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Sex differences and Tat expression affect dopaminergic receptor expression and response to antioxidant treatment in methamphetamine-sensitized HIV Tat transgenic mice

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Abstract

Methamphetamine (Meth) abuse is a common HIV comorbidity. Males and females differ in their patterns of Meth use, associated behaviors, and responses, but the underlying mechanisms and impact of HIV infection are unclear. Transgenic mice with inducible HIV-1 Tat protein in the brain (iTat) replicate many neurological aspects of HIV infection in humans. We previously showed that Tat induction enhances the Meth sensitization response associated with perturbation of the dopaminergic system, in male iTat mice. Here, we used the iTat mouse model to investigate sex differences in individual and interactive effects of Tat and Meth challenge on locomotor sensitization, brain expression of dopamine receptors (DRDs) and regulatory adenosine receptors (ADORAs). Because Meth administration increases the production of reactive oxygen species (ROS), we also determined whether the effects of Meth could be rescued by concomitant treatment with the ROS scavenger N-acetyl cysteine (NAC). After Meth sensitization and a 7-day abstinence

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James Kesby made the in vivo administrations.

Svetlana Semenova participated in the conception and design.

Maria Cecilia Garibaldi Marcondes obtained funding, designed all the experiments, made the perfusions and dissections, analyzed data, supervised students and wrote the manuscript.

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period, groups of Tat+ and Tat- male and female mice were challenged with Meth in combination with NAC. We confirmed that Tat expression and Meth challenge suppressed DRD mRNA and protein in males and females' brains, and showed that females were particularly susceptible to the effects of Meth on D1-like and D2-like DRD subtypes and ADORAs. The expression of these markers differed strikingly between males and females, and between females in different phases of the estrous cycle, in a Tat -dependent manner. NAC attenuated Meth-induced locomotor sensitization and preserved DRD expression in all groups except for Tat+ females. These data identify complex interactions between sex, Meth use, and HIV infection on addiction responses, with potential implications for the treatment of male and female Meth users in the context of HIV, especially those with cognitive disorders.

Keywords

Human immunodeficiency Virus; neuroHIV; methamphetamine; Tat; dopamine receptors; reactive oxygen species; N-acetyl cysteine

1. Introduction

Use of the highly addictive and easily accessible drug methamphetamine (Meth) is common among individuals with, or at risk for, human immunodeficiency virus (HIV) infection¹⁻⁴. Recent studies have reported that Meth use is increasing among females with social risk factors^{5,6}. Studies in humans and experimental animal models have identified marked sex differences in several aspects of Meth and other addictive substance use, such as motivators, addictive behaviors, use patterns, and responses⁷⁻⁹. In animal models of Meth self-administration, females have been reported to acquire sensitization more rapidly, to self-administer higher quantities, and to show higher motivation than males¹⁰⁻¹⁴. One hypothesis suggests that steroid hormones may play an important role in defining responses to Meth^{15,16}.

HIV infection and Meth co-exist as risk factors for neurological disorders and addictive behaviors, and sex differences in the toxic effect of Meth on the brain, especially dopaminergic system neurons, have been described^{8,17-19}. However, interactions between HIV infection and sex differences in the response to Meth, including effects on brain biochemistry, have not been sufficiently examined. The development of animal models of HIV infection in the brain has enabled detailed investigations of the precise interactive effects of factors of HIV infection, including of particular viral proteins, and Meth use at the molecular level. The iTat transgenic mouse model, in which expression of the HIV-1 transactivator of transcription (Tat) protein can be specifically induced in brain astrocytes, has been used to investigate some of the neurological consequences of HIV infection (neuroHIV)²⁰. We previously used this model to demonstrate that the effect of Meth on addictive behaviors and dopaminergic markers is enhanced in male Tat+ compared with male control (Tat-) mice. However, little is known about the extent to which sex differences and Tat expression affect the dopaminergic response to Meth, including the production of dopamine (DA), expression of DA receptors (DRDs) and the DRD regulatory adenosine receptors (ADORAs), and downstream events. Such information may provide vital clues

about the molecular events underlying sex differences in Meth use and responses to treatment, especially in HIV-infected individuals.

The inflammatory environment induced by HIV infection of brain cells, especially innate immune cells, can be exacerbated by drugs of abuse, leading to enhanced viral replication^{21,22}. While the mechanisms by which drugs of abuse act on immune cells vary considerably, one effect common to several psychotropic drugs, is induction of reactive oxygen species (ROS) such as superoxide, peroxide, and hydroxy radicals^{23,24}. ROS are oxygen byproducts that play key roles in normal cell physiology as regulators of epigenetic signaling; however, they are highly reactive molecules and accumulation of excessive levels can cause damage to cellular components²⁵. An imbalance between the natural cellular antioxidant system and ROS levels leads to oxidative stress. The physiological roles of ROS include signaling for transcriptional activation of stress-responsive genes that function in repair pathways, and activation of genes that affect inflammation and behavior^{26,27}. Interestingly, administration of ROS scavenger compounds can attenuate drug-seeking and locomotor outcomes in rodent models of Meth self-administration²⁸. We showed that treatment with the antioxidant N-acetyl cysteine (NAC) can prevent and rescue the development of acute hyperthermia following administration of a single dose of Meth to naïve wild type mice^{29,30}. NAC is a cysteine pro-drug that directly reacts with ROS³¹ and increases glutathione levels^{32–35}. NAC also increases the activity of the cysteine-glutamate antiporter system x(c)- to regulate glutamate levels and transmission³⁶. Meth is considered to be one of the strongest inducers of ROS-associated neurotoxicity among drugs of abuse^{28,37}. Notably, HIV infection also induces ROS production, which has critical effects on pathogenesis³⁸, and in terms of interactions with Meth. While Meth use is largely associated with behaviors that increase the risk of HIV exposure³⁹, it is clear that Meth-induced ROS production could enhance the risk of neuropsychological impairment in HIV-infected individuals^{40,41}. Thus, there is a need to determine whether interactions exist between sex and HIV infection not only in the development of addiction associated behavioral responses, but also in the molecular changes in the dopaminergic system induced *via* alterations in ROS levels.

In the present study, we employed iTat transgenic mice to investigate the consequences of sex differences and Tat expression on locomotor activity, DRD expression, and ADORA expression following acute Meth administration to Meth-sensitized mice. We also examined whether therapeutic targeting of ROS by co-administration of NAC at the time of Meth challenge can modulate changes in the DA system. Our results show that Tat expression differentially affects the behavior and brain biochemistry of males and females in response to Meth; Meth and Tat expression have important and distinct interactive effects on the expression of DRD subtypes and their regulatory ADORA molecules; and NAC administration modulates Meth-associated locomotor behaviors, mirrored by changes in DRD expression, in sex- and Tat-dependent manners. These findings have implications for our understanding of sex-related differences in the response to Meth of HIV-infected individuals, and could contribute to better therapeutic management strategies specifically tailored to males and females.

