Published in final edited form as:

Brain Res. 2019 May 15; 1711: 16–22. doi:10.1016/j.brainres.2019.01.010.

Sex differences in memory and intracellular signaling after methamphetamine binge treatment.

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Abstract

Methamphetamine is a neurotoxic psychostimulant known to cause cell death and terminal degradation of dopaminergic neurons in the striatum concomitant with memory deficits. However, most of the research studies have not examined the influence of sex on these changes. In this study we compared the effects of a binge regimen of methamphetamine (four injections of 4mg/kg) on male, female, and ovariectomized (OVX) female Sprague-Dawley rats. We show that male and OVX female animals had a deficit in a novel object recognition task, while intact females did not show this deficit. Neurochemical analysis of the same animals indicated higher levels of FosB protein in caudate-putamen (CPu) and nucleus accumbens (NAc) of the male animals than intact or OVX females. Methamphetamine also increased Bcl-2 protein levels in CPu of all the cohorts. We did not find a significant effect of methamphetamine on the dopamine neuron markers tyrosine hydroxylase (TH) or dopamine transporter (DAT) 7 days after methamphetamine administrations. Our behavioral and neurochemical studies indicate that methamphetamine differentially affects male and female animals and shows sex differences in memory and molecular mechanisms in the striatum of these animals.

Keywords

Methamphetamine; neurotoxicity; sex differences

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1. Introduction

Methamphetamine is a potent psychostimulant that is widely abused, with an estimated 6.5% of the United States population having used methamphetamine in their lifetime (SAMHSA, 2017). Its addictive and neurotoxic potentials are significant causes for concern (reviewed by Halpin, Collins, & Yamamoto, 2014).

The behavioral consequences of these changes have been shown in a variety of contexts. Methamphetamine abusers show impairments in several cognitive domains, including attention, executive function, and multiple memory processes (Potvin et al., 2018). Similar behavioral deficits exist in rodents, which are often used as drug abuse models. In a “binge” model, in which several injections are given over the course of a single day, rats are impaired on a novel object recognition (NOR) task (Bisagno, Ferguson, & Luine, 2002). These memory deficits have been shown to correlate with lower levels of dopamine transporter (DAT) in striatum in both abstinent human users (McCann et al., 2008) and rats subjected to binge-like forced administration paradigms (Belcher, Feinstein, O’Dell, & Marshall, 2008).

In general, less research has been done on female than on male animals (Clayton, 2016). However, both behavioral and neurological responses to methamphetamine appear to differ by sex. Compared with males, female rats show higher responding across a variety of addiction markers, including higher drug intake during extended access self-administration and a greater degree of meth-primed reinstatement after a period of abstinence (Reichel, Chan, Ghee, & See, 2012). Female rats also exhibit greater methamphetamine-induced locomotor increases and stereotypic behavior (Milesi-Halle, McMillan, Laurenzana, Byrnes-Blake, & Owens, 2007). In contrast, female mice have been shown to be more resistant to methamphetamine-induced depletions of dopamine and its metabolites (Bourque, Liu, Dluzen, & Di Paolo, 2011).

Female and male mice also show opposing patterns in expression cell-survival pathways in striatum: while a large dose of methamphetamine sufficient to cause toxicity increases the levels of Bcl-2, a pro-survival/anti-apoptotic marker, in males, it does not impact Bcl-2 expression in females (Bourque et al., 2011). Previous studies have shown increases in FosB in response to methamphetamine treatment, while FosB knockouts show increased susceptibility to neurotoxic insult (Kuroda, Ornthalai, Kato, & Murphy, 2010). It is not clear whether FosB expression in response to methamphetamine differs by sex, but given its role in cell survival and its sexually dimorphic expression in response to other psychostimulants such as cocaine (Zhou et al., 2016), we believe this is worthy of investigation.

One possible explanation for these differences lies in the different levels of gonadal hormones present between sexes. Estrogens and their receptors have been implicated in a wide variety of neuroprotective processes and pathways (Baez-Jurado et al., 2018). Introducing estrogen or progesterone to ovariectomized rats lacking circulating gonadal hormones ameliorates cognitive deficits induced by methamphetamine (Ghazvini et al., 2016). One proposed mechanism for the neuroprotective effects seen in females is the activation of the PI3K/AKT and ERK pathways via the G protein-coupled estrogen receptor

(GPER), resulting in upregulation of anti-apoptotic cascades (Bourque et al., 2011). Given the observations of sex differences in humans and rodents in response to neurotoxic insult, female gonadal hormones likely play a role in neuroprotection against methamphetamine.

