40-h drug-free periods between each dosing regimen, and then challenged with a multiple high-dose METH regimen (4 injections, 8.0 mg/kg/injection, s.c.; 4-h intervals) or saline vehicle, 66 h after the last pretreatment regimen. Vesicular immunoreactivity was assessed 2h after the last challenge injection. Data represent the mean optical density and vertical lines represent 1 S.E.M. *Values are significantly different ($p \leq 0.05$). Number of rats per group, n = 6.

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Figure 1

A

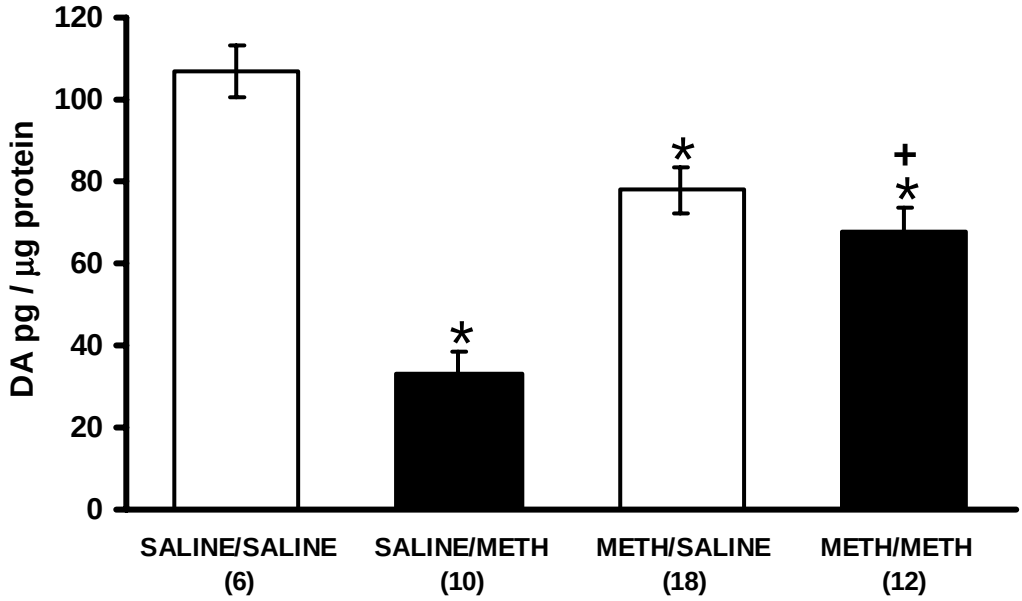

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Figure 2

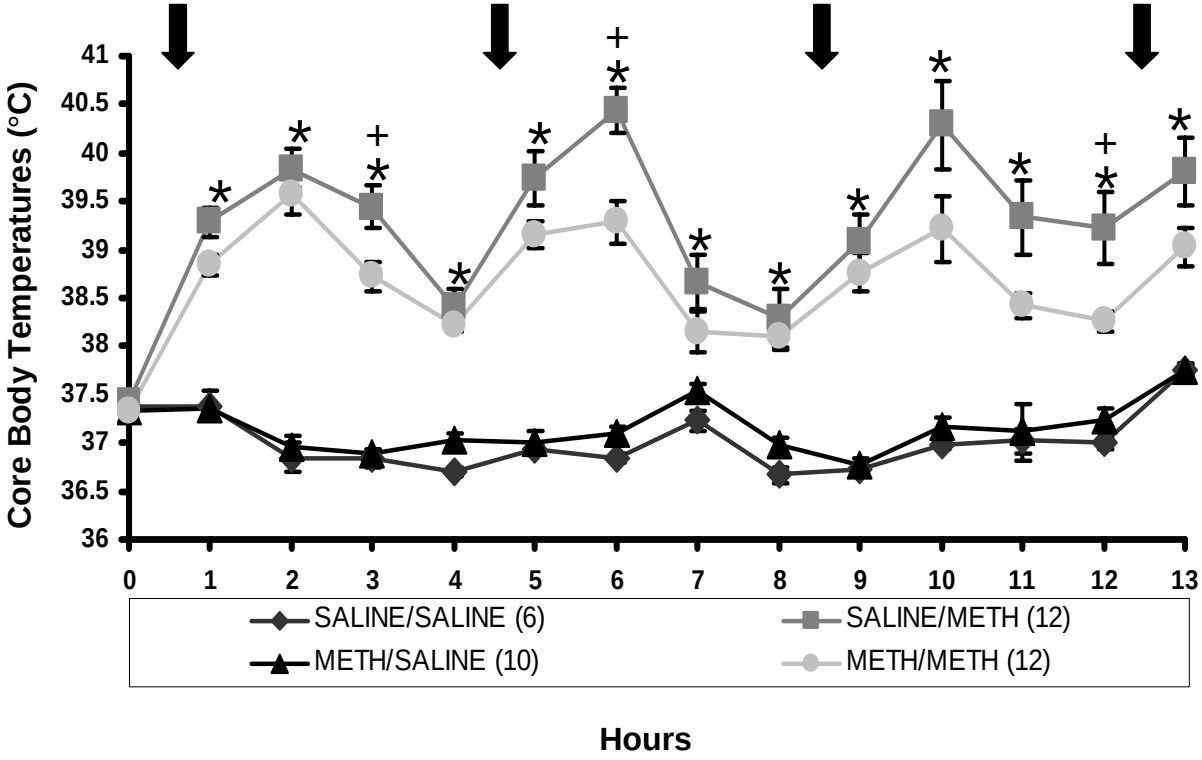

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Figure 3

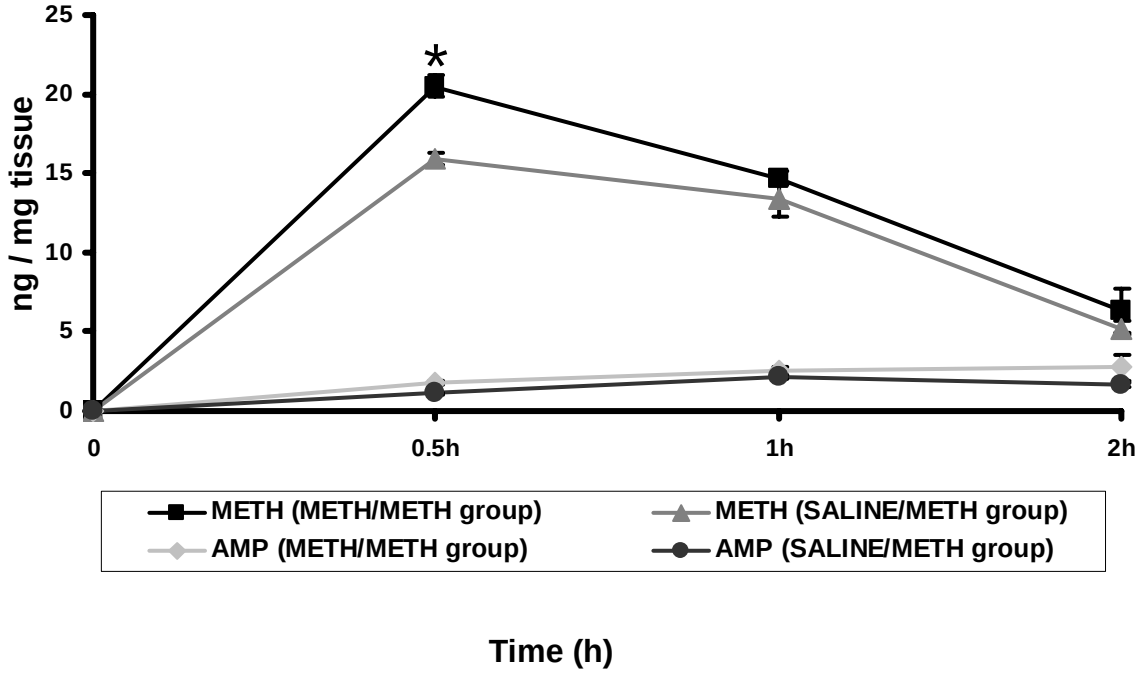

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Figure 4

