Methamphetamine Administration Increases Proceptive Behavior by Activating Src Kinase and Upregulating Progesterone Receptor in the Medial Amygdala

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Abstract

Methamphetamine, a psychostimulant drug of abuse, increases sexual motivation both in humans and in rodent models. The activation of dopamine type-1 receptors (D₁Rs) within the medial amygdala, in the presence of ovarian hormones (EB+P), are both necessary and sufficient for increases in proceptive, or sexually motivated, behaviors. Here, we demonstrate that methamphetamine increases progesterone receptor expression in the medial amygdala independently of D₁R activation, and that lentiviral overexpression of the progesterone receptor was able to recapitulate the methamphetamine-induced enhancement of proceptive behaviors. Furthermore, we found that within the medial amygdala, these progesterone receptors show an increase in phosphorylation of serine 294 of the progesterone receptor in a region-specific manner. The involvement of this phosphorylation site suggests a role for cytosolic kinases, which may be responsible for enhanced progesterone receptor action. The phosphorylation of serine 294 is blocked by D₁R antagonists, and by inhibiting cSrc and ERK1/2, downstream of D₁R signaling, we identified that Src and ERK1/2 are required for enhanced proceptive behavior. Taken together, we propose that within the medial amygdala, methamphetamine enhances progesterone receptor's sensitivity to its cognate ligand via interaction with cSrc kinase and ERK1/2, as well as an increase total progesterone receptors, thus leading to enhanced proceptive behaviors in the rat.

INTRODUCTION

Women's sexual motivation, especially the underlying neural mechanisms mediating it, remains poorly understood despite years of study on gonadal steroids and neural circuitry {Blaustein, 2008 #127}. Clinical self-surveys have implicated Methamphetamine as a libido-enhancing drug in women. (Methamphetamine acts as a psychostimulant, affecting the brain via increased catecholamine release {Fleckenstein, 2007 #286} {Sulzer, 2005 #55} for review).), Women who abuse Methamphetamine report the drug makes them more confident in, and increases their motivation for, sexual activities {Rawson, 2002 #50} {Semple, 2004 #46} {Mansergh, 2006 #28}³. This observation has also been made in rodent models. Work in our lab and others has shown that Methamphetamine administration increases proceptive behaviors in ovariectomized (OVX), hormonally primed (estradiol and progesterone treated) rats {Holder, 2010 #24; Holder, 2010 #80; Holder, 2015 #195} {Guarraci, 2010 #228; Guarraci, 2014 #324} {Rudzinkas, 2016 #226}. Female sexual behavior in the rat has been most strongly linked to a few hormone-concentrating regions of the brain, including but not limited to the ventromedial nucleus of the hypothalamus (VMN) and the medial amygdala, especially in the posterior dorsal regions. Within these regions, concentrations of estradiol and progesterone that rise and fall with the estrous cycle can precisely modulate sexual behavior {Tennent, 1980 #214}. These behavioral changes are namely the onset of behavioral estrous and receptivity, which is primarily driven by high levels of estradiol, but also proceptive or paracopulatory behaviors that are dependent almost entirely on high levels of progesterone subsequent to priming with estradiol to manifest. It is generally accepted that ovarian hormones (estradiol and progesterone, EB+P) and their action on the neural substrate is responsible for female-specific sexual behaviors in the rat {Erskine, 2002 #216; Erskine, 1989 #222} {Blaustein, 2008 #212}.

There is a discrete population of behaviorally relevant neurons within the posteriodorsal medial amygdala that respond to the combined methamphetamine and EB+P stimulus, thus resulting in the expression of enhanced proceptivity {Williams, 2017 #383}. We have previously demonstrated that

methamphetamine's effects on proceptive behavior require both the dopamine type-1 receptors (D₁R) and ligand-bound progesterone receptors within the medial amygdala {Holder, 2015 #195}. Furthermore, in the absence of circulating ovarian hormones, methamphetamine can increase progesterone receptor in the medial amygdala. Therefore, we believe the behaviorally relevant cells within the medial amygdala are acting as a drug-sex nexus, integrating the appropriate methamphetamine-induced cues with progesterone receptor signaling {Holder, 2015 #195}.