The present study sought to assess differences between male and female rats, as well as ovariectomized (OVX) females lacking gonadal hormones, in the behavioral effects of methamphetamine and markers of neurotoxicity. The injection paradigm used (“binge” administration, 4 injections over one day) has been shown in previous studies to induce neurotoxic effects (Belcher, O’Dell, & Marshall, 2005). In addition to the caudate-putamen (CPu), the nucleus accumbens (NAc) has been shown to suffer DA depletion associated with methamphetamine-induced learning and memory deficits in rats (Gutierrez, Williams, & Vorhees, 2018). The hippocampus (HIP) has also been shown to be degraded by high-dose methamphetamine use, and is known to underlie some tasks involving object recognition behaviors (Warburton & Brown, 2015). Therefore, we selected these three areas for study. By including intact female as well as OVX female animals, we intend to assess whether previously observed changes in males exist in hormonally unperturbed females as well as whether these changes are dependent on gonadal hormones.

2. Results

2.1 Novel object recognition

A 2×3 (drug x sex) ANOVA revealed main effects for drug ($F(1,30)=7.73$ $p=.009$) and sex ($F(2,30)=5.13$, $p=.012$), as well as a significant interaction ($F(2,30)=3.57$, $p=.041$). As shown in Figure 1, during the novel object phase of testing, methamphetamine reduced object recognition ($p=.026$) and OVX females ($p=.007$), but not intact females ($p>.05$). No significant differences between groups were seen in total time exploring during testing ($p>.05$); see Figure 2.

2.2 TH levels in CPu, NAc, and HIP.

As shown in Figure 3, although there was a main effect of sex in NAc ($F(2,12)=5.258$, $p=.023$), none of the areas showed a significant drug effect within sex comparisons. In NAc, males showed higher TH expression than OVX females ($p=.023$). CPu and HIP did not show main effects of sex. TH levels varied by sex with females and OVX lower than males, but not by drug ($F(1,12)=1.447$, $p=0.252$).

2.3 DAT levels in CPu, NAc, and HIP

As shown in Figure 4, CPu DAT levels differed by sex ($F(2,12)=9.352$, $p=0.004$), with males higher than females ($p=.008$) or OVX ($p=.007$). However, drug-treated animals overall did not differ significantly from saline ($F(1,12)<0.001$, $p>.05$). Pairwise comparisons revealed that drug-treated males showed higher DAT levels than drug female ($p=.024$) and drug OVX ($p=.042$) animals. Similarly, NAc DAT levels did not differ by drug ($F(1,12)=0.673$, $p>.05$) but did differ by sex ($F(2,12)=9.259$, $p=.004$), with male saline higher than female saline ($p=.002$) and OVX saline ($p=.038$). However, in HIP, no significant differences existed by sex ($F(2,12)=0.224$, $p>.05$) or drug ($F(1,12)=0.016$, $p>.05$).

2.4 Bcl-2 levels in CPu, NAc, and HIP

As shown in Fig 5, CPu Bcl-2 levels showed a main effect for sex ($F(2,12) = 10.18, p=.003$) and drug ($F(1,12) = 12.7, p=.004$). Specifically, methamphetamine-treated animals showed higher Bcl-2 levels than their saline counterparts within sex, and OVX levels were lower than males ($p=.004$) and females ($p=.009$). Although in NAc Bcl-2 levels were not affected by methamphetamine treatment ($F(1, 12) = 1.409, p>.05$), levels did differ by sex ($F(2,12) = 38.18, p<.001$). Females had higher levels of Bcl-2 than OVX ($p=.002$) and males higher than either females ($p=.004$) or OVX ($p<.001$). HIP Bcl-2 levels were not impacted by sex ($F(2, 12) = 3.247, p>.05$) or drug ($F(1, 12) = 0.021, p>.05$).

2.5 FosB levels in CPu, NAc, and HIP

As summarized in Figure 6, in CPu, there was a main effect on FosB expression for both sex ($F(2, 12) = 30.31, p<.001$) and drug ($F(1, 12) = 8.563, p=.013$), with drug-treated animals expressing higher levels overall. Male FosB levels were higher than intact females ($p=.001$) and OVX ($p<.001$). NAc FosB levels were not impacted by drug administration ($F(1,12) = 0.443, p>.05$), but did differ by sex ($F(2, 12) = 16.84, p=.0003$), with saline males significantly higher than saline OVX ($p=.007$) and drug males significantly higher than drug OVX ($p=.002$). However, in HIP there were no significant differences between sexes ($F(2, 12) = 1.519, p>.05$) or drug/saline conditions ($F(1, 2) = 0.169, p>.05$).

3. Discussion

Our studies show that a binge methamphetamine administration impairs object recognition memory in male animals. We also show the novel finding that this deficit does not exist in intact females, but is present in OVX females lacking gonadal hormones. This is consistent with previous research that has found that exogenous estrogen and progesterone treatment of OVX females prevents spatial memory deficits related to methamphetamine exposure (Ghazvini et al., 2016). Our findings further clarify the role of sex in methamphetamine-induced recognition memory deficits and provide additional evidence for the sex differences in the behavioral effects of psychostimulants.

Previous research has shown that the striatum is particularly affected by methamphetamine abuse in humans, affecting motor and memory functions (Volkow et al., 2001). FosB is an immediate early gene that is induced in the striatum by psychostimulants (Chandra & Lobo, 2017) as well as by neurotoxic insult to dopamine neurons outside of the striatum (Patterson, Kim, Goudreau, & Lookingland, 2016). We show here that a binge treatment of methamphetamine also induces FosB protein in striatum, and that males show higher expression of FosB than either intact or OVX females. While previous research has focused on its rapid increase in expression, we show that this change persists for at least one week after methamphetamine administration in this paradigm, and that its expression both at basal levels and in response to methamphetamine differs by sex. Male expression of FosB was higher than either intact or OVX females, though ovariectomy did not seem to alter expression, indicating a hormone-independent effect. The increase in FosB seen here may be due to elevations in the level of FosB, a stable isoform that has been shown to persist for longer and has been implicated in the addictive effects of psychostimulants (Cornish, Hunt,

Robins, & McGregor, 2012). Our work extends the role of FosB in stimulant-induced behavioral dysfunction.

Bcl-2 is an anti-apoptotic, pro-survival protein that is antagonized by pro-apoptotic proteins in many conditions of cellular distress (Cui & Placzek, 2018). We found increases in Bcl-2 across sexes in CPu, though OVX (both saline and drug) showed lower levels than either males or intact females. Likewise, in striatum, OVX animals showed lower expression of Bcl-2 in NAc than males, with females intermediate between the two. This may indicate a unique deficit in OVX animals beyond merely the absence of estrogen-mediated cell survival signaling as in males. In this way, OVX females are not as directly analogous to males as they appear to be in their behavior.

The changes we observed in Bcl-2 and FosB levels in CPu after methamphetamine administration are consistent with previous results in mice, which showed higher Bcl-2 levels in methamphetamine-treated males (Bourque et al., 2011). However, we did not observe differences between males and intact females, though OVX levels were lower overall. This is in contrast to previous research using a bolus (40mg/kg) dose of methamphetamine in mice, which showed that males showed a greater increase in Bcl-2 than females in response to the drug (Dluzen et al., 2011). The difference in dosing regimen may account for the disparity between our results and those previously described.

We did not observe any neurochemical alterations in the hippocampus. Others have reported that the particular behavioral task here (novel object recognition) is less dependent on the hippocampus than other more spatially-demanding tasks (Barker & Warburton, 2011). Methamphetamine has also been shown to have toxic effects in striatum following a methamphetamine binge administration, resulting in greater impairment to recognition memory than spatial tasks (Bisagno et al., 2002), and similar striatal changes in humans correlate with memory deficits (Volkow et al., 2001). In accordance with our behavioral findings, the hippocampus may be more critical to memory for the spatial object-in-place (OIP) task (Reichel, Ramsey, Schwendt, McGinty, & See, 2012) while the recognition-based NOR task may depend on the dorsal striatum (Qiao, Wang, Ma, Li, & Liang, 2017). Our results provide further evidence for the behavioral dissociation of hippocampal and striatal memory in object recognition tasks.

Previous studies have shown TH and DAT reductions as an indicator for the toxic effects of methamphetamine (Chiu & Schenk, 2012). This could indicate a sparing of presynaptic dopamine terminals or a recovery by the time of sacrifice. We did not find an effect of methamphetamine on the TH or DAT 7 days after methamphetamine administrations. We did, however, observe increases in Bcl-2 in males, intact females, and OVX females. In the short term, increases in pro-apoptotic BAX and decreases in anti-apoptotic Bcl-2 have been observed (Raineri et al., 2012); we show an increase in Bcl-2 one week after last injection, possibly indicating a compensatory survival mechanism one week after methamphetamine treatment. The changes in Bcl-2 and FosB, but not TH or DAT, indicate that the observed disruption is possibly in the postsynaptic targets of DA neurons in striatum rather than presynaptic terminal degradation. Additional timepoints should be studied to clarify these possibilities.

Estrogen exerts its effects on cell survival in a number of ways that may be differentially altered by methamphetamine: the effects of methamphetamine on body temperature, DAT functioning, and dopamine release are all impacted by estrogen (Dluzen & McDermott, 2002). The mechanisms involved are varied and a continued focus of research. The G protein-coupled estrogen receptor (GPER1) is upstream of the MAPK/ERK and PI3K/mTOR pathways, both of which have been shown to be sexually dimorphic, and GPER1 is upregulated in male mice after high-dose methamphetamine treatment (Bourque et al., 2011). The estrogen receptors ER α and ER β are also implicated in the higher survival of neurons in females in response to other neurotoxic insults (Cheng, Hung, & Chu, 2016). It remains to be seen which of these are most important for the sex differences in memory we document here, as well as the mechanisms by which they exert their effects.

Based on our molecular findings, other factors beyond current hormonal milieu (e.g. organizational effects) should be investigated. That OVX did not directly mirror male animals in their molecular indicates a difference beyond the presence or absence of estrogen and progesterone. For example, the presence of estrogen during early life has effects on the functioning of striatal DA neurons in adulthood, and neonatal treatment of female rats with estradiol results in higher striatal levels of DA and enhanced responding to stimulants in adulthood, long after hormone levels return to baseline (Cruz et al., 2014). These organizational effects may persist in our adult OVX animals. Androgens may also account for some of the sex differences observed, as these may exacerbate many of the neurotoxic mechanisms described, particularly by increasing dopamine release induced by methamphetamine (Dluzen & McDermott, 2006). Androgens are present and likely synthesized to some degree in the mesocorticolimbic system, including NAc, though this area of research is sparse and the significance of these chemicals in normal and dysfunctional states is not well understood (Tobiansky, Wallin-Miller, Floresco, Wood, & Soma, 2018). Future studies involving castrated male animals might be instructive in this regard. Our results add to the body of research showing sex differences in the response to methamphetamine, and underline the importance of the consideration of sex in treatment options, especially those involving pharmacological interventions.

4. Experimental Procedure

4.1. Animals

Adult (8-week) Sprague-Dawley male and female rats, as well as females having undergone ovariectomy in adulthood were obtained from Charles River, Raleigh, NC. Females were freely-cycling. Animals were housed individually with no food or water restriction and maintained on a standard 12/12h light/dark cycle. All activities were approved by the Hunter College/CUNY Institutional Animal Care and Use Committee and were in compliance with the NIH Guide for the Care and Use of Laboratory Animals.

4.2. Drugs and antibodies

Methamphetamine HCl was obtained from Sigma. Antibodies for Bcl-2 (#2876) and FosB (#2251) were obtained from Cell Signaling Technologies (Beverly, MA). Antibodies for TH (#25269), DAT (#14002), and α -tubulin (#8035) were obtained from Santa Cruz

Technologies (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies (anti-mouse and anti-rabbit) were obtained from Amersham/GE Healthcare.

4.3. Drug administration

After one week of acclimation after arrival at the animal facility, animals were handled for one week to reduce anxiety. Animals were randomly assigned to receive either methamphetamine or saline. Each animal received four intraperitoneal injections of 5 mg/kg bodyweight methamphetamine or physiological saline, with injections separated by two hours. Animals were monitored for signs of distress for several hours; none expired or required euthanasia.

4.4. Novel object recognition

Novel object recognition was conducted in a black open-topped box measuring 32 × 45cm with 30cm high walls. Three days after the injections, animals were acclimated to the testing apparatus. For three successive days, animals were placed individually into the apparatus for ten minutes at a time to habituate to the arena before any objects were introduced. Seven days after injection, animals were given the novel object recognition task. The task consists of two phases. During the first phase, animals are placed in the box with two identical objects and allowed to explore for five minutes, then returned to their home cage. One hour later, animals are placed into the same box with one object replaced by a different object (the novel object). Objects included cans and bottles approximately 7cm in diameter and 10–20cm high. Interaction time with the original and the novel objects was recorded using a digital video camera and scored by three independent observers naïve to experimental condition, and their scores were averaged. Interaction was defined as an animal facing and engaging with the object within 1 cm or whisking the object. Animals were excluded from analysis if they did not interact with either object during either the first or second phase, resulting in a final N of 5 for male saline, 6 for male drug, 8 for female saline, 6 for female drug, 6 for OVX saline, and 5 for OVX drug. Because of high inter-subject differences in absolute time spent with objects, data was converted to percentages of time spent with the novel object relative to total time spent exploring both objects combined. The day after the behavioral testing (eight days after injection), animals were exposed to carbon dioxide and sacrificed by rapid decapitation. Brains were extracted and snap frozen, then stored at –80°C for later use.

4.5. Protein preparation and Western blot

Brain areas selected for study were dissected on a cold plate and stored at –80°C until homogenization. Punches of brain tissue were taken from 1mm coronal sections, consulting a rat brain atlas for location (Paxinos & Watson, 2007). Tissue was homogenized in RIPA buffer containing protease inhibitor cocktail and phosphatase inhibitors using a Polytron handheld homogenizer (Kinematica, Luzern, Switzerland). After 30 minutes on dry ice, homogenates were centrifuged for 15min at 13,000 rpm at 4°C. Supernatants were collected and stored at –80°C. Protein concentration was determined using a BCA protein concentration kit (Pierce). Extracts equivalent to 50µg of protein were combined with Laemmli buffer with 1% β-mercaptoethanol and loaded onto precast Biorad 12% mini protean TGX gels for electrophoresis. Proteins were then transferred to PVDF membranes

(Immobilon) for blotting. Membranes were incubated with blocking solution (5% sucrose in Tris-buffered saline with added Tween-20) for 30 minutes, then incubated overnight with gentle rocking at 4°C with primary antibodies (1:1000 concentration). After washing with Tris-buffered saline, membranes were incubated with HRP-conjugated secondary antibody for 30 minutes, followed by a one minute exposure to enhanced chemiluminescent substrate (Pierce/Thermo Scientific, Rockford IL). The resulting signal was captured using a UVP Chemi Doc-It imaging system and Visionworks Software and analyzed using ImageJ (NIH). Results are expressed in arbitrary densitometric units, relative to α -tubulin.

4.6 Analysis

Comparisons were made using two-way analysis of variance (ANOVA), with additional pairwise comparisons where appropriate using Tukey's multiple comparison correction. For all comparisons, an alpha level of .05 was used, corrected for multiple comparisons where necessary.

Acknowledgments

We thank Anastassiya Shamina and Vladimir Golosnoev for technical assistance. This work was supported by DA12136, MD007599 and PSC-CUNY to SJ and VQ.

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Highlights

- Methamphetamine binge induces memory deficits in male and ovariectomized rats
- Female rats do not show memory deficits after methamphetamine treatment
- Males show greater induction of FosB following methamphetamine treatment

FIG 1:

Novel object recognition seven days after methamphetamine binge dosage. Data are expressed as percent of total exploration time (old plus new object) spent exploring novel object (mean + SEM; n=5–8 per group; *: significant difference between saline and drug ($p < .05$); #: significant difference from female ($p < .05$)).

FIG 2:
Total exploration time of both objects in the test phase of the novel object paradigm. No significant differences were found by drug or sex. Data expressed as time in seconds (mean + SEM; n=5–8 per group).

FIG 3:
TH levels in A) caudate-putamen B) nucleus accumbens and C) hippocampus.
Representative blots are inset. Results are presented in arbitrary densitometric units.†:
significant difference ($p < .05$) from male; $n=3$ per group.

FIG 4:
 DAT levels in A) caudate-putamen B) nucleus accumbens and C) hippocampus.
 Representative blots are inset. Results are presented in arbitrary densitometric units. †:
 significant difference ($p < .05$) from male; $n=3$ per group.

FIG 5:
 Bcl-2 levels in A) caudate-putamen B) nucleus accumbens and C) hippocampus.
 Representative blots are inset. Results are presented in arbitrary densitometric units. †:
 significant difference ($p < .05$) from male; #: significant difference ($p < .05$) from female; *:
 significant difference between drug and saline ($p < .05$); $n = 3$ per group.

FIG 6:
FosB levels in A) caudate-putamen B) nucleus accumbens and C) hippocampus. Representative blots are inset. Results are presented in arbitrary densitometric units. †: significant difference ($p < .05$) from male; #: significant difference ($p < .05$) from female; *: significant difference between drug and saline ($p < .05$); $n = 3$ per group.