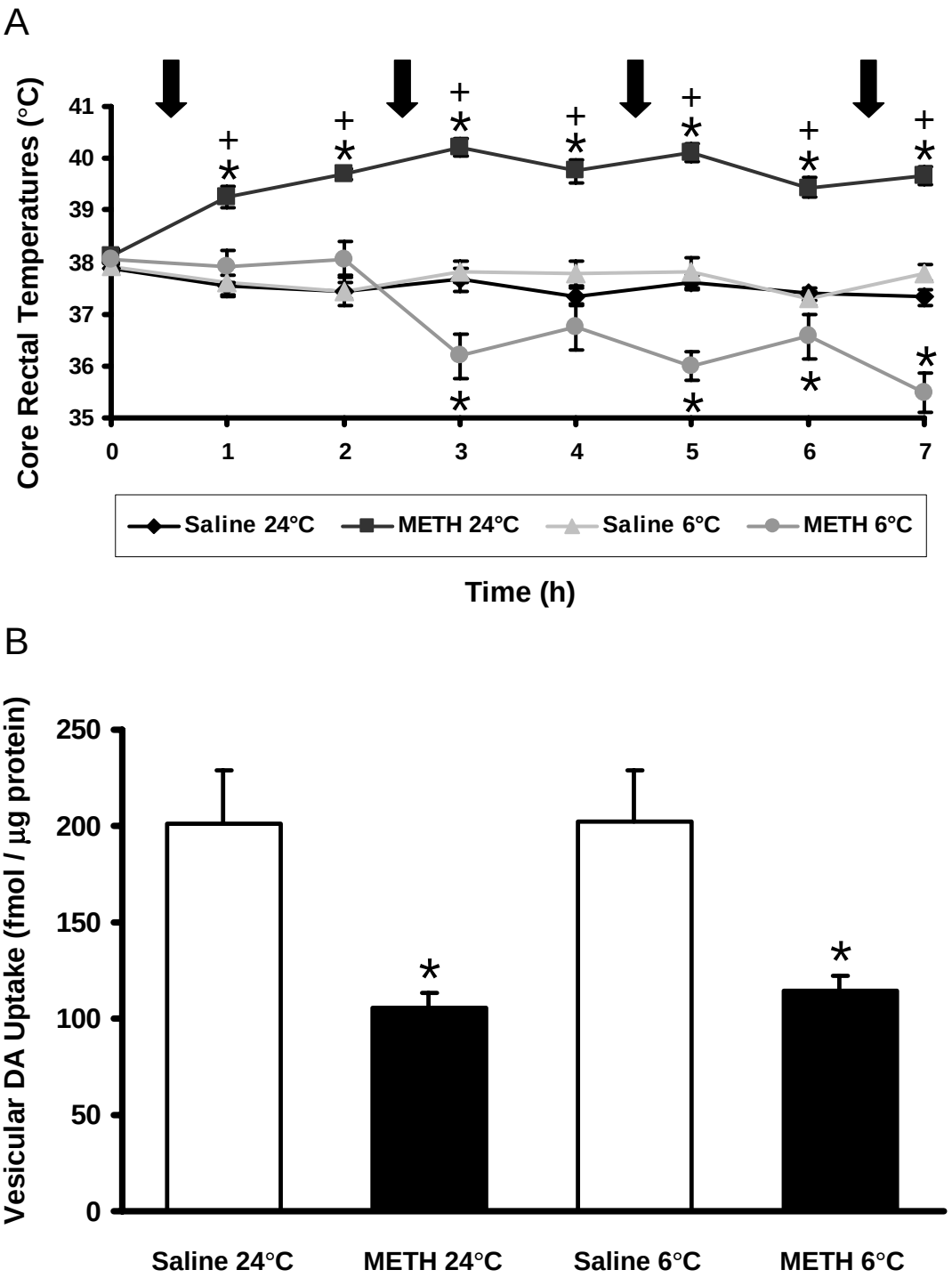

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Figure 5

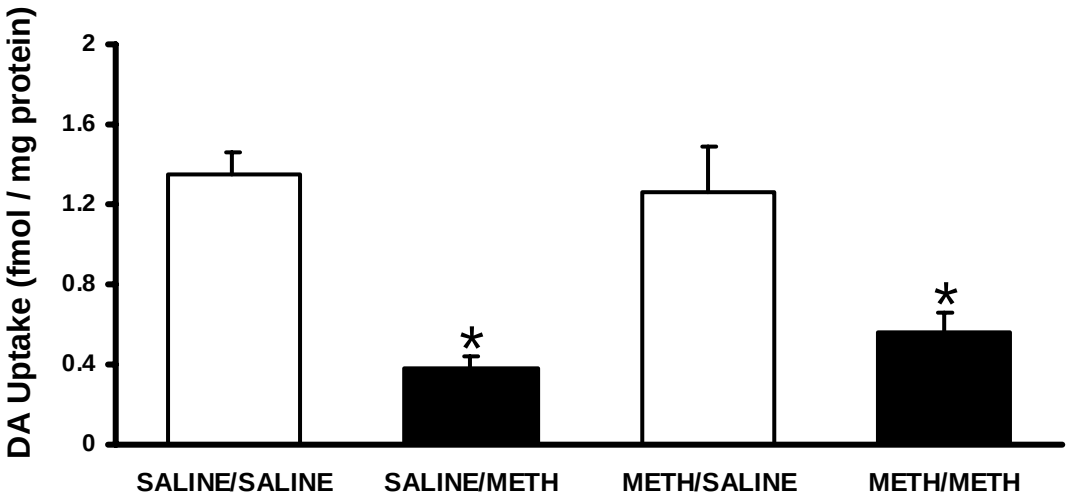

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Figure 6

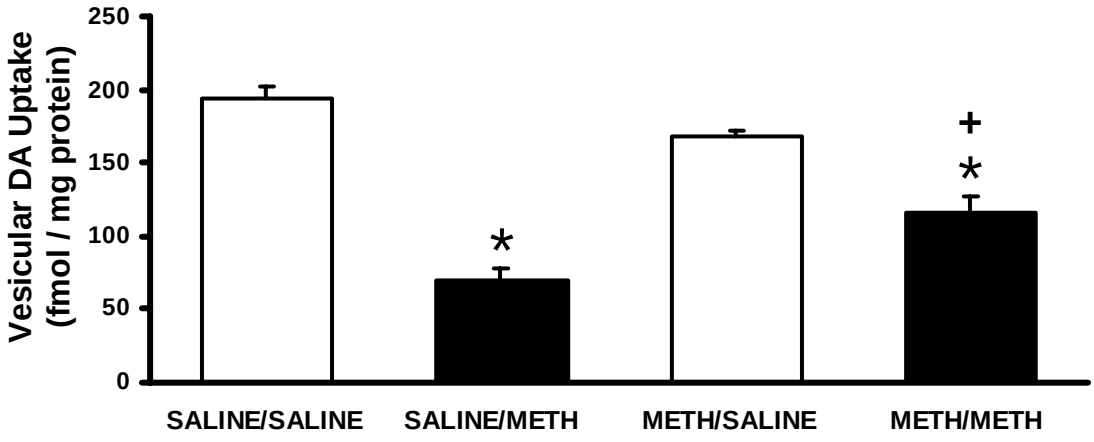

Figure 7