The specific intracellular mechanisms involving the interactions of progesterone receptor and the downstream mechanisms of methamphetamine have remained elusive. Within recent years, both progesterone receptor and progesterone metabolites have been shown to interact with or initiate kinase cascades leading to both reflexive (receptive) and appetitive (proceptive) sex behaviors in the rat {Gonzalez-Flores, 2010 #193;Gonzalez-Flores, 2008 #186;Gonzalez-Flores, 2009 #187;Lima-Hernandez, 2012 #188;Lima-Hernandez, 2014 #327}. Specifically, there is a robust effect of ERK1/2 and Src family kinase inhibition on reducing hormonally-driven female sexual behaviors{Gonzalez-Flores, 2010 #157;Gonzalez-Flores, 2008 #186;Gonzalez-Flores, 2009 #187;Lima-Hernandez, 2012 #188;Lima-Hernandez, 2014 #327}. Other studies have demonstrated the ligand-independent activation{Auger, 2001 #6;Olesen, 2008 #39;Olesen, 2007 #40;Olesen, 2005 #16} and upregulation of progesterone receptors {Quadros, 2002 #56;Wagner, 1998 #32} in the brain. Whether either or both of these mechanisms can underlie proceptive behaviors remains unexplored. Here, we test the hypothesis that methamphetamine, increases progesterone receptor expression and/or amplifies progesterone receptor signaling cascades in the posteriodorsal medial amygdala to increase proceptive behaviors.

METHODS

Animals

Adult female Sprague-Dawley rats (250-275g) were ordered from Charles River and housed in the Laboratory Animal Facility of the Health Sciences Facilities at the University of Maryland, School of Medicine under a reversed 12 h:12 h dark:light cycle (lights off at 0600h) with food and water available

ad libitum. All animals were bilaterally OVX under isoflurane anesthesia and allowed a 7–10 day recovery period following surgery. All procedures were approved by the University of Maryland, Baltimore Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

Stereotaxic Surgeries

At the time of OVX, animals were placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA) under isoflurane anesthesia and an incision was made to expose the skull. The animals receiving drug infusions directly into the posteriodorsal medial amygdala had chronic indwelling 26-gauge guide cannulae (Plastics One, Roanoke, VA) bilaterally implanted (3.0 mm posterior, ± 3.55 mm lateral, and 7.0 mm ventral from Bregma) and affixed to the skull using dental acrylic. For experiments using SCH23390, the D₁R antagonist, rats were cannulated intracerebroventricularly (*icv*), and a 26-gauge cannula was stereotactically implanted into the right lateral ventricle of the rat using the following coordinates in relation to Bregma: -1.0 mm anteroposterior (AP); ± 1.5 mm mediolateral (ML) and -4.2 mm dorsoventral (DV) to the top of the skull. Dummy stylets were placed in the guide cannulae in order to keep them unobstructed. Animals receiving shRNA or lentivirus injections had holes drilled into the skull (3.0 mm posterior, ± 3.55 mm lateral from Bregma) and a Hamilton Syringe and needle was slowly lowered into the posteriodorsal medial amygdala (8.8 mm ventral from skull surface). All stereotaxic coordinates were determined using a standard brain atlas {Paxinos, 2005 #149}.

Hormone & Drug Treatments

Following the recovery period, animals received subcutaneous injections of 17 β -estradiol benzoate (EB) dissolved in sesame oil (100 ug/ml); 5ug on the first day, followed by 10ug of EB 24 hours later. 48 hours after the first injection of EB, the rats received a subcutaneous injection of progesterone dissolved in sesame oil (500 ug, 0.1 ml). Rats receiving oil control injections received a volumetric equivalent of sesame oil. This paradigm mimics the natural rise in hormones across the estrous cycle and has been

shown to induce female receptivity {Todd, 2005 #258} {Hadjimarkou, 2008 #257} . Experimental rats who received methamphetamine (5 mg/kg) received intraperitoneal (IP) injections daily by body weight concurrent with hormone or oil injections. Animals who received control injections received a volumetric equivalent of saline vehicle. Injections of the ovarian hormones (EB+P) and methamphetamine were administered between 0800 and 0900 h.

Sexual Behavior

The behavioral tests were conducted under dim red light in the dark phase of the light cycle between 1300 and 1600 h, approximately 4 h after the last methamphetamine administration. Experimental females were placed in a 50 cm × 38 cm × 25 cm Plexiglas observation chamber with a sexually naive male who had been acclimated to the behavioral box for 5 minutes prior. Each behavioral test was recorded by a video camera and was completed after 15 min had elapsed. During the tests, the investigator remained at a consistent location approximately 0.5 m away from the observation chambers during all trials. A single observer per experiment was blinded to the treatment groups and scored the receptive and proceptive sexual behaviors as previously described. Briefly, the number of proceptive behaviors (hops, darts, and ear wiggling) and lordosis number and intensity (degree of spinal curvature, 0-3) that occurred in 15 minutes was quantified {Holder, 2010 #24}.

Microinfusion experiments

All animals were hormonally primed as previously described. Animals received a single infusion of the Src family kinase (SFK) inhibitor 3-(4-chlorophenyl)-1-(1,1-dimethyl)-1H-pyrazolo[3,4-d]pyrimin-4-amine (PP2; 70 ng/side) (Cayman Chemical, Ann Arbor, MI), the D₁R antagonist SCH23390 (200 ng), or the ERK1/2 inhibitor SCH772984 (10 ug/side) five minutes prior to hormone and methamphetamine or saline injections. During the microinfusions, the dummy stylets were removed and replaced by 33 gauge microneedles that project 1mm below the guide cannulae. These microneedles were attached via polyethylene tubing to a 25 µl Hamilton syringe (700 series, Hamilton, Reno, NV) loaded into a BASi

Bee pump attached to a Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, IN). Infusions occurred over 5 minutes and the injectors remained in place for an additional 5 minutes to ensure diffusion away from the injector tips. Control infusions were done with equivalent volume and concentration of vehicle.

Cannula Placement verification

Following sexual behavior testing, the animals were transcardially perfused under ketamine/xylazine anesthesia (90 mg/kg, IP) with 0.9% saline containing 2% sodium nitrate, followed by 4% paraformaldehyde in 0.5 M KPBS. Brains were stored overnight in a 4% paraformaldehyde solution and then submerged in 30% sucrose in KPBS. After the brains had equilibrated with the sucrose solution, the brains were frozen on dry ice and stored at -80°C until processed for Nissl staining. Brains were sectioned (35 μm) in the coronal plane in a cryostat and stored in a cryoprotectant solution (ethylene glycol/glucose in sodium phosphate buffer) until processed for Cresyl Violet. The sections were rinsed in KPBS and mounted serially on 2% gelatin-coated glass slides. They were rehydrated in dH_2O , stained in a 0.5% Cresyl Violet (Chroma) solution containing 1 M sodium acetate, and 1 M acetic acid and coverslipped. A placement was deemed appropriate when the needle track was located within sections that corresponded to plates 56–60 in a standard brain atlas {Paxinos, 2005 #149} and fell dorsolateral to the optic tract.

shRNA infusions

All animals received 2 μL injections of Src shRNA (10^8 TU/mL SMARTvector Lentiviral Rat Src UBC-TurboGFP, Clone V3SVRN04_18913577, Dharmacon, Lafayette, CO) or scramble control (2.45×10^8 TU/mL SMARTvector Non-targeting UNC-TurboGFP Control Particles, VSC10240, Dharmacon, Lafayette, CO) at time of OVX. Infusions occurred over 10 minutes and the needle was left in place an additional 5 minutes to allow for diffusion away from needle tip. Animals were given two weeks recovery time to allow for full expression of cSrc shRNA prior to treating with ovarian hormones and

methamphetamine or saline and observation of behavior. Knockdown of Src was verified two ways. First, coronal sections of the posteriodorsal medial amygdala were collected at 35 μ m, fixed on a slide, coverslipped and expression of GFP was visualized to determine injection placement. Second, 300 μ m sections adjacent to those collected for fluorescence were obtained and fresh-frozen punches of the posteriodorsal medial amygdala were collected for western blot and analyzed for the expression of phosphorylated (pY416) and total Src.

Western Blotting

Animals used for protein analysis were euthanized by CO₂ asphyxiation 15 minutes or 4 hours after progesterone administration. Brains were collected on dry ice and sections containing the posteriodorsal medial amygdala and VMN, were micro punched for the region of interest and stored at -80C until homogenization. 1.5-2mm punches of frozen posteriodorsal medial amygdala or VMN were sonicated in RIPA buffer (50mM Tris hydrochloride, pH 8; 150mM sodium chloride, 1% Nonidet-P40 [Sigma], 0.5% sodium deoxycholate [Sigma], 0.1% sodium dodecyl sulfate [Quality Biological Inc., Gaithersburg, MD] in water). Protease and phosphatase inhibitors were added just prior to use (1:300 each; Sigma). Samples were then centrifuged and supernatants collected for analysis. A Coomaassie Blue kit was used to measure protein concentration of each sample (Pierce, Rockford, IL). Homogenates were electrophoresed on 8-16% gradient Tris-Glycine gel (Invitrogen, Carlsbad, CA) and blotted onto a polyvinylidene fluoride (PVDF) membrane (LC2005, Novex, Carlsbad, CA). Western blots were blocked with 5% bovine serum albumin and incubated overnight at 4C with anti-phospho-Src (pTyr416) antibodies (1:1000), anti-Src (1:1000), anti-phospho-ERK (Thr202/Tyr204) (1:1000), anti-ERK, or blocked with 1% milk for 1 hour and incubated with anti-phospho-serine 294-progesterone receptor (pSer294-progesterone receptor), anti-phosphoserine 345-progesterone receptor (pSer345-progesterone receptor), anti-GAPDH (1:1m) (Cell Signaling), or anti-total progesterone receptor antibodies (A-2 Monoclonal progesterone receptor, Santa Cruz) at 1:1000 overnight at 4C. The blots were incubated with an HRP-linked secondary antibody (Cell Signaling) at 1:2000 for 1 hour and visualized with a chemiluminescent signal (Cell Signaling, Danvers, MA) on BLU-

C radiography film (Stellar Scientific, Baltimore, MD). The films were then scanned into a computer at 1200 dpi, and the scanned images were analyzed using NIH Image software (<http://rsb.info.nih.gov/niimage>). The optical densities were measured for each individual band. Band values were normalized to GAPDH.

Progesterone Receptor Lentivirus

To test the functional role of upregulated progesterone receptor in the medial amygdala, lentivirus was designed containing an ubiquitin-progesterone receptor-GFP-construct to overexpress progesterone receptor. The virus used a class II lentivirus shuttle vector. At the KpnI and AgeI enzyme sites, the following primers were used to clone in the full length progesterone receptor mRNA sequence for rats under the ubiquitin promotor:

Forward - 5'-CACGGTACCATGACTGAGCTGCAGGCAAAGGAT-3'

Reverse - 5'-CACACCGGTTCACTTTTTGTGAAAGAGG-3'

The cloned vector was then sent to the UPenn Viral Vector core for packaging. Female rats (n=20) were OVXed and stereotactically injected with 2ul in the medial amygdala of either the lentivirus containing progesterone receptor-GFP (titer of 1×10^9 TU/mL), or a control lentivirus expressing only GFP (Qiagen). The OVX and infusion surgery was completed two weeks prior to sex behavior testing, to allow for optimal expression of the lentiviral vector. Hormone injections occurred on Day 14, 15, and 16 after the surgery. Immediately after the test, female rats were injected with 0.5 ml ketamine/xylazine (88.9 mg/mL/11.1 mg/mL), and their brains collected via perfusion as described above. While blinded to treatment groups, using a fluorescent microscope, an experimenter determined whether cells in the medial amygdala expressed GFP on either side of the brain. Only 1 side of the bilateral medial amygdala needed to have GFP-positive cells to be considered a “hit”. Rats with GFP completely outside of the medial amygdala were considered “misses”. Control-GFP rats (n=6) were compared in a Student’s t-test and an ANOVA to the misses (n=6) and the hits (n=8). The misses were not significantly different from the control rats on any statistical test, thus the control and miss groups were collapsed to increase the power

of the experiment. To further verify expression of GFP in the medial amygdala (relative to the background of the tissue), IHC was run on the remaining sections for GFP. Prior to primary antibody incubation, tissue was washed in 0.1M glycine for 30 min. It was then transferred to 0.5% Triton-KPBS, 1% H₂O₂, and 5% NGS (Vector Laboratories, Burlingame, CA) for 1hr. The tissue was incubated in GFP primary antibody (1:40,000 dilution, Abcam) at in KPBS at 4°C for 48hrs. The tissue was developed according to the standard protocol, using a biotin-linked anti-chicken secondary antibody (Vector). At this dilution, no sections which had not received a viral injection had any visible staining.

Data Analysis

Student's t-test or two-way ANOVA followed by a Tukey's post-hoc comparison was used to analyze each data set using the GraphPad Prism 6 program (San Diego, CA) on a Dell computer running Microsoft Windows 7 Pro. Effect sizes were calculated using an effect size calculator available at <http://www.cognitiveflexibility.org/efficientsize/>. Power analyses were calculated using the free sample size calculators at <http://powerandsamplesize.com/>.

RESULTS

D₁R Antagonist, SCH23390, does not significantly decrease progesterone receptor.

Initially we hypothesized that methamphetamine, acting through the D₁R, could directly increase progesterone receptor in the posteriodorsal medial amygdala. To test this hypothesis, rats were treated with methamphetamine and EB+P, and SCH23390 (a D₁R antagonist) or vehicle. T -tests showed no significant differences between the progesterone receptor levels of the SCH23390 and vehicle treated rats. (Figure 1).

Progesterone receptor overexpression in the medial amygdala in the absence of methamphetamine increases proceptivity in female rats

Virally-transfected rats were subjected to sex behavior testing and their behavior was quantified for 15 minutes. In progesterone receptor-GFP virus treated rats, a statistically significant increase in overall

proceptivity (total hops, darts, solicitations, and ear wiggles) (Figure 2A) as well on specific proceptive events quantified, such as hops and darts (Figure 2B) progesterone receptor was observed relative to control rats (rats treated with GFP virus or rats that received the progesterone receptor-GFP virus but the needle missed the medial amygdala). Female rats injected with the progesterone receptor-encoding virus showed significantly increased lordosis intensity (Figure 2C). There were no significant changes to rejection behaviors (Figure 2D).

Progesterone receptor shows increased phosphorylation at Ser294, but not at Ser345 in the posterio-dorsal medial amygdala, but not the VMN following methamphetamine and treatment with ovarian hormones.

Fifteen minutes after the final administration of oil or ovarian hormones and methamphetamine or saline, fresh frozen posteriodorsal medial amygdala tissue was collected for phospho-progesterone receptor quantification by western blotting. Methamphetamine increased phosphorylation of Ser294 of the progesterone receptor in the posteriodorsal medial amygdala (Figure 3B). In contrast, methamphetamine had no effect on phosphorylation at Ser345-progesterone receptor in the posteriodorsal medial amygdala (Figure 3C). As predicted, methamphetamine administration significantly increased total progesterone receptor protein abundance (Figure 3D). Conversely in the VMN, there were no significant differences in phosphorylation levels of Ser294 or pSer345, or total progesterone receptor levels between groups, supporting an important role for the posteriodorsal medial amygdala independent of the VMN (Figure 3E-H).

D₁R Antagonist, SCH23390, does significantly decrease phospho-progesterone receptor at Ser 294.

While inhibition of the D₁R with SCH23390 did not affect total progesterone receptor protein expression (Figure 1), we predicted that D₁R may still play a role in regulating phosphorylation status of progesterone receptor⁶. Accordingly, we investigated whether the D₁R activation altered progesterone receptor phosphorylation at Ser294 at the 4 hour (behavior) timepoint, using the same brain homogenates used for

total progesterone receptor. Inhibition of D1R with SCH23390 decreased phosphorylation of the progesterone receptor at Ser294 in the posteriodorsal medial amygdala (Figure 4). These data show that while the D1R is not required for the upregulation of progesterone receptor protein it is involved in its phosphorylation.

ERK1/2 shows increased phosphorylation at 15 minutes after the combined treatment of Methamphetamine and Ovarian hormones in the Posteriodorsal Medial Amygdala but not the VMN.

ERK1/2 phosphorylation levels were evaluated as a marker of ERK1/2 activity. Both EB+P and methamphetamine increased ERK 1/2 phosphorylation in the posteriodorsal medial amygdala (Fig. 5A,B). In contrast in the VMN, another hormonally responsive brain region (Fig. 5C,D), there was no significant effect of EB+P or methamphetamine on ERK1/2 phosphorylation, although there was a trend for increased phosphorylation following methamphetamine ($p=0.059$; Fig. 5E).

Methamphetamine-facilitated increases in proceptive behavior is blocked by SCH772984

To determine if ERK1/2 was required for increases in proceptive behavior following methamphetamine administration to hormone-primed rats, we infused a specific, ATP-competitive ERK1/2 inhibitor, SCH772984. SCH772984 fully inhibited the methamphetamine-induced proceptive behaviors (Figure 6A), but no effect on lordosis quotient or score (receptive behaