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Title Page

Relationship between temperature, dopaminergic neurotoxicity and plasma drug concentrations in methamphetamine-treated squirrel monkeys

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Running Title Page

Running title: Temperature, neurotoxicity and drug concentrations.

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Abbreviations: AMPH - Amphetamine
METH - Methamphetamine
DA - Dopamine
DOPAC - Dihydroxyphenylacetic acid
5-HT - Serotonin
5-HIAA - 5-Hydroxyindoleacetic acid
WIN35,428 - 2-beta-carbomethoxy-3beta-(4-fluorophenyl)tropane (beta-CFT).

Abstract

To examine the relationship between temperature (ambient and core), dopaminergic neurotoxicity and plasma drug (methamphetamine, METH) and metabolite (amphetamine, AMPH) concentrations, two separate groups of squirrel monkeys (n=4-5 per group) were treated with METH (1.25 mg/kg, given twice, 4 hr apart) or vehicle (same schedule) at two different ambient temperatures (26 °C and 33 °C). Core temperatures and plasma drug concentrations were measured during the period of drug exposure; striatal monoaminergic neuronal markers in the same monkeys were determined one week later. At the temperature range examined, the higher ambient temperature did not significantly enhance METH-induced hyperthermia or METH- induced dopaminergic neurotoxicity, although there were trends toward increases. Acute METH-induced increases in core temperature correlated highly and directly with subsequent decreases in striatal dopaminergic markers. Squirrel monkeys with the greatest increases in core temperature (and largest dopaminergic deficits) had the highest plasma drug metabolite (AMPH) concentrations. There was substantial inter-animal variability, both with regard to elevations in core temperature and plasma drug concentrations. Pharmacokinetic studies in six additional squirrel monkeys revealed comparable individual differences in METH metabolism. These results, which provide the first available data on the within-subject relationship between temperature (ambient and core), plasma concentrations of METH (and AMPH) and subsequent dopaminergic neurotoxic changes, suggest that, as in rodents, core

temperature can influence METH neurotoxicity in primates. In addition, they suggest that inter-animal differences presently observed in thermal and neurotoxic responses to METH may be related to individual differences in drug metabolism.

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Introduction

Methamphetamine (N-methyl-β-phenylisopropylamine, METH) is an amphetamine (AMPH) analog with high potential for abuse (Kalant, 1966; Miller and Hughes, 1994). Indeed, county law enforcement agencies in the US recently identified METH abuse as their primary drug problem (Kyle and Hansell , 2005). In addition to problems related to its abuse *per se*, METH has neurotoxic potential toward brain dopamine (DA) and serotonin (5-HT) neurons. In particular, animals given repeated doses of METH develop long-lasting depletions of DA and 5-HT, their major metabolites, their membrane transporters, their rate-limiting biosynthetic enzymes, and their vesicular transporters (see Gibb et al., 1994; Lew et al., 1997; Cho and Melega, 2002; McCann and Ricaurte, 2004). Anatomic studies indicate that reductions in presynaptic DA and 5-HT axonal markers are related to destruction of DA and 5-HT axon terminals (Ricaurte et al., 1982; Ricaurte et al., 1984a; 1984b; Sonsalla et al., 1996, Fukumura et al., 1998).

To date, neurotoxic effects of METH have been documented in mice, rats, guinea pigs, cats, vervet monkeys, rhesus monkeys and baboons (see Seiden and Ricaurte, 1987; Lew et al., 1997; Melega et al., 1997; Villemagne et al., 1998). Evidence of possible METH-induced DA neurotoxicity is also available in humans (McCann et al., 1998; Volkow et al., 2001; Sekine et al., 2001). The profile of METH-induced neurotoxic changes varies according to species. In

mice, neurotoxic effects of METH primarily involve DA neurons, with 5-HT neurons generally showing smaller or no lasting effects (Seiden and Ricaurte, 1987; O'Callaghan and Miller, 1994; Callahan et al., 1998; Fumagalli et al., 1998; Gluck et al., 2001). In rats, METH-induced neurotoxicity typically involves both DA and 5-HT neurons, with 5-HT neurons typically more severely affected (Ricaurte et al., 1980; Hotchkiss and Gibb, 1980). In non-human primates, as in mice, DA neurons tend to be more affected than 5-HT neurons (Villemagne et al., 1998). The basis for these species differences in neurotoxicity profile is unknown.

The precise mechanism by which METH produces a distal axotomy of brain DA neurons remains to be elucidated. However, studies over the last decade have demonstrated that temperature (ambient and core) can markedly influence METH-induced DA neurotoxicity in rodents (mice and rats). Specifically, higher temperatures enhance neurotoxicity, whereas lower temperatures typically afford neuroprotection (Bowyer et al., 1992; 1994; Albers and Sonsalla, 1995; Ali et al., 1994; O'Callaghan and Miller, 1994; Farfel and Seiden, 1995; Callahan et al., 1998; Miller and O'Callaghan, 1994; 2003). In contrast to the sizeable literature in rodents, only one study has evaluated the influence of temperature on METH-induced neurotoxicity in non- human primates (Melega et al., 1998). Based on the observation that hypothermia (secondary to MK801) did not afford protection against METH-induced DA neurotoxicity in vervet monkeys, these authors concluded that rodents and primates might possess different

regulatory factors with regard to temperature and METH neurotoxicity (Melega et al., 1998).

Little is presently known about the relationship between temperature, neurotoxicity and plasma METH concentrations. In fact, to our knowledge, there are no studies which have measured each of these parameters in the same animal. As such studies could yield insight into the mechanisms of METH neurotoxicity, the present study was undertaken to evaluate the relationship among temperature (ambient and core), plasma concentrations of METH and its metabolite, AMPH, during the period of drug exposure, and measures of dopaminergic axon terminal integrity one week later. In a separate group of squirrel monkeys, we also characterized the pharmacokinetic profile of METH and its metabolite (AMPH).

Methods

Animals: Adult squirrel monkeys (*Saimiri sciureus*) of both genders, ranging in weight from 0.69 to 1.3 kg, were used. Animals were housed in standard steel cages in a colony room maintained at an ambient temperature of 26 ± 1^{o}C and 20-40 % humidity, with free access to food (New World Primate Diet) and water. The colony room was maintained on a 14:10 cycle (14 hr light:10 hr dark), with lights on at 7AM and off at 9PM. The facilities for housing and care of the animals are accredited by the American Association for the Assessment and Accreditation of Laboratory Animal Care. Animal care and experimental manipulations were approved by the Institutional Animal Care and Use Committee (IACUC) at the Johns Hopkins University School of Medicine. The committee recognized that an ambient temperature of 33°C exceeded the maximum temperature recommended for nonhuman primates by the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* but approved an explicit exception for this study. Only animals on the relevant protocol were exposed to an ambient temperature of 33°C.

Drugs and Chemicals: *d*-Methamphetamine was obtained through the National Institute on Drug Abuse drug supply program (Bethesda, MD, USA), and its authenticity was confirmed by means of gas chromatography/mass spectroscopy (GC/MS). Doses were expressed as the base weight. For GC/MS determinations, racemic amphetamine and methamphetamine were

obtained from Lipomed (Cambridge, MA), racemic-D_8-amphetamine (AMPH-D_8) and racemic- D_8-methamphetamine (METH-D_8) were purchased from Cerelliant (Round Rock, TX), and heptafluorobutyric anhydride (HFBA) was purchased from Alltech (Deerfield, IL). Clean Screen with Clean Thru tips solid-phase extraction columns (6 cc) were obtained from United Chemical Technologies (Bristol, PA). [^{3}H]WIN35,428 was purchased from New England Nuclear (Boston, MA). [^{3}H]DTBZ was obtained from Amersham Life Science (Buckinghamshire, England). Other drugs and chemicals were obtained from the following source: dopamine hydrochloride, DOPAC, 5-hydroxytryptamine (5-HT) creatinine sulphate complex, 5-hydroxyindoleacetic acid dicyclohexylammonium salt (5-HIAA), sodium octyl sulphate and ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, USA), sodium phosphate, citric acid, phosphoric acid, and perchloric acid (J.T. Baker, Phillipsburg, NJ, USA).

Drug administration: *d*-Methamphetamine (or vehicle) was administered orally. Oral administration was accomplished by orogastric gavage, with the animal gently restrained in a plexiglass chair during the procedure. Dose selection was based upon pilot studies and published reports indicating that two doses of METH given hours apart have the potential to produce lasting effects on brain DA neurons in non-human primates (Melega et al., 1998). We

ultimately selected a dose of 1.25 mg/kg, given twice at a 4-hr interval, because pilot studies showed that this dosage regimen is well tolerated and produced a modest reduction in striatal DA neuronal markers one week later, thus leaving room for a possible enhancement by a higher ambient temperature (33^{o}C instead of 26^{o}C). Drug (or vehicle) administration was performed at 11AM and 3PM, so that assessment of effects on core temperature took place during a relatively stable period of the circadian temperature cycle (see Results).

Overall design: Two different groups (n=4-5 per group) of drug-naïve animals were used to test the effects of increased ambient temperature on METH-induced changes in core temperature and subsequent DA neurotoxicity (i.e., 5 monkeys received vehicle and METH at 26^{o}C and 4 different monkeys received vehicle and METH at 33^{o}C) (*Table 1*). The rationale for the temperatures selected (26^{o}C and 33^{o}C) was as follows: An ambient temperature of 26^{o}C was selected because it is considered to be in the thermoneutral range for the squirrel monkey (Stitt and Hardy, 1971; Robinson and Fuller, 1999); an ambient temperature of 33°C was used as a ?warm' environment because it is toward the high end of the thermoneutral range for the squirrel monkey (Stitt and Hardy, 1971; Robinson et al., 1993), and because studies in rodents indicate that a 7^{o}C increase in ambient temperature increases METH neurotoxicity (Callahan et al., 1998).

First, 24-hr baseline core temperatures for each monkey were collected at the ambient temperature in which animals would subsequently receive vehicle and METH (at least one week after implantation of the telemetry transmitter - see below). Then, on day 1, the effect of vehicle administration on core temperature was determined; vehicle was administered by gavage at 11AM and 3PM. On day 2, the effect of METH on core temperature was determined; like the vehicle, METH was administered by gavage at 11AM and 3PM. As indicated above, METH was given at a dose of 1.25 mg/kg, with a 4 hr interval between the first and second dose (total dose 2.5 mg/kg). Throughout the period of METH (vehicle) exposure, core temperature was monitored non-invasively by means of telemetry, as described below. As a precautionary measure, at both ambient temperatures, core temperatures of squirrel monkeys were prevented from exceeding 41°C (by means of ventilation in a cooler environment). Immediately prior to the second dose, and two hours after second dose of METH, blood samples were collected for subsequent determination of plasma drug concentrations. On day 9 (i.e., one week after METH treatment), the animals were sacrificed for measurement of monoamine neuronal markers. Thus, for each animal, we collected data on the acute temperature response, plasma drug concentration, and subsequent regional brain DA/5-HT neuronal markers. As detailed in *Table 1*, due to technical issues, we were unable to collect core temperature data in one animal treated at 26^{o}C and drug plasma concentration data in a different animal treated at 33^{o}C.

Pharmacokinetic study: For this experiment, designed to assess inter-animal differences and characterize the pharmacokinetics of METH (and its metabolite, AMPH) in squirrel monkeys, six different squirrel monkeys were used. All of these animals had participated in prior studies involving stimulant drug administration; however, none had received any other drug for at least 4 weeks prior to this study (as previously noted, all other experiments reported in this paper used drug-naïve animals). METH was administered at a dose of 1.25 mg/kg, p.o.. Blood samples were collected at 5, 15 , 30, 60, 120, 180, 240, 300, and 360 min after METH administration. At each time point, the animal was briefly anesthetized with isoflurane, to facilitate blood sampling. Plasma concentrations of both METH and AMPH were determined. Pharmacokinetic parameters [peak plasma concentrations (C_{max}), times of peak plasma concentration (T_{max}), area under the concentration-time curve (AUC), and the elimination half-lives (t½)] were determined using the software program WinNonlin^{TM} (Pharsight Co., Mountain View, CA). Data were fitted to a noncompartmental model with first order output and elimination.

Core Temperature Measurement: Core temperature was measured non-invasively using a telemetry system (Data Sciences International Inc., St. Paul, MN) designed to monitor physiological parameters from conscious, freely moving animals. Briefly, a small transmitter

(Model TA10TA-F40) was implanted in the peritoneal cavity. This device permits continuous, on-line monitoring of core temperature. For implantation of the transmitter, squirrel monkeys were anesthetized with isoflurane. The sterile transmitter was inserted into the peritoneum. Monkeys were allowed a minimum of 7 days to recover from surgery before undergoing any experimental manipulation or data collection. Core temperature data were collected using a commercially available software package (Dataquest A.R.T., version 2.3, St. Paul MN). Core temperature was sampled every 1.5 seconds (40 times per minute), but 30 minute means were used for analysis. Average 1 min values that changed by more than 1^{o}C in a 1 minute period were excluded from the analysis because core temperature measurements were sensitive to the quality of radioreception.

Plasma sampling: For collection of blood samples used in the determination of plasma drug concentrations, animals were removed temporarily from their home-cages and anesthetized briefly with isoflurane, as below. While under isoflurane anesthesia, animals were placed in a supine position on a heating pad [maintained at 37^{o}C by circulating warm water via a heat therapy pump (Gaymar Industries Inc., Orchard Park, NY, USA)], on a steel surgical table. Blood samples were collected immediately prior to and 2 hr after the second METH dose (i.e., 4 and 6 hr after the first dose of METH). These time points were chosen based on the

pharmacokinetics of METH in the squirrel monkey, with an eye toward obtaining estimates of peak plasma drug concentrations of METH and AMPH after the second dose of METH. At each time point, approximately 0.5 to 1.0 ml of blood was withdrawn from the femoral vein using a disposable, sterile 3 ml syringe and 23 G 1" needle. Blood samples were dispensed into 4 ml Vacutainer Plus haematology (lavender) tubes, containing 7.2 mg K_2EDTA solution (Becton-Dickinson, Franklin Lakes, NJ, USA), and stored on ice for up to 30 min, until centrifuged. Samples were centrifuged at 1100 x g for 10 min at 4^{o}C (Sorvall RC5B Plus, Kendro Laboratory Products, Newtown, CT, USA), plasma was withdrawn using a 5 3/4 Pasteur pipette and decanted into a 1.5 ml microcentrifuge tube. Sodium metabisulphite (250 mM) was added at a volume of 30 μL per ml of plasma to minimize oxidation of the compounds of interest. Samples were vortexed for 10 s, then stored at -20 ^{o}C until further processed.

Determination of plasma METH and AMPH concentrations: Sample preparation and derivatization were carried out as previously described by Peters et al. (2003a), with minor changes. A volume of 0.1 mL of internal standard (IS) containing 1.5 μg/mL of each METH-d_8 and AMPH-d_8 was added to 0.1 mL of plasma diluted with 2 mL of purified water. The analytes were eluted with 1.2 mL of a mixture of methylene chloride, 2-propanol, and ammonium hydroxide (80:20:2 by volume), and derivatization was carried under heat (60^{o}C) for

30 minutes (instead of using microwave irradiation). Analysis was performed using a Agilent Technologies Model 6890N Gas Chromatograph system combined with an Agilent Technologies Model 5973 inert Electron Impact Mass Spectrometer and an Agilent ChemStation Software version Rev.D.0100. The GC conditions were as follows: spitless injection mode; column, HP-5MS (30 m x 0.25 mm i.d.), 5% phenylmethylsiloxane, 250 nm film thickness; injection port temperature, 250^{o}C; carrier gas, helium; flow-rate, 1.0 mL/min; column temperature, initially

80^{o}C, increased to 180^{o}C at 12^{o}C/min, final time 1 min. The MS conditions were as follows: transfer line heater, 280^{o}C; source temperature, 230^{o}C; electron ionization (EI) mode; ionization energy, 70 eV; selected-ion monitoring (SIM) with the following program: solvent delay 3 min; time window A, 3.00-5.50 min, *m/z* 96, 126, 243 (target ion) for AMPH-d_8 and *m/z* 91, 118, 240 (target ion) for AMPH; time window B, start at 5.50 min, *m/z* 123, 213, 261 (target ion) for METH-d_8 and *m/z* 118, 210, 254 (target ion) for METH. Quantification was carried out by comparison of peak area ratios (analyte vs IS) with calibration curves in which peak area ratios of spiked calibrators were plotted against their concentrations. The concentrations of the calibration curves ranged from 5 ng/mL to 400 ng/mL.

Brain dissection: One week after drug treatment, animals were sacrificed under deep

sodium pentobarbital anesthesia (60 mg/kg; i.p.), and the brain was removed from the skull in a cold room (4^{o}C). Regional dissection of the brain was performed at 4^{o}C using the Emmers and Akert (1963) atlas as a guide. Brain regions of interest were isolated from coronally cut sections (approximately 4-5 mm thick) by means of free dissection over ice. Brain tissue was wrapped in aluminum foil, then stored in liquid nitrogen until assay.

HPLC monoamine assays: Concentrations of DA and 5-HT and their major metabolites were measured by reverse phase high performance liquid chromatography (HPLC) coupled with electrochemical detection as described previously (Yuan et al., 2001).

DAT and VMAT binding: The density of DAT and VMAT-type 2 binding sites in regional brain homogenates was determined using previously described methods Villemagne et al. (1998).

Statistics: Temperature data were analyzed using ANOVA for repeated measures. Neurochemical data were evaluated by means of one-way ANOVA. When statistical differences were observed, *post hoc* comparisons were performed using LSD multiple range test. Pearson product moment correlations were calculated to evaluate the relationship among drug plasma concentrations, temperature responses, and subsequent DA deficits. Significance was set

at $P < 0.05$. Data analysis was performed using the Statistical Program for the Social Sciences (SPSS for Windows, Release 10.5).

Results

In keeping with previous reports (Fuller et al., 1979; Robinson and Fuller, 1999), squirrel monkeys exhibited diurnal variation in their core temperature (*Fig. 1*). On average, core temperature was higher when lights were on (7AM to 9PM) than when lights were off (9PM to 7AM). Under the conditions of our study, the 24 hr mean core temperature was 37.9^{o}C, with a standard deviation of 0.93^{o}C. Core temperature was relatively stable between 8AM and 7PM.

Having identified a relatively stable period of the circadian core temperature cycle in our squirrel monkeys (8AM to 7PM), we proceeded to assess the effect of increased ambient temperature on METH-induced hyperthermia. This was done at 26^{o}C and 33^{o}C, using separate groups (n=4-5 per group) of animals to test the effects of METH at each ambient temperature. As shown in *Fig. 2*, METH increased core temperature at both 26^{o}C and 33^{o}C. Mean core temperature increases appeared greater at 33^{o}C than 26^{o}C, but the difference did not achieve statistical significance. As can be seen in the figure, there was substantial inter-animal variability in thermal response, both in monkeys treated with METH at 26^{o}C and in those treated at 33^{o}C. Per pre-determined protocol (see Methods), two of the four monkeys treated at 33^{o}C were moved to a cooler environment (as a precautionary measure) when they reached a core

temperature of 41^{o}C. Both of these animals looked perfectly fine despite their elevated core temperature.

Next , we examined the effect of increased ambient temperature on METH-induced DA neurotoxic changes. Dopaminergic neuronal markers were determined one week after METH treatment. On average, monkeys treated at 33^{o}C had larger dopaminergic deficits than those treated at 26^{o}C (*Fig. 3*). However, like elevations in core temperatures, dopaminergic deficits induced by METH at the two ambient temperatures (26^{o}C and 33^{o}C) were not significantly different. Again, substantial inter-animal variability was noted, at both ambient temperatures . Indeed, as shown in *Fig. 3*, one monkey treated at 26^{o}C (unfortunately the animal in which core temperature data are not available, see Methods and *Table 1*) showed greater than 90% deficits in DA neuronal markers one week after METH treatment.

Regional brain 5-HT and 5-HIAA were not significantly affected by prior treatment with METH, at either 26^{o}C or 33^{o}C, even in animals with large dopaminergic deficits (*not shown*).

Analysis of the relationship between acute increases in core temperature and subsequent decreases in dopaminergic neuronal markers showed that the two variables were highly

correlated (*Fig. 4)*. Similarly high correlations were observed when the AUC, rather than the T_{max}, of the temperature response was considered. Thus, regardless of the ambient temperature during METH treatment (26^{o}C or 33^{o}C), squirrel monkeys with the greatest increases in core temperature developed the largest DA deficits.

To begin to identify the basis for the substantial inter-animal variability described above, we examined the relationship among drug plasma concentrations, acute increases in core temperature and subsequent decreases in dopaminergic markers. Given the study design, this was feasible in each animal. Plasma concentrations of METH, determined 2 hr after the second dose (i.e., 6 hr after the first dose of METH) did not correlate with either METH-induced hyperthermia or METH-induced DA neurotoxic changes (*not shown*). In contrast, plasma concentrations of METH's metabolite, amphetamine (AMPH), correlated highly and directly with acute increases in core temperature (*Fig. 5*). In addition, there were significant correlations between plasma AMPH concentrations and dopaminergic neuronal markers (*Fig.6*).

To determine if the inter-animal differences detailed above might, at least in part, be related to individual differences in METH metabolism, we examined the pharmacokinetic profile of METH and its metabolite (AMPH) in a separate group of squirrel monkeys (n=6). As shown

in *Fig. 7* and *Table 2*, individual differences in METH metabolism are substantial, with wide ranges in various pharmacokinetic parameters including C_{max} and $T_{1/2}$ values.

Brief anesthesia with isoflurane, used to facilitate blood sampling, had a tendency to transiently decrease core temperature (10-15 min) in the squirrel monkey (*not shown*).

Discussion

The present study provides the first available data on the within-subject relationship between temperature (ambient and core), plasma concentrations of METH (and AMPH) and subsequent dopaminergic neurotoxicity. Results indicate that plasma concentrations of AMPH, acute increases in core temperature, and subsequent decreases in dopaminergic neuronal markers are highly correlated. In addition, they reveal substantial inter-animal variability in both the thermal and neurotoxic responses to METH, and suggest that, at least in part, these response differences may be related to individual differences in METH metabolism. Each of these findings is discussed, in turn, below.

Indication that core temperature can influence METH-induced DA neurotoxicity in non-human primates comes from the highly significant correlation presently observed between increases in core temperature and decreases in striatal dopaminergic markers (*Fig. 4*). Virtually identical observations have been made in rodents (Bowyer et al., 1994; Albers and Sonsalla, 1995). Thus, in both rodents and non-human primates, increases in core temperature are associated with augmented METH-induced DA neurotoxicity. At first glance, this conclusion may seem at odds with that of Melega and colleagues (1998) stating that the effects of temperature on METH neurotoxicity might be species-dependent (see Introduction). However, these authors based their conclusion on the observation that decreased core temperature

(secondary to MK-801-induced anesthesia) did not afford protection against METH-induced DA neurotoxicity in vervet monkeys. However, a similar effect has been reported in rodents. Specifically, Albers and Sonsalla (1995) found that decreased core temperature (secondary to reserpine) did not afford protection against METH-induced DA neurotoxicity in mice. Thus, in both rodents and primates, drug-induced decreases in core temperature do not always afford protection against METH-induced DA neurotoxicity. The fact that increases in core temperature are not essential for the expression of METH-induced DA neurotoxicity (either in rodents or primates) does not detract from the fact that, in general, increases in core temperature are associated with increased METH neurotoxicity. Indeed, these seemingly discrepant facts serve as a reminder that the mechanism by which core temperature influences METH neurotoxicity is unknown. That said, it is clear that core temperature can influence METH neurotoxicity, and that the expression of DA neurotoxicity after amphetamines (METH and AMPH) is critically dependent upon the function of the DAT (Ricaurte et al., 1984b; Marek et al, 1990; Pu et al., 1994; Fumagalli et al., 1998). As we have previously shown that increases in temperature lead to increases in DAT function (Xie et al, 2000), it is possible that, at least in part, increased core temperature enhances METH-induced DA neurotoxicity by amplifying a DAT-dependent neurotoxic cascade (Callahan et al., 2001). Alternatively, elevations in core temperature could enhance neurotoxicity via alternate mechanisms. For example, core temperature elevations could lead to increased formation of reactive oxidative species (Cubells et al., 1994; Giovanni et al.,

1995; Yamamoto and Zhu, 1998) or amplify other molecular events thought to underlie the neurotoxic mechanism of action of METH and related drugs (see Bowyer and Holson,1995; Seiden and Sabol, 1996).

Under the present experimental conditions, ambient temperature did not produce significant effects on thermal or neurotoxic responses to METH in squirrel monkeys. This contrasts with previous observations in rodents (see Introduction). However, factors that (alone or in combination) may have contributed to the lack of a significant effect of ambient temperature in squirrel monkeys include the limited range of ambient temperatures tested (26- 33^{o}C), the high degree of inter-animal variability (see below), and the relatively small sample size necessarily employed. In addition, our decision (for safety reasons) not to allow any monkey to exceed a core temperature of 41^{o}C may have played a role, because two of the monkeys treated at an ambient temperature of 33^{o}C (whereas none of those treated at 26^{o}C) reached a core temperature of 41^{o}C and had to be moved to a cooler environment. Thus, in effect, our study design may have inadvertently introduced a ceiling effect that disproportionately affected the animals treated at the warmer ambient temperature. Finally, there may be innate differences between rodents and primates in thermoregulation.

At both ambient temperatures tested (26ºC and 33ºC), we observed substantial inter-animal variability, both with respect to thermal and neurotoxic responses to METH. The basis for these individual differences is unknown. However, it may be relevant that monkeys with the greatest increases in core temperature (and largest DA deficits) had the highest plasma drug concentrations. This observation raises the question of which of these two factors (drug concentration or core temperature) has primacy, and whether they are directly related. For example, it is possible that increased plasma drug concentrations lead to increased core temperature, and that these factors, individually or in combination, act to increase dopaminergic neurotoxicity. Alternatively, increases in core temperature may somehow influence drug metabolism or clearance of drug, and thereby lead to higher plasma drug (and metabolite) concentrations (and more pronounced neurotoxic effects). However, to our knowledge, there is no evidence that increased core temperature, *per se*, inhibits METH or AMPH metabolism, clearance or cytochrome P_{450} activity. Indeed, Clausing and colleagues (1995) have shown that hypothermia (rather than hyperthermia) leads to higher amphetamine concentrations. Thus, in light of these considerations, we presently, favor the view that drug concentrations (at relevant sites of action) are the principal determinants of increases in core temperature and neurotoxicity, and that the inter-animal variability presently observed may be related, as least in part, to individual differences in drug metabolism. In a preliminary way, this view is supported by our

demonstration that there are substantial inter-animal differences in pharmacokinetic parameters of METH and AMPH in squirrel monkeys (*Fig. 7 and Table 2*). To our knowledge, these are the first such data reported.

While there was a highly significant correlation between plasma concentrations of AMPH and increases in core temperature (as well as significant or near significant correlations between plasma AMPH concentrations and decreases in various DA neuronal markers), there was no correlation between plasma concentrations of METH and the above mentioned outcome measures (core temperature and dopaminergic deficits). The better predictive value of plasma AMPH, as compared to METH, may be related to the fact that, in the squirrel monkey, METH is rapidly converted AMPH in the periphery (*Fig. 6*), thus minimizing the amount of METH reaching the central compartment (brain). As the neurotoxic effects of AMPH are largely restricted to brain DA neurons, whereas those of METH involve both DA and 5-HT neurons (see Introduction), rapid conversion of METH to AMPH in the periphery may account for the absence of brain 5-HT deficits in the present study.

The observation that two oral doses of METH, on the order of those used by some humans (Cho and Melega, 2002), are sufficient to produce DA neurotoxic changes in squirrel monkeys are in keeping with our own earlier findings in baboons (Villemagne et al., 1998), as

well as those of Melega and colleagues (1998) in vervet monkeys. Of note, however, these studies did not allow for the development of tolerance, which can diminish METH-induced neurotoxic changes (Schmidt et al., 1985; Gygi et al., 1996; Stephans and Yamamoto, 1996; Riddle et al., 2002; Johnson-Davis et al., 2003). This issue notwithstanding, the present results indicate that multiple high doses of METH are not always necessary for the production of dopaminergic neurotoxicity, and demonstrate that plasma levels of AMPH associated with dopaminergic neurotoxicity in non-human primates are on the order of those in some METH and AMPH users (Peters et al., 2003b; Nakashima et al., 2003).

Several limitations of the current study should be mentioned. First, the range of ambient temperatures studied was limited, and may not have included a transition point at which ambient temperature's influence would have been significant. Second, the sample size was small (n=4-5 per group), a necessary feature of primate studies. A larger sample size might have resulted in improved power to draw conclusions regarding potential effects of ambient temperature. Third, there is a possibility that isoflurane anesthesia, used to facilitate plasma sampling, might have influenced the effect of altered core temperatures. However, previous studies suggest that isoflurane does not influence the degree of neurotoxic injury produced following treatment with other amphetamine neurotoxins (Mechan et al., 2005), and indeed, in the current study, significant toxicity was observed despite the transient hypothermic effect of anesthesia. Finally, the study

design did not include a neurochemical group of controls that was warmed to 33°C. However, previous studies in rodents have not demonstrated monoaminergic neurotoxicity following transient exposure to increased temperature (Bowyer et al., 1994; Albers and Sonsalla, 1995), and given the valuable animal resource under study, the addition of such a control group seemed inappropriate.

In conclusion, results from the present study, the first to explore within subject relationships among temperature (ambient and core), plasma drug concentrations, and dopaminergic neurotoxicity, underscore the fundamental importance of core temperature in METH neurotoxicity across species (rodents and primates). In addition, they show that there are substantial inter-animal differences in thermal and neurotoxic responses to METH, and suggest that such differences may, at least in part, be related to individual differences in METH metabolism. As similar inter-individual differences have been noted in humans (Cook et al., 1992;1993; Schepers et al., 2003), additional studies are needed to identify their basis and potential clinical consequences.

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Footnotes

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Legends for Figures

Fig. 1. Record of 24-hr mean (± s.e.m.) core temperature in squirrel monkeys (n=8) housed in a colony room maintained on a 14:10 hr light:dark cycle (lights on: 07:00 hr) at an ambient temperature of 26 ± 1^{o}C. Note diurnal variation with progressive decrease in core temperature after lights are turned off at 9PM and gradual increase beginning approximately 2 hr before lights come back on at 7AM.

Fig. 2. Effect of METH (1.25 mg/kg, p.o., given twice, 4 hr apart) on core temperature in squirrel monkeys maintained at an ambient temperature of 26^{o}C or 33^{o}C during the period of drug exposure. Separate groups of animals (n=4 per group) were used to assess the effects of METH at each ambient temperature. Core temperature was measured non-invasively by means of telemetry as described in *Methods*. METH (or saline) was administered at the times indicated by the arrows.

Fig. 3. Effect of METH (1.25 mg/kg, p.o., given twice, 4 hr apart) on striatal dopaminergic neuronal markers in squirrel monkeys maintained at an ambient temperature of 26^{o}C or 33^{o}C during the period of drug exposure. Dopaminergic markers were measured one week after METH treatment. Filled symbols represent mean values (± s.e.m.). Control values were 9.3 ± 0.5

for DA, 1041 ± 61 DPM for [^{3}H]WIN35,428 binding, and 3517 ± 164 DPM for [^{3}H]DTBZ.

Fig. 4. Relationship between increases in core temperature seen acutely and decreases in DA concentrations observed one week later in the caudate (A, C) and putamen (B, D). Shown are the results for all monkeys (i.e., those treated with METH at an ambient temperature of 26^{o}C and 33^{o}C) on which data are available. Circles are from animals treated at 26^{o}C; squares are from animals treated at 33^{o}C. *R* = Pearson correlation coefficient.

Fig. 5. Relationship between plasma AMPH concentrations and increases in core temperature after METH administration to squirrel monkeys. Shown are results for all animals (i.e., those treated with METH at an ambient temperature of 26^{o}C and 33^{o}C) on which data are available. Circles are from animals treated at 26^{o}C; squares are from animals treated at 33^{o}C. *R*=Pearson correlation coefficient.

Fig. 6. Relationship between plasma amphetamine concentration (C_{max}) and striatal dopaminergic deficits in squirrel monkeys administered METH one week previously. METH was administered orally at a dose of 1.25 mg/kg, x 2, 4 hr interval. For determination of plasma

drug concentrations, blood was collected 6 hr after the first dose of METH (2 hr after second dose). Shown are results for all monkeys (i.e., those treated with METH at an ambient temperature of 26^{o}C and 33^{o}C) for which results were available. Filled circles are from animals treated at 26^{o}C; filled squares are from animals treated at 33^{o}C; open circle is from animal treated at 26^{o}C on which temperature data is unavailable. *R* = Pearson correlation coefficient. *Designates $p < 0.05$.

Fig. 7. Mean plasma concentration versus time profiles of METH (A) and AMPH (B) in squirrel monkeys administered a single dose of METH at a dose of 1.25 mg/kg, p.o. (gavage). Results shown represent the mean ± s.e.m. (n = 6).

Table 1. Squirrel monkeys used in temperature, neurotoxicity and drug concentration studies, along with outcome measures available on each animal.

Monkey #	**Ambient Temperature**	**Core Temperature data**	**METH/AMPH concentration data**	**Dopaminergic marker data**
484	26ºC	No	Yes	Yes
377	26ºC	Yes	Yes	Yes
410	26ºC	Yes	Yes	Yes
471	26ºC	Yes	Yes	Yes
349	26ºC	Yes	Yes	Yes
412	33ºC	Yes	No	Yes
411	33ºC	Yes	Yes	Yes
405	33ºC	Yes	Yes	Yes
346	33ºC	Yes	Yes	Yes

Table 2. Pharmacokinetic parameters of METH and AMPH in separate group of squirrel monkeys administered single dose of METH (1.25 mg/kg, p.o.). Note that for one of the six squirrel monkeys, the $T_{1/2}$ of amphetamine could not be determined because its concentration had not decreased sufficiently within the period of drug sampling.

Methamphetamine

	N	*Mean*	*SD*	*Min*	*Median*	*Max*	*Range*	*CV%*
$T_{1/2}$ (hr)	6	1.35	0.5	0.64	1.34	2.15	1.5	35.7
Tmax (hr)	6	0.7	0.3	0.5	0.5	1	0.5	38.7
Cmax (ng/ml)	6	136.4	86.5	56.4	118.4	293	236.6	117.1
AUC (ng/ml x hr)	6	338.9	332.4	76.4	239.3	970.2	893.9	98.1
Vol dist (mL/kg)	6	10785.7	6272.7	3337.9	10272	19857.9	16520	58.2
Clearance (mL/hr/kg	6	6926.7	5528.5	1077.1	5695.8	16037.2	14960.1	79.8

Amphetamine

	N	*Mean*	*SD*	*Min*	*Median*	*Max*	*Range*	*CV%*
$T_{1/2}$ (hr)	5	5.98	5.8	2.24	3.33	16.15	13.91	97.1
Tmax (hr)	6	2	1.5	1	1.5	5	4	77.5
Cmax (ng/ml)	6	130.5	25.3	89	130.2	157.2	68.2	19.4
AUC (ng/ml x hr)	6	558.2	107	427.7	577	680.3	252.6	19.1
Vol dist (mL/kg)	5	7307.6	1563.4	5661.1	6969	9819.8	4158.6	21.4

Clearance (mL/hr/kg	5	1288.3	724.7	421.5	1179	2335.6	1914.1	56.2

Fig. 1

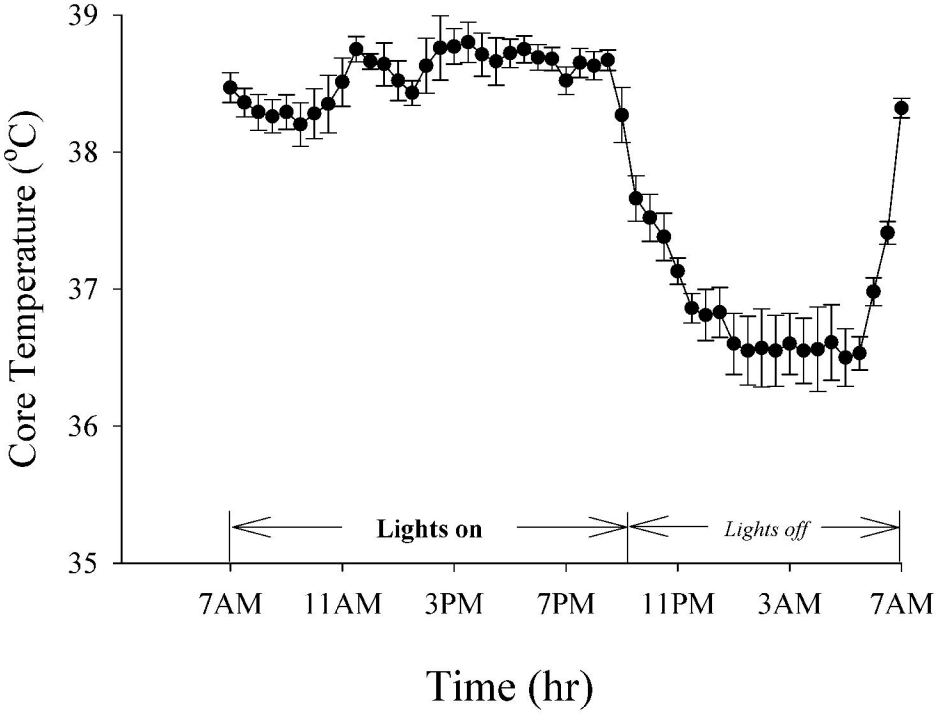
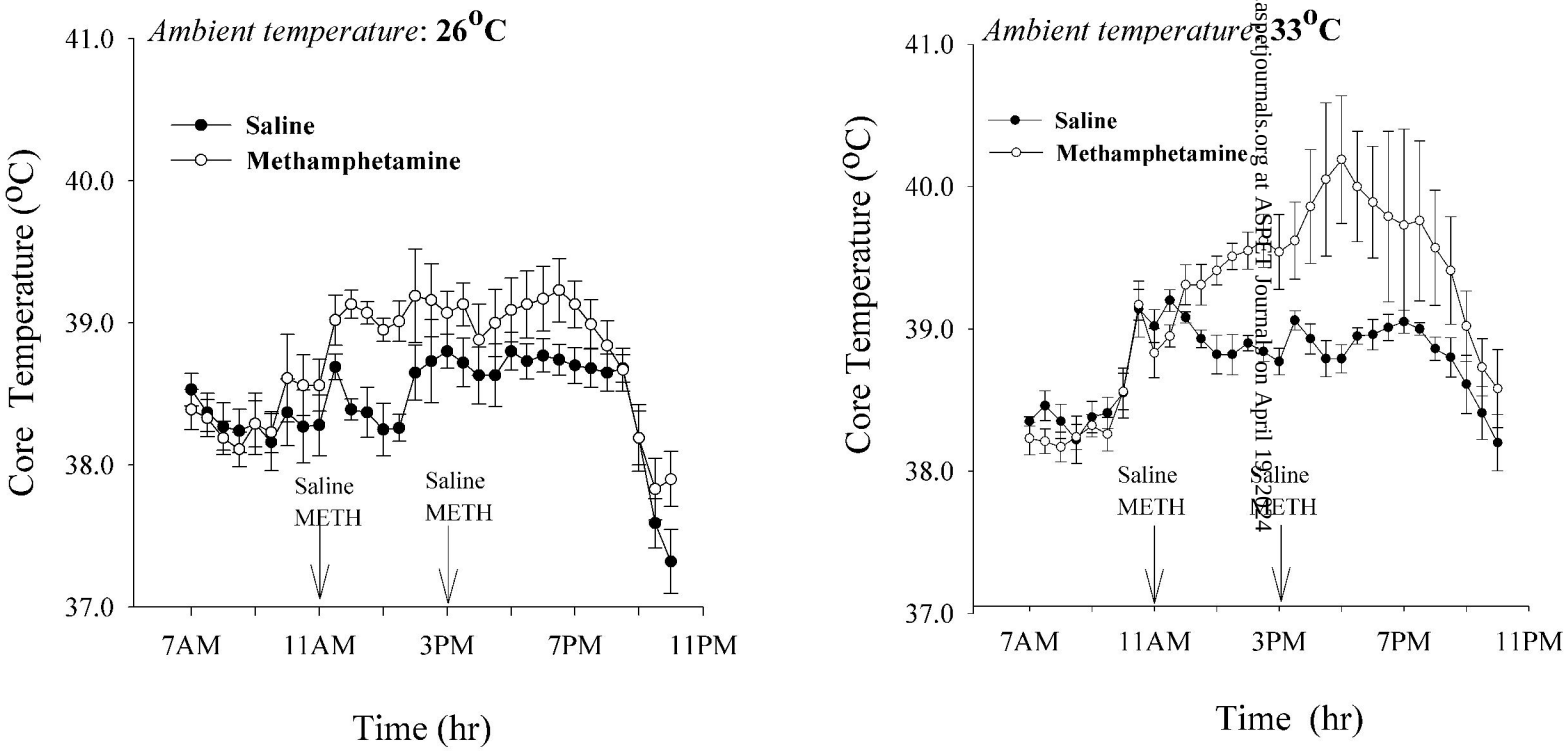


Fig.2



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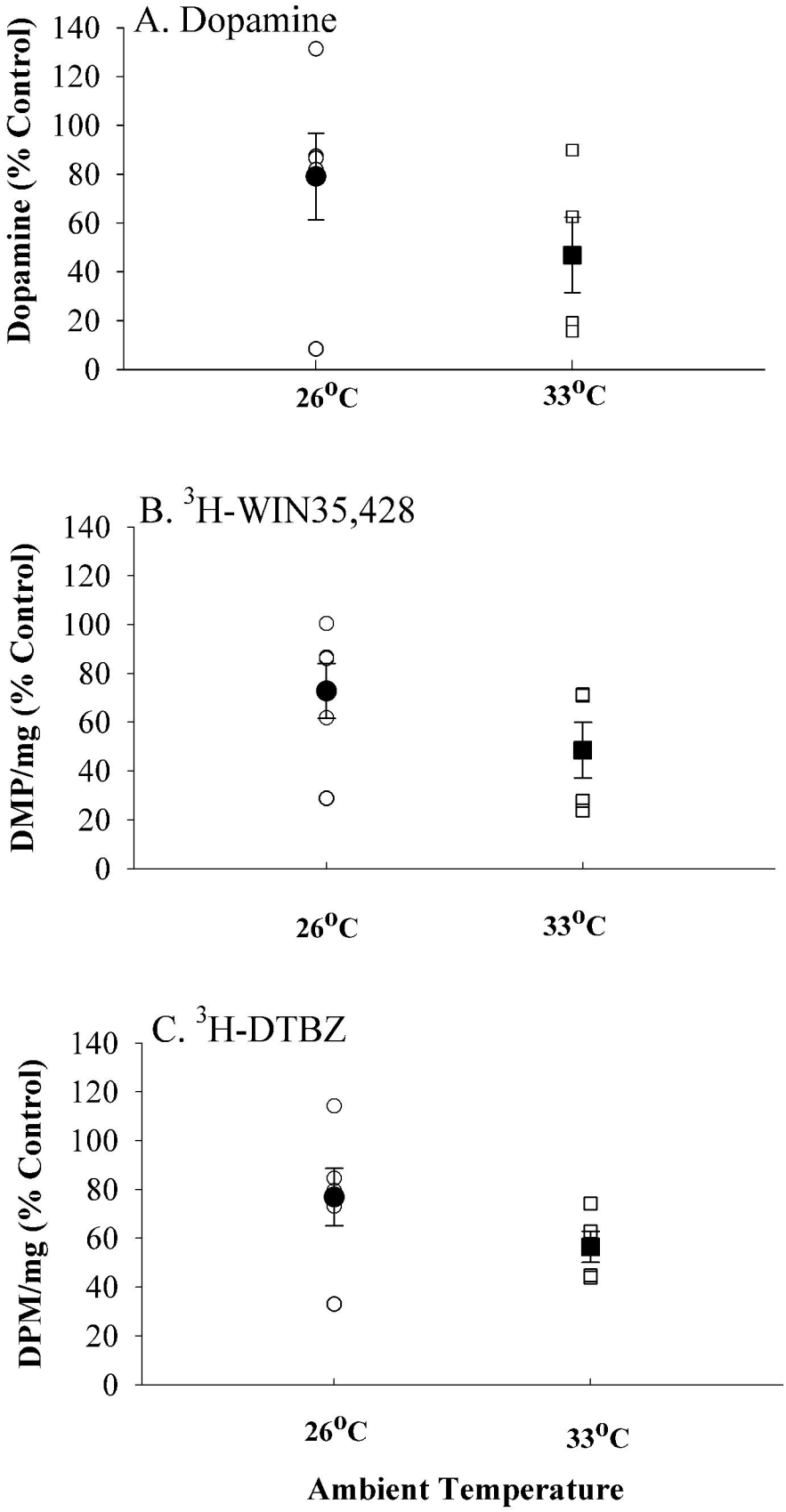

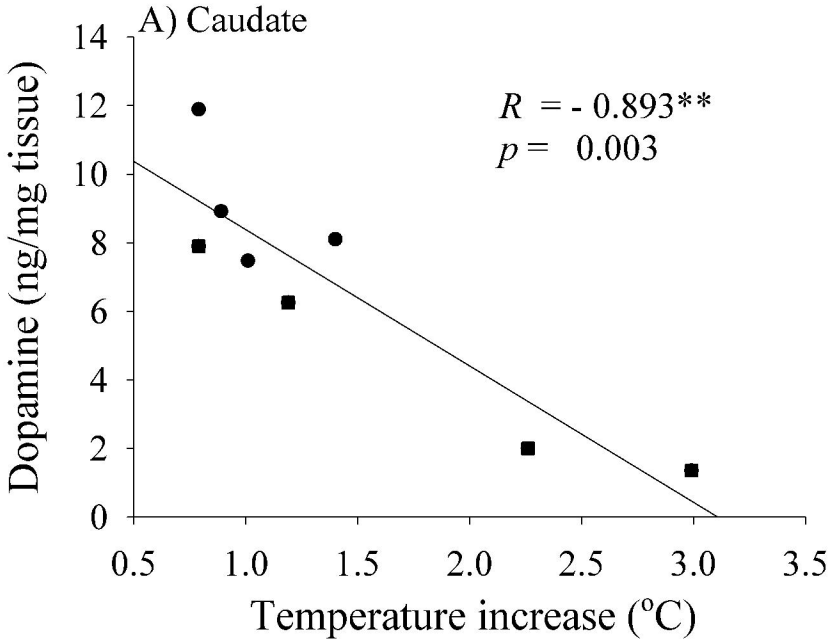

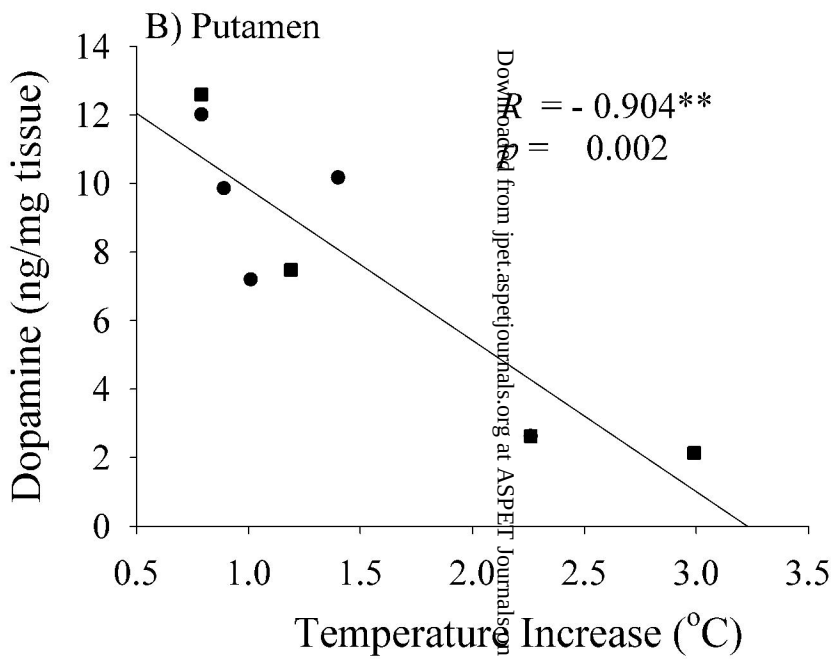

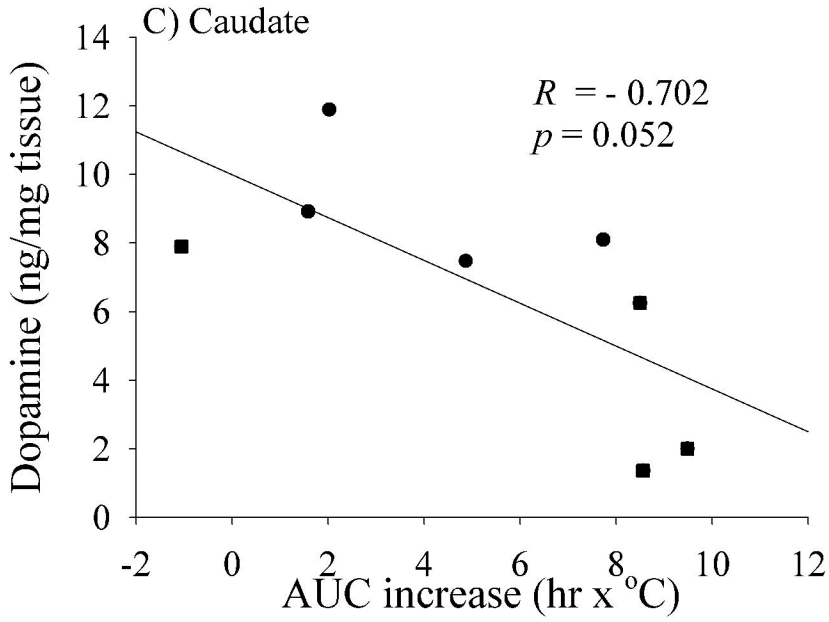

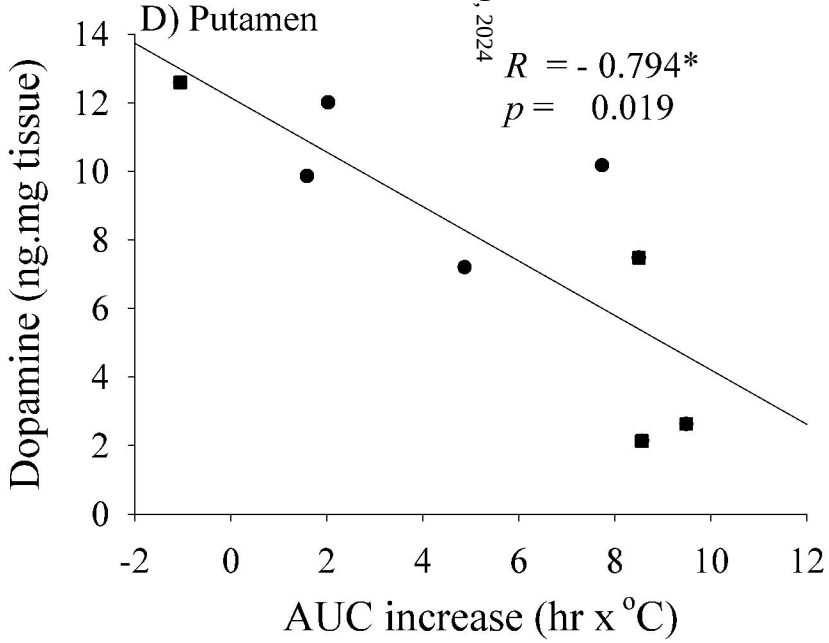

Fig. 5

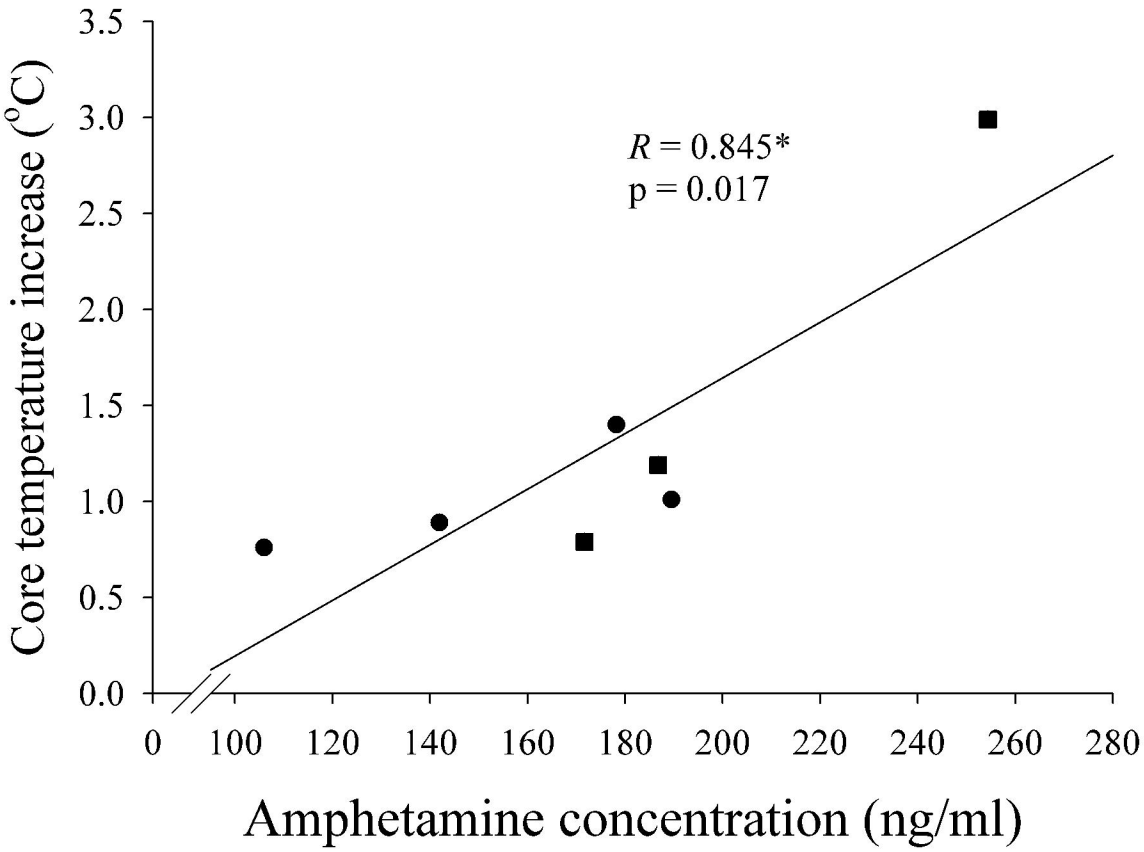

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Fig. 6

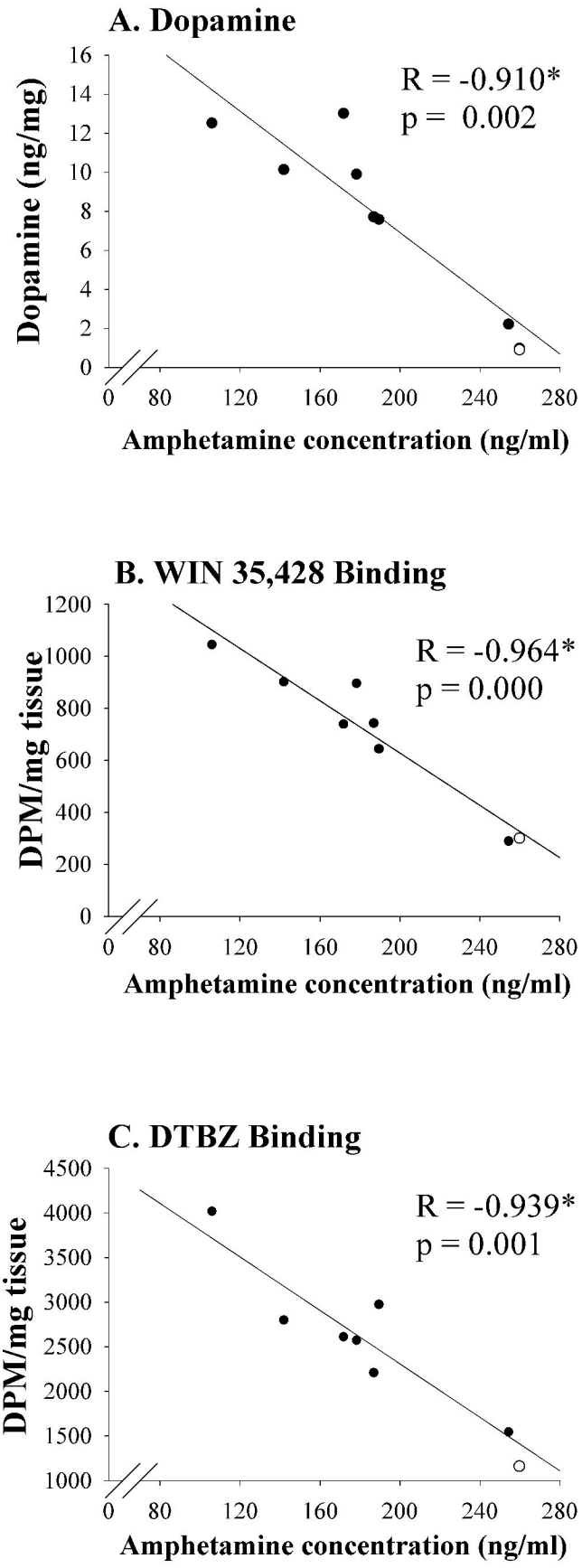

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Fig. 7