2. Material and Methods

2.1. Animals:

Male and female mice (3–5 months old) harboring the GFAP promoter-controlled Tet-binding protein with the TRE promoter-Tat protein transgene were used. iTat transgenic mouse colonies with a C57BL/6J background were obtained by generating two transgenic lines; Teton-GFAP (Tat[−]) and TRE-Tat86 (Tat⁺), followed by cross-breeding as described⁴². The mice were housed in groups of 2–4 in a humidity- and temperature-controlled animal facility on a 12 h/12 h reverse light/dark cycle (lights off at 7:00 AM) with *ad libitum* access to food and water. Locomotor activity testing was conducted during the dark phase between 8:00 AM and 5:00 PM, and mice from all groups were tested concurrently. All of the experiments were conducted in accordance with the guidelines of the American Association for the Accreditation of Laboratory Animal Care and National Research Council's Guide for the Care and Use of Laboratory Animals, and the study was approved by the San Diego Biomedical Research Institute and University of California San Diego Institutional Animal Care and Use Committees.

2.2. Doxycycline (Dox) regimen:

All mice (Tat[−] and Tat⁺) were administered 100 mg/kg Dox (doxycycline hyclate; Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection once a day for 7 days. This regimen was previously shown to induce Tat effectively^{43–45}. Specifically, Tat expression increases during Dox administration and wanes significantly over 14 days after the termination of Dox treatment⁴⁵. The behavioral response to Tat induction is extinguished by day 21⁴⁵. Mice received Dox injections in the evening (5:00 PM) beginning the day before initiation of the Meth sensitization procedure.

2.3. Meth sensitization and NAC injection:

Meth sensitization consisted of an acquisition phase and a challenge phase as previously described^{20,46}. For acquisition, mice received an intraperitoneal injection of 2 mg/kg Meth (methamphetamine hydrochloride; Sigma-Aldrich) once a day for 7 days. After a 7-day washout period, mice were then challenged with 1 mg/kg Meth or an equivalent volume of saline (Sal). Injections of NAC (Sigma-Aldrich) or vehicle (Sal) were administered at the same time as the Meth or Sal challenge. Thus, groups (n=8–14, see figure legends) of male Tat[−], male Tat⁺, female Tat[−], and female Tat⁺ mice were treated with: (i) Sal challenge plus Sal treatment (Sal group), (ii) Meth challenge plus Sal treatment (Meth group), (iii) Sal challenge plus NAC treatment (Sal/NAC group), and (iv) Meth challenge plus NAC treatment (Meth/NAC group). Behavioral responses were examined 5 minutes after challenge. Twenty-four hours later, females were examined for estrous cycle phase, and blood and brain tissue were collected (see below).

2.4. Locomotor activity testing:

Locomotor activity was tested in four open field arenas (60 × 60 cm) equipped with infrared beams (Med Associates, St. Albans, VT, USA) to calculate total traveled distance. Mice

were acclimated to the testing room for at least 1 h before the assessments, and tests were performed in the dark for a period of 30 min.

2.5. Determination of estrous cycle phase and tissue harvesting.

Mice were anesthetized with 1–3% isoflurane/oxygen 24 h after challenge. Estrous cycle phase was determined in female mice using a vaginal cytology method by lavage, as described⁴⁷. Blood was collected by cardiac puncture and the mice were then perfused with ice-cold PBS containing 0.2% EDTA. The brains were harvested and the two hemispheres were divided; one was placed in 10% buffered formalin for 48 h and then in 70% ethanol for histological procedures, and the other was immediately dissected. Areas of the brain such as hippocampus, caudate-putamen, and nucleus accumbens, were separated in eppendorfs and then snap frozen in dry ice and ethanol for molecular studies. We note that the hemisphere designated for molecular analysis was lacking the hippocampal and nucleus accumbens regions, which were excised and used for another published study²⁰. Therefore, for the present study, RNA was extracted from the caudate-putamen of one hemisphere and the histological analysis was performed on sections of the whole contra-lateral hemisphere.

2.6. Measurement of serum progesterone level.

Sera were prepared from blood samples collected at sacrifice, and progesterone levels were quantified by ELISA (#582601, Cayman Chemical, Ann Harbor, MI, USA). Assays were performed with two serum dilutions per sample, each in duplicate.

2.7. qRT-PCR.

Total RNA was extracted from the caudate-putamen using a Nucleospin RNA kit (Macherey-Nagel, Duren, Germany). RNA integrity was verified using an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and RNA concentration was measured using a Nanodrop spectrophotometer. RNA was reverse transcribed using SuperScript III Reverse Transcriptase (Invitrogen, Waltham, MA). qPCR was performed using RT2 SYBR Green ROX FAST Mastermix (Qiagen, Valencia, CA, USA) in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). mRNA expression levels were quantified by the ddCT method and the results were normalized to the mean level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S housekeeping genes. Primers were purchased from Qiagen (Table 1).

2.8. Immunohistochemistry (IHC).

Brain samples fixed in buffered formalin/ethanol were embedded in paraffin, cut into 5 μ m sections, and mounted on glass slides. Rehydrated sections were treated with 3% hydrogen peroxide in absolute methanol and then placed in 0.01 M citrate solution, pH 6.39, in a humidified heated chamber. Sections were blocked with Phosphate Buffered Saline (PBS) containing 5 g/L casein (Sigma-Aldrich) and 0.5 g/L thimerosal (Sigma-Aldrich) and then incubated with anti-mouse DRD1 antibody (NLS43, Novus Biologicals, Littleton, CO, USA) or anti-mouse DRD2 antibody (orb154598, Biorbyt, San Francisco, CA, USA) diluted in casein buffer overnight at 4°C. After washing, the sections were incubated with biotinylated goat anti-rabbit IgG (1:300; Vector Labs, Burlingame, CA, USA), and staining

was visualized with ABC biotin/avidinperoxidase kit and Nova Red (both from Vector Labs). Sections were counterstained with Gill's hematoxylin. Images were captured using an Axiovert 200 inverted microscope (Carl Zeiss, Oberkochen, Germany) with Axio Vision software (version 4.8.1; Carl Zeiss). Automated scanning of stained sections was performed using a digital pathology scanner (Leica Biosystems, Buffalo Grove, IL, USA) with Aperio Imagescope (Leica Biosystems) for visualization and conversion of images to .tiff format. Image analysis was performed using Fiji/ImageJ (National Institutes of Health, Bethesda, MD, USA). Stained cells were identified and sorted from background by color threshold. A negative binary mask was then obtained to measure the percentage of the section containing positively stained cells, normalized to the total section area.

2.9. Statistical analysis.

Behavioral data analyses were performed with IBM SPSS Statistics 20 (Armonk, NY, USA). Multivariate analysis was performed using non-repeated measures Brown–Forsythe analysis of variance (ANOVA) corrected with Welch factor, followed by Tamhane's T2 multiple comparisons. Data not meeting the assumption of homogeneity of variance were analyzed using Greenhouse–Geiser adjusted degrees of freedom. Molecular markers were analyzed using Prism 8 (GraphPad, San Diego, CA, USA). Data were analyzed using ANOVA and mixed models, with Tat expression, and challenge, sex, and estrous cycle phase as factors. When appropriate, post hoc multiple comparisons were performed using Bonferroni's test. The results are expressed as the mean $\pm$ SD, unless indicated in the legends. Size effects and variation are reported in the text. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of Tat expression, sex, and challenge on locomotor activity and response to NAC in Meth-sensitized mice

To determine how Tat expression, sex, and reduction in ROS affects the response of mice to Meth, we first examined the effects on locomotor activity. Groups of Tat[−] and Tat⁺ mice were sensitized to Meth and then challenged with vehicle (Sal) or Meth co-administered with Sal or NAC. Locomotor activity was examined 24 h later. Locomotor activity was significantly increased by challenge with Meth compared with Sal regardless of sex, and it was further enhanced by Tat expression in both males and females (Figure 1A). Co-administration of NAC during challenge significantly attenuated Meth-induced locomotor sensitization in Tat[−] females and males and Tat⁺ males, but not in Tat⁺ females (Figure 1A). Thus, locomotor sensitization was significantly affected by sex in Tat⁺ mice ($p = 0.0024$) but not in Tat[−] mice, and the response to NAC, which suggests an involvement of ROS in mechanisms associated with addictive behaviors, was also sex dependent.

3.2. Determination of estrous cycle phase in females

To more precisely investigate the effects of sex on DRD and ADORA expression in Meth-sensitized and -challenged mice, we determined the estrous cycle phase of each female by vaginal cytology at 24 h after challenge, the same time as collection of brain tissues for molecular analyses. Of the 62 females analyzed (Tat⁺ and Tat[−]), 54% were in estrus, 35% in

diestrus, 6% in metestrus, and 5% in proestrus. Serum progesterone levels at the time of cytology were variable but were consistently and significantly higher in females in diestrus compared with the other phases, regardless of Tat expression (Figure 1B), which was consistent with our cytology-based results and previous reports⁴⁸. Due to the small number of females in proestrus (n=2) and metestrus (n=3), they were excluded from the following molecular analyses, and the results are reported only for females in estrus or diestrus.

3.3. Transcriptional changes in ADORA genes

qRT-PCR analysis of ADORA mRNA levels in the brains of mice sacrificed at 24 h post-challenge revealed a significant overall effect of Tat expression, with untreated Sal-challenged Tat+ animals expressing lower levels than the Tat- counterparts (F6, 126 = 56.4, $p < 0.0001$). We also detected differences between Tat- and Tat+ mice in the effects of Meth challenge, sex, estrous cycle phase, and NAC as described below for each ADORA subtype.

3.3.1. Effects of Tat expression on ADORA1 transcription and its interactions

with sex—For ADORA1 expression (Figures 2A and 2B), there were significant main effects of sex and estrous cycle (F2, 126=6.4361, $p=0.0004$), Tat expression (F1, 126=19.776, $p<0.0001$), and their interactions (F2, 126=20.335, $p<0.0001$). The effect of Tat expression was significant in males ($p<0.0001$) and females in diestrus ($p=0.0027$), but not in females in estrus ($p=0.1495$). Moreover, the expression of ADORA1 in females in diestrus had an interaction with Tat expression and with challenge ($p<0.0001$).

ADORA1 was expressed at lower levels in Tat+ compared with Tat- females in diestrus following Sal ($p<0.0001$) and Meth ($p<0.0001$) challenges. Moreover, there was a significant increase in ADORA1 levels in NAC-treated Tat+ animals compared with Tat- animals after challenge with either Sal or Meth ($p<0.0001$), indicating that ROS may regulate ADORA1 transcription in Meth-sensitized mice, regardless of challenge. There were significant effects of Tat, challenge, and sex interaction, which in males were due to differences in Meth/NAC as a challenge ($p<0.000001$), and in females in diestrus were due to both Meth and Sal challenge ($p=0.000575$) in multiple comparisons.

3.3.2 Effects of sex on ADORA1 transcription and its interactions with challenge

—In Tat- animals (Figure 2A) there was no main effect of sex ($p=0.086$) on ADORA1 transcription. However, females in estrus differed significantly from both males ($p=0.0066$) and females in diestrus ($p<0.0001$) in multiple comparisons. The effect of challenge was significant in females in estrus ($p<0.001$) but not in males or females in diestrus. In multiple comparisons, there was no effect of Meth, whereas NAC co-administration downregulated ADORA1 in females in estrus after challenge with either Meth ($p<0.0001$) or Sal ($p=0.0001$).

In Tat+ animals (Figure 2B), the main effect of sex accounted for 15.9% of the total variance ($p<0.0001$). Females differed significantly from males in ADORA1 expression ($p<0.0001$), and females in estrus differed significantly from females in diestrus ($p=0.0173$). The interaction of sex and challenge was significant, with a size effect of 2.44 between males and females (F2, 80=42.37, $p<0.0001$) (Figure 2B). Multiple comparisons revealed that males and females in diestrus exhibited similarly reduced ADORA1 transcription compared with

females in estrus, with both Sal and Meth challenge ($p<0.0001$). In contrast, NAC had different effects in males and females; thus, ADORA1 levels were decreased in females in estrus ($p=0.0011$) but increased in females in diestrus ($p=0.000052$) after Sal/NAC compared with Sal challenge. Meth/NAC challenge decreased ADORA1 expression in males ($p=0.013422$) and females in diestrus ($p=0.026$) when compared with Meth challenge, but it had no effect in females in estrus (Figure 2B).

Collectively, the data in Sections 3.3.1 and 3.3.2 indicate that ADORA1 expression in the brain of Tat⁻ and Tat⁺ mice is affected not only by sex but also by estrous cycle phase, with higher expression in females in estrus, and males and females in diestrus exhibiting similar ADORA1 transcription, regardless of the challenge. NAC attenuated the differences in ADORA1 expression associated with sex and estrous cycle in Sal-challenged Tat⁻ and Tat⁺ animals and in Meth-challenged Tat⁻ animals. *One exception is in the context of Tat, Meth/NAC modifies the response of females in diestrus, causing ADORA1 expression to increase compared to males and females in estrus, and also compared to Tat⁻.* Overall, these data suggest that sex differences in ADORA1 expression occurring in Meth-sensitized animals, can be attributed at least partly to ROS-mediated effects, particularly in females.

3.3.3 Effects of Tat expression on ADORA2A transcription and its

interactions with sex—We detected a significant effect of Tat on ADORA2A transcription (Figures 2C and 2D) in mixed models ($F_{1, 126}=72.45$, $p<0.0001$) and a significant interaction between Tat and sex ($F_{2, 126}=32.78$, $p<0.0001$). Similar to ADORA1, the effect of Tat on ADORA2 was detectable following Sal challenge, with Tat⁺ males and females in diestrus expressing significantly lower levels than the corresponding Tat⁻ animals ($p<0.0001$). Conversely, ADORA2A expression in females in estrus was higher in Tat⁺ than Tat⁻ animals ($p<0.0001$).

3.3.4 Effects of sex on ADORA2A transcription and its interactions with challenge

—We identified a main effect of sex and estrous cycle on ADORA2A expression ($F_{2, 126}=149.62$, $p<0.0001$), accounting for 37.22% of the total variance. There was a significant interaction with challenge ($F_{14, 126}=25.848$, $p<0.0001$) that accounted for 14.22% of the total variance, and main effects of challenge ($F_{3, 126}=32.76$, $p<0.0001$) and of interaction with Tat expression ($F_{6, 126}=26.085$, $p<0.0001$).

In Tat⁻ mice (Figure 2C), mixed models identified effects on ADORA2A expression of sex ($F_{2, 46}=44.64$, $p<0.0001$), challenge ($F_{3, 46}=33.474$, $p<0.0001$), and their interaction ($F_{6, 46}=4.38$, $p=0.0016$). Bonferroni's post hoc test revealed that ADORA2A expression was lower in females in diestrus compared with males and females in estrus following Sal challenge ($p=0.0002$). In males, ADORA2A expression was decreased by Meth compared with Sal challenge ($p=0.013$) but this was not detected in females regardless of estrous cycle phase. Also in males, NAC co-administration increased ADORA2A expression in response to Meth challenge ($p=0.0002$), while in females in diestrus, NAC co-administration increased ADORA2A in response to both Sal and Meth challenge ($p<0.0001$).

In Tat⁺ mice (Figure 2D), an effect of sex and estrus cycle ($F_{2, 80}=47.81$, $p<0.0001$) and an effect of the interaction between sex and challenge ($F_{6, 80}=18.94$, $p<0.0001$) on ADORA2A

transcription was detected. Multiple comparisons revealed that ADORA2A was significantly upregulated in females in estrus compared with males ($p<0.00001$) or females in diestrus ($p=0.004$) following Sal challenge (Figure 2B). However, Meth challenge increased ADORA2A in males ($p=0.00006$) but had no effect in females, regardless of estrous cycle phase. Compared with Sal alone, co-administration of Sal/NAC increased ADORA2A in males ($p<0.00001$) but not in females (Figure 2B) compared to Sal alone; whereas co-administration of Meth/NAC did not affect Meth-induced expression of ADORA2A in males and in females in estrus but increased it in females in diestrus ($p=0.036$).

The data in Sections 3.3.3 and 3.3.4 indicate that ADORA2A transcription is affected by sex and estrous cycle, with the level of expression being highest in females in estrus followed by males and females in diestrus. This holds true for Tat⁻ animals challenged with Sal or Meth and for Tat⁺ animals challenged with Sal, but in Tat⁺ animals challenged with Meth, ADORA2A expression is upregulated to a level similar to that seen in females in estrus. In both Tat⁻ and Tat⁺ animals, NAC co-administration with the challenge decreased or abolished the sex differences, suggesting that ROS-regulated pathways contribute to the response differences due to sex and estrous cycle phase.

3.3.5 Effects of Tat expression on ADORA2B expression and its interactions

with sex—With respect to ADORA2B expression (Figures 2E and 2F), there were effects of Tat expression ($F_2, 126=53.478, p<0.0001$), of sex and estrous cycle (size effect=2.44, $F_3, 126=42.697, p<0.001$), and of their interaction ($F_{15}, 126=27.067, p<0.001$). The effect of Tat was characterized by significantly lower ADORA2B expression in Sal-challenged Tat⁺ compared with Tat⁻ animals, regardless of sex or estrous cycle phase ($p<0.001$). The interactions between Tat and sex were detectable in mixed models and further identified in multiple comparisons. For instance, Meth challenge caused an increase in ADORA2B expression in Tat⁺ males ($p<0.0001$) and a decrease expression in Tat⁺ females in diestrus ($p<0.0169$), compared with the corresponding Tat⁻ animals, whereas ADORA2B expression was comparable in Meth-challenged Tat⁻ and Tat⁺ females in estrus. NAC co-administration with Meth increased ADORA2B expression in Tat⁻ females ($p<0.0001$), but decreased it in Tat⁺ females, regardless of estrous cycle phase ($p<0.003$).

3.3.6 Effects of sex on ADORA2B expression and its interactions with

challenge—Main effects of sex, including estrous cycle phase ($F_2, 126=68.738, p<0.0001$) and of challenge ($F_7, 126=55.059, p<0.0001$) on the expression of ADORA2B were detected, and there was also an effect of the interaction between sex and challenge ($F_{14}, 126=24.514, p<0.0001$).

In Tat⁻ mice (Figure 2E), ADORA2B transcription was significantly decreased in males ($p=0.007$) and increased in females in estrus ($p<0.001$) by Meth compared with Sal challenge, but there was no effect in females in diestrus. NAC co-administration had no effect on ADORA2B expression in Sal-challenged mice, whereas NAC plus Meth increased ADORA2B expression in males ($p=0.015$) and females in diestrus ($p<0.0001$) but had no effect on expression in females in estrus, compared with Meth alone. Thus, although NAC elevated ADORA2B expression in Meth-challenged males, the expression level was higher

in females, regardless of estrous cycle phase, than in males challenged with either Meth/Sal or Meth/NAC.

In Tat+ mice (Figure 2F), we identified effects of sex ($F_{2, 80}=19.77$, $p<0.0001$), challenge ($F_{3, 80}=23.24$, $p<0.0001$), and sex and challenge interaction ($F_{6, 80}=16.92$, $p<0.0001$). In Sal-challenged mice, ADORA2B was expressed at higher levels in females, regardless of cycle phase, than in males. However, Meth challenge increased ADORA2B expression in males ($p<0.0001$) but not in females, when compared with Sal challenge. NAC increased ADORA2B expression in Sal-challenged males ($p<0.00001$) but had no effect in females, whereas NAC increased ADORA2B expression in Meth-challenged females in diestrus ($p=0.0373$) (Figure 2C).

Overall, the data presented in Sections 3.3.5 and 3.3.6 indicate that ADORA2B expression was influenced by sex, challenge, and their interaction. Meth challenge caused a significantly higher increase in ADORA2B expression in females in estrus compared with females in diestrus. With respect to Tat expression, Sal-challenged Tat+ males had lower ADORA2B expression than Tat- males, whereas Meth- challenged Tat+ males had higher expression levels compared with Sal-challenged Tat+ or Meth-challenged Tat- males. As observed for the other ADORA molecules, NAC co-administration with Sal or Meth abolished the sex differences in ADORA2B expression.

3.4 Transcriptional changes in DA receptors

We analyzed the transcription of two groups of DA receptors: the D1-like receptors DRD1 and DRD5 (Figure 3) and the D2-like receptors DRD2, DRD3, and DRD4 (Figure 4) in Tat- and Tat+ male and female mice. As described below, DRD expression changes in response to Meth and rescue by NAC were influenced by Tat expression, sex, and estrous cycle phase.

3.4.1 Effects of Tat expression, challenge, and sex on D1-like DRD (DRD1 and DRD5) transcription—For DRD1 expression (Figures 3A and 3B), we detected a main effect of Tat expression ($F_{2, 126}=63.687$, $p<0.0001$) and effects of sex and estrous cycle ($F_{7, 126}=195.63$, $p<0.0001$) and Tat and sex interaction ($F_{14, 126}=13.569$, $p<0.0001$). Lower DRD1 expression was observed in Tat+ than Tat- animals, which was more pronounced in females regardless of estrous cycle, with or without NAC co-administration.

In Tat- mice (Figure 2A), there were significant effects of sex and estrous cycle ($F_{2, 46}=76.23$, $p<0.0001$), challenge ($F_{3, 46}=9.899$, $p<0.0001$), and the interaction $F_{6, 46}=2.339$, $p=0.0497$). DRD1 expression in males and females in estrus was not significantly affected by challenge. Expression was lower in females in diestrus than in males and females in estrus, regardless of the challenge, and the expression level was further decreased by Meth in females in diestrus, as detected in multiple comparisons ($p=0.0012$). Co-administration of NAC with Meth challenge significantly increased DRD1 expression compared with Meth alone in females in diestrus ($p<0.0001$), although the expression level did not reach the level detected in males or females in estrus.

In Tat+ mice (Figure 2B), we also identified effects of sex ($F_{2, 80}=61.20$, $p<0.0001$), challenge ($F_{3, 80}=7.789$, $p=0.0001$), and their interaction ($F_{3, 80}=4.9$, $p=0.0003$). Multiple

comparisons revealed that females in estrus or in diestrus both had lower DRD1 expression than did males, regardless of challenge. For instance, DRD1 levels in Sal-challenged females in diestrus and in estrus were comparable, and both were significantly lower than the levels in Sal-challenged males ($p=0.000537$). NAC co-administration with Sal significantly increased DRD1 expression in males ($p<0.0001$) and females in diestrus ($p=0.00053$), but not in females in estrus. Meth challenge increased DRD1 expression in males compared with Sal ($p<0.00001$) but the levels remained low in females, regardless of estrous cycle phase ($p<0.0001$). NAC co-treatment reversed the Meth effect on DRD1 levels in males, but had no additional effects in females. Females had lower DRD1 expression levels than males, and the levels were not significantly influenced by challenge.

These data indicate that DRD1 expression was influenced by Tat expression, sex, and estrous cycle. Thus, in the absence of Tat, expression in males and females in estrus was similar and higher than the expression in females in diestrus, regardless of challenge. In contrast, in Tat-expressing mice, DRD1 expression was higher in males than females in estrus or in diestrus. Also in Tat+ mice, NAC increased DRD1 transcription levels upon Sal challenge and decreased it upon Meth challenge. NAC also increased DRD1 expression in Sal-challenged, but not Meth-challenged, females in diestrus.

Regarding DRD5 expression (Figures 2C and D), a main effect of Tat ($F_1, 126=46.042$, $p<0.0001$) was characterized by lower DRD5 expression in Tat+ than Tat- animals, regardless of sex, estrous cycle, or challenge.

In Tat- mice, there were effects of sex and estrous cycle ($F_2, 46=31.64$, $p<0.0001$), challenge ($F_3, 46=5.29$, $p=0.0031$), and their interaction ($F_6, 46=4.173$, $p=0.0018$). DRD5 expression was higher in females, regardless of estrous cycle phase, than in males. Upon Meth challenge, DRD5 expression was increased significantly in females in estrus ($p=0.0053$), modestly increased in females in diestrus, and unchanged in males.

In Tat+ mice, we also identified main effects of sex and estrous cycle ($F_2, 80=11.62$, $p<0.0001$), challenge ($F_3, 80=22.22$, $p<0.0001$), and their interaction ($F_6, 80=5.87$, $p<0.0001$). DRD5 expression was significantly higher in Sal-challenged females, regardless of estrous cycle phase, compared with males ($p<0.0001$) (Figure 3E). In contrast to Tat- animals, Meth challenge elevated DRD5 expression in Tat+ males, but not females (Figure 3D). Overall, Tat expression markedly reduced DRD5 expression, especially in females challenged with Meth.

3.4.2 Effects of Tat expression, challenge, and sex on D2-like DRD (DRD2, DRD3, DRD4) transcription—We detected a main effect of Tat expression ($F_1, 146=16.192$, $p<0.0001$) and an effect of sex and estrous cycle ($F_2, 146=87.052$, $p<0.0001$) on DRD2 expression.

In Tat- mice (Figure 4A), there were effects of sex and estrous cycle ($F_2, 46=42.34$, $p<0.0001$), challenge ($F_3, 46=10.55$, $p<0.001$), and their interaction ($F_6, 46=5.527$, $p=0.0002$). Sal-challenged females in diestrus had significantly lower levels of DRD2 compared with similarly treated males ($p=0.0078$) or females in estrus ($p=0.012$). Meth

challenge reduced DRD2 expression in females in estrus to levels below that of Sal-challenged females in estrus ($p<0.0001$) or males ($p<0.0001$), and this was reversed by co-administration of NAC with Meth ($p=0.0051$). In contrast, challenge had no significant effect on DRD2 expression in females in diestrus.

In Tat+ mice (Figure 4B), we detected effects of sex and estrous cycle (F_2 , $80=32.32$, $p<0.0001$), challenge (F_3 , $80=10.77$, $p<0.0001$), and their interaction (F_6 , $80=12.67$, $p<0.0001$). Upon Sal challenge, DRD2 expression was significantly higher in females in estrus compared with either males or females in diestrus ($p=0.0063$, Figure 4B). Under all other challenge conditions, expression of DRD2 was lower in females, regardless of estrous cycle phase, than in males ($p<0.0001$, Figure 4B). Compared with Sal, Meth challenge increased DRD2 in males ($p=0.004$) and decreased it in females in estrus ($p=0.0412$). NAC co-administered with Sal elevated DRD2 levels in males ($p<0.00001$) and in females in diestrus ($p<0.000005$), whereas NAC co-administered with Meth decreased DRD2 expression in males ($p=0.0182$) but not females.

Overall, Meth challenge decreased the expression of DRD2 in females, and this effect was further increased by Tat expression, regardless of estrous cycle phase. Moreover, Meth/NAC challenge increased DRD2 in Tat-, but not Tat+, females.

The expression of DRD3 (Figures 4C and 4D) was significantly affected by Tat expression (F_1 , $146=14.738$, $p<0.0001$), accounting for 13.332% of the total variance, but there were no main effects of challenge or sex.

In Tat- animals (Figure 4C), expression of DRD3 was affected by challenge and NAC only in males. For instance, DRD3 expression levels were significantly increased by Meth/NAC compared with Meth in males ($p=0.001$) and compared with Sal/NAC- or Meth/NAC-challenged females in estrus ($p=0.03$). DRD3 expression in Sal/NAC-challenged males was not significantly different than in Sal-challenged males but was significantly higher than in females in estrus, regardless of the challenge ($p=0.0011$).

In Tat+ mice (Figure 4D), DRD3 expression was significantly affected by sex (F_2 , $80=146.4$, $p<0.0001$), challenge (F_3 , $80=18.63$, $p<0.0001$), and their interaction (F_6 , $80=11.72$, $p<0.0001$) (Figure 4C). After Sal challenge, DRD3 expression was lower in females in diestrus than in males or females in estrus ($p=0.00112$), and Meth challenge further reduced the expression in both males ($p=0.0026$) and females in diestrus ($p<0.000001$). NAC administered with Sal at challenge increased DRD3 expression in males ($p<0.000001$) and females in diestrus ($p=0.000537$), but did not affect the levels in females in estrus. NAC and Meth co-administration elevated DRD3 expression in males compared with Meth alone ($p<0.000001$), but not in females.

In summary, DRD3 expression changes in Tat- mice were largely limited to the influence of NAC in males, particularly the effect of NAC on Meth-challenged mice. In Tat+ mice, however, DRD3 expression was more variable in females than in males, and only Sal and Sal/NAC challenge resulted in sex-dependent differences in expression.

In the case of DRD4 (Figures 4E and 4F), we did not identify a main effect of Tat expression ($F_{1, 146}=8.5665$, $p<0.061$) but there was a main effect of sex and estrous cycle ($F_{2, 146}=3.954$, $p=0.0216$).

In Tat- mice (Figure 4E), there was an effect of sex and estrous cycle ($F_{2, 46}=8.671$, $p<0.0001$) particularly in Sal-challenged animals, in that females in estrus and diestrus both expressed higher DRD4 levels than did males ($p<0.0001$ and $p=0.0048$, respectively). Meth challenge increased DRD4 expression in males compared with Sal ($p<0.0001$) and compared with Meth-challenged females, regardless of estrous cycle phase ($p<0.0001$). In males, Meth/NAC reduced the expression of DRD4 compared with Meth alone.

In Tat+ mice (Figure 4F), there were no significant effects of challenge, sex, or estrous cycle on DRD4 expression. Upon Meth challenge, DRD4 expression in males was higher than in females in diestrus ($p=0.044$) or in estrus ($p=0.0179$). In females in diestrus, NAC/Meth challenge resulted in elevated DRD4 expression compared with Sal alone ($p<0.0001$) or Meth alone ($p<0.0001$).

Overall, the data presented in Sections 3.4.1 and 3.4.2 indicate that DRD4 transcription was not affected by Tat expression but was affected by sex and estrous cycle, particularly upon Meth challenge, regardless of Tat expression. However, Meth/NAC challenge caused a significant decrease in DRD4 expression compared with Meth alone in Tat- mice, whereas a similar decrease detected in Tat+ mice did not reach the level of statistical significance in multiple comparisons.

3.2. 3.5. Effects of Tat expression, challenge, and sex on DRD1 and DRD2 protein expression in the brain

To verify the mRNA results, we next examined DRD1 and DRD2 protein expression by IHC staining of the contra-lateral brain hemisphere from the same mice used for the transcriptional analysis. For technical reasons, mRNA levels were examined in tissue from the caudate-putamen, whereas we stained protein in sections of the entire hemisphere (see Methods). The images in Figures 5 and 6 are focused on the hippocampal area, but expression levels were quantified in sections of the whole hemisphere. We note that the caudate and hippocampus are adjacent structures, and the hippocampus not only receives direct and indirect projections from the caudate but also plays an important role in CNS impairment following HIV infection and drug abuse^{6,49}. Importantly, we found that the Tat, sex, and challenge-specific differences in whole-brain and hippocampal DRD1 (Figure 5) and DRD2 (Figure 6) protein expression were consistent with the patterns observed for DRD1 and DRD2 mRNA levels.

The main significant effect for DRD1 expression (Figure 5A and 5B) was derived from Tat expression ($F_{3, 80}=10.91$, $p=0.0017$), and the main significant effects for DRD2 expression were derived from Tat expression ($F_{4, 80}=15.36$, $p=0.0006$) and the interaction between sex, challenge, and NAC ($F_{12, 80}=7.587$, $p=0.007$). Multiple comparisons revealed that differences between Tat+ and Tat- mice could be attributed to females challenged with Sal ($p=0.0323$) or with Meth ($p=0.0363$). Overall, DRD1 and DRD2 expression levels throughout the brain were consistent with the transcriptional patterns in the caudate (Figures

5B and 6B). Females showed higher susceptibility to Meth as a challenge, and a lower response to NAC in the context of Tat expression. The distribution of positive cells suggested that DRD1 and DRD2 expression was disrupted by Tat expression and decreased by Meth challenge in both male and female mice. Meth challenge substantially decreased the expression of both receptors in Tat⁻ males and females and in Tat⁺ males. NAC treatment was effective in preventing the effects of Meth challenge on DRD1 and DRD2 protein expression in all animals except Tat⁺ females, in which NAC was without effect.

4. Discussion

The main goal of this study was to investigate whether sex influences the interactions between Tat expression and Meth sensitization with respect to transcriptional changes of dopaminergic molecule expression in the brain. We previously showed that Tat expression enhances the locomotor behaviors of male mice using the same Meth sensitization and challenge paradigm employed here²⁰. Another goal of the present study was to determine whether the Tat⁻ and sex-dependent differences in locomotor enhancement and molecular alterations induced by Meth might be mediated by changes in ROS levels in the brain. The results obtained in this iTat mouse model of neuroHIV have important implications for understanding the effects of sex differences in Meth-associated behaviors and the response to therapy of HIV-infected individuals.

We found that locomotor activity following Meth sensitization was significantly increased by Tat expression in the brain, which confirmed our previous observations in males²⁰ and extended them to show similar responses in females. However, Tat expression had differential influences on the locomotor response of females and males in the presence of NAC. Thus, NAC promoted a partial but significant decrease in locomotor sensitization in Tat⁻ males and females and Tat⁺ males, but not in Tat⁺ females. This finding suggests that mechanisms dictating interactions between Meth and Tat that may be influenced by sex.

Locomotor activity in response to Meth in females was likely independent of estrous cycle, even though the estrous cycle phase was determined 24 hours after the behavioral testing. On the other hand, locomotor patterns were clearly influenced by Tat expression, while the response to NAC was influenced by sex and Tat expression interactions. Tat⁺ females differed from Tat⁻ females and from males in general, by not responding to NAC with an attenuated sensitization. By determining estrous cycle at termination, we aimed at correlating differences between Tat⁺ and Tat⁻ males and females, and to address whether female hormones such as progesterone can play a role. The interactions between progesterone levels and dopaminergic activity and baseline levels have been suggested⁵⁰. We found that females in diestrus phase had variable but higher levels of progesterone compared with females in other phases, which is consistent with the results of McLean and coworkers⁴⁷. However, a second peak of progesterone may occur at the end of proestrus^{47,51-53}, although we did not detect such a peak. Potential reasons may be that progesterone levels can be influenced by factors, such as genetics and diet^{54,55}, which were not controlled for in our experiments. We found that the estrous cycle affected the expression of DRDs, as did the interaction between estrous cycle and Tat expression. This observation suggests that the estrous cycle impacts the dopaminergic system. A gap in our

study is that hormone levels were not collected during behavior tests, which will be addressed in the future.

In our previous work with male iTat mice, we observed that DRD1 and DRD2 expression in the brain was affected by Tat rather than by Meth, while the expression of ADORAs was not influenced by Tat but by Meth challenge²⁰. In the present study, we found that the estrous cycle was an additional factor in the complex interaction between neurotransmitters and behaviors, and exhibited important interactions with Tat expression. For instance, DRD1 expression in Tat⁻ females in estrus was similar to that in Tat⁻ males and higher than Tat⁻ females in diestrus, whereas in Tat⁺ animals, both female groups had significantly lower DRD1 expression than did males, regardless of challenge. Moreover, NAC did not significantly affect DRD1 expression in females. DRD2 transcription was similar in Tat⁻ males and females; however, the expression was significantly decreased by Meth challenge in the females in estrus or diestrus compared with the males, and this difference was abolished by NAC. In contrast, DRD2 expression in Tat⁺ mice varied between females and males and between females in estrus vs diestrus, and NAC had no effect on DRD2. Tat⁺ females in diestrus also exhibited changes in DRD3 transcription upon Meth challenge that were refractory to NAC.

IHC staining revealed differences in the expression and distribution of DRD1 and DRD2 proteins in whole sagittal hemisphere sections that were consistent with the changes in receptor transcription detected in the caudate-putamen. IHC staining provided an opportunity to examine expression throughout the brain, particularly the hippocampus, which is a caudate-adjacent region and receives direct and indirect projections from the caudate^{56–60}. Neurons in the hippocampus are also known to be severely damaged by HIV infection and Meth abuse^{49,61}. We detected strong and well-organized neuronal DRD expression in the hippocampus, with changes in response to challenge, in a sex-dependent manner that were representative of other areas of the brain, consistent with our previous results in the caudate²⁰. For technical reasons, we were unable to detect DRD3 in brain tissue. However, variation in dopamine receptor expression across the estrous cycle and between sexes has been described^{62–64}, and transcription of inflammatory molecules in the brain also varies with estrous cycle phase⁶⁵.

ADORAs and DRDs form heterodimeric complexes that regulate DA signaling. For instance, ADORA1A negatively regulates DRD1 while ADORA2A can antagonize DRD2^{66–68}. The complexity of the interactions between Tat expression and estrous cycle detected here were evident in the ADORA transcriptional patterns. Our study investigated ADORA expression only at the transcriptional level, which cannot be directly extrapolated to function. However, our results demonstrate that our current understanding of the dynamics of sex differences in drug abuse-related behaviors and the impact of HIV infection is currently insufficient to address neurological disorders resulting from their interaction with a unified therapeutic strategy.

DA levels in the brain are known to differ between sexes. For instance, an *in vitro* study using striatal tissue from gonadectomized female and male mice and intact male mice showed that co-infusion of estrogen, but not testosterone, significantly reduced Meth-

induced increases in DA production, suggesting that differential neuromodulatory effects that may contribute to sex differences in the response to Meth⁶⁹. These observations have been further validated in rodent models of behavior and neuroprotection^{70–76}. Here, we showed that the expression of DRDs may be affected by Tat expression in a sex-dependent manner. In our model, progesterone levels may explain some of the sex differences, particularly the strong sensitivity of females in diestrus to DRD transcriptional changes in the presence of Tat. However, further studies will be necessary to more directly assess the contribution of progesterone and other sex hormones to differences in the dopaminergic system caused by HIV and in the context of drug abuse.

We previously showed that administration of NAC can prevent and reverse select effects of acute Meth administration, such as hyperthermia, in drug-naïve mice^{29,30}, suggesting that ROS may signal some of the Meth-induced molecular changes, particularly those associated with mitochondrial activity. Here, we co-administered NAC with the challenge to test the hypothesis that ROS may participate in signaling drug sensitization behaviors and DA receptor changes. We found that NAC treatment was more effective in attenuating the effects of Meth challenge in Tat– compared with Tat+ animals and in Tat+ males compared with Tat + females. Consistent with this, ROS production and the oxidative stress response have been reported to be influenced by sex in experimental models and in humans^{77–79}. Our results additionally suggest that antioxidant therapies may be beneficial for Meth abuse, but their efficacy is likely to be affected by sex and additional factors such as HIV infection.

We have shown here that Tat expression, sex differences, and their interactions influence the effects of Meth on locomotor activity and brain expression levels of DRDs, particularly DRD1 and DRD2, at the mRNA and protein level. Our results are supported by previous studies on gender differences in the response to amphetamines, which showed a reduced DA response and a pervasive comorbidity of depression among female compared with male Meth users⁸. Measurable gender differences have been reported in areas with high number of women that are Meth abusers and HIV -infected, especially regarding higher incidence of risk behaviors and lower age of initiation, which is significant in women compared to men^{80,81}. Our findings are relevant to understanding sex differences in HIV-associated neurocognitive disorders. Sex differences in the prevalence and profile of HIV-associated neurocognitive deficits may be explained by biopsychosocial risk factors that are more prevalent in HIV-infected women⁸². Collectively, our results suggest that HIV infection, Meth sensitization, and sex differences, independently and in combination, play important roles in the development of addictive behaviors, including effects on the dopaminergic system that may explain trends in cognitive deficits. Our results also suggest that therapeutic strategies targeting ROS signaling pathways may be helpful in the treatment of Meth-addicted HIV-infected males, but may have more limited benefits in females.

5. Conclusions

We have shown that expression of the HIV peptide Tat leads to enhanced Meth-induced locomotor sensitization and changes in the expression of molecules of the DA system in a sex-dependent manner. The results also suggest vulnerabilities in Tat+ females, in which Meth-induced changes in DA receptors are not prevented by treatment with NAC and

suppression of ROS. The interactive effects of Tat, sex, estrous cycle phase, and Meth challenge on expression of DRD1 and DRD2 may explain, at least in part, the observed sex differences in Meth responses.

Authorship and Acknowledgements

EJB performed the qRT-PCR analyses. HK performed the IHC analyses. LAB participated in the study design, trained EJB and HK, supervised the experiments, and wrote the Material and Methods section. AR participated in discussions and performed statistical tests. JK performed mouse phenotyping assays and executed the Meth sensitization regimen. SS participated in the study design, performed the challenges and NAC treatments, and helped in writing of the manuscript. MCGM obtained funding, designed the experiments, supervised the research, performed statistical analysis, and wrote the manuscript. We thank Anne O'Rourke for the hard work of editing this manuscript, Christine Auciello (San Diego Biomedical Research Institute) for administrative assistance, and Bruno Conti (The Scripps Research Institute) for exciting discussions and for providing laboratory space during part of this study.

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Highlights

1. Induction of HIV Tat expression in the brains of iTat transgenic mice, a model of neuroHIV, replicates changes in the transcription of dopaminergic system genes observed in HIV-infected humans.
2. Tat induction enhances locomotor sensitization in the iTat model of methamphetamine (Meth) sensitization and challenge.
3. Male and female Tat- and Tat+ mice differ in their response to Meth and to the beneficial effects of treatment with the reactive oxygen species scavenger N-acetyl cysteine (NAC).
4. NAC decreases locomotor behaviors in Meth-challenged Tat- males and females and Tat+ males, but Tat+ females are refractory to this treatment.
5. Tat expression and sex differences differentially affect brain expression of dopamine receptor (DRD) subtypes and DRD regulatory adenosine receptor (ADORA) subtypes in iTat mice in response to Meth and NAC.

Figure 1. Effect of sex and NAC treatment on locomotor activity of Meth-sensitized Tat- and Tat+ mice.

(A) Motor activity of male and female Tat- and Tat+ mice was recorded for 30 min starting 5 minutes after challenge with Meth or saline (Sal). NAC at 100 mg/kg or Sal vehicle were administered at the same time as the challenge. Data are presented as the mean $\pm$ SEM of $n=10-19$ mice/group. Main effects: Tat ($F_{1, 129}=90.16$, $P<0.0001$), NAC ($F_{1, 129}=13.2$, $p<0.05$), METH ($F_{1, 129}=304.8$, $P<0.0001$), and NAC $\times$ Meth $\times$ Sex interaction ($F_{1, 129}=5.7$, $P<0.05$). * $p<0.05$ by ANOVA followed by Bonferroni's post hoc test. (B) Serum progesterone levels were determined by ELISA at 24 h after challenge. Mean $\pm$ SD of duplicate samples.

Figure 2. Effect of Tat expression, sex, and estrous cycle phase on ADORA transcription in the brains of Meth-challenged and NAC-treated mice.

Groups of Tat- (A, C, E) and Tat+ (B, D, F) were Meth sensitized, underwent a 7-day washout period, and were then challenged with saline alone (Sal), Meth alone, Sal plus NAC (Sal/NAC), or Meth plus NAC (Meth/NAC). At 24 h after challenge, the mice were euthanized and samples of caudate-putamen were analyzed by qRT-PCR for the expression of (A, B) ADORA 1, (C, D) ADORA 2a, and (E, F) ADORA 2b mRNA. Receptor mRNA levels were normalized to the mean GAPDH mRNA and 18S rRNA levels. * $p < 0.05$ in mixed models and Bonferroni's multiple comparisons. Comparisons between Tat+ and Tat- mice are described in the text.

Figure 3. Effect of Tat expression, sex, and estrous cycle phase type 1 DRD (DRD1 and DRD5) transcription in the brains of Meth-challenged and NAC-treated mice.

Groups of Tat- (A, C) and Tat+ (B, D) mice were challenged as described for Figure 2. At 24 h after challenge, the mice were euthanized and samples of caudate-putamen were analyzed by qRT-PCR for the expression of (A, B) DRD1 and (C, D) DRD5 mRNA.

Receptor mRNA levels were normalized to the mean GAPDH mRNA and 18S rRNA levels.

* $p < 0.05$ in mixed models and Bonferroni's multiple comparisons. Comparisons between Tat + and Tat- mice are described in the text.

Figure 4. Effect of Tat expression, sex, and estrous cycle phase on type 2 DRD (DRD2, DRD3, and DRD4) transcription in the brains of Meth-challenged and NAC-treated mice. Groups of Tat- (A, C, E), and Tat+ (B, D, F) mice were challenged as described for Figure 2. At 24 h after challenge, the mice were euthanized and samples of caudate-putamen were analyzed by qRT-PCR for the expression of (A, B) DRD2, (C, D) DRD3, and (E, F) DRD4 mRNA. Receptor mRNA levels were normalized to the mean GAPDH mRNA and 18S rRNA levels. * $p < 0.05$ in mixed models and Bonferroni's multiple comparisons. Comparisons between Tat+ and Tat- mice are described in the text.

Figure 5. Quantification of DRD1 protein expression in the brains of Meth-challenged and NAC-treated mice by immunohistochemistry.

Groups of male and female Tat- and Tat+ mice were challenged as described for Figure 2.

(A) At 24 h after challenge, serial sagittal sections of one hemisphere were stained with anti-DRD1 antibody. Images show 20x magnification of the hippocampal region, and rectangles containing cells of interest are additionally shown in the smaller images. (B) Quantification of DRD1 protein expression. Stained serial sagittal sections of one hemisphere were imaged and digitized using ImageJ. DRD1 expression is presented as the percentage area of positive staining normalized to the total brain area. Mean $\pm$ SEM of $n=7-14$ males and females/group. * $p<0.05$ by 2-way ANOVA and mixed effects analysis with Bonferroni's post hoc test. Females in estrus showed the same expression pattern as animals in other estrous cycle phases.

Figure 6. Quantification of DRD2 protein expression in the brains of Meth-challenged and NAC-treated mice by immunohistochemistry.

Groups of male and female Tat- and Tat+ mice were challenged as described for Figure 2 (A) At 24 h after challenge, serial sagittal sections of one hemisphere were stained with anti-DRD2 antibody. Images show 20x magnification of the hippocampal region, and rectangles containing cells of interest are additionally shown in the smaller images. (B) Quantification of DRD2 staining. Stained serial sagittal sections were imaged and digitized using ImageJ. DRD2 expression is presented as the percentage area of positive staining normalized to the total brain area. Mean $\pm$ SEM of $n=7-14$ males and females/group. * $p<0.05$ by 2-way ANOVA and mixed effects analysis with Bonferroni's post hoc test. Females in estrus showed the same expression pattern as animals in other estrous cycle phases.

Table 1.

Primers used in this study

<i>Mus musculus</i> Gene	Qiagen Catalog Number
GAPDH	PPM02946E
18S	PPM57735E
DRD1	PPM04267A
DRD2	PPM04288A
DRD3	PPM04829A
DRD4	PPM04830C
DRD5	PPM04828A
ADORA1	PPM04290B
ADORA2a	PPM03472F
ADORA2b	PPM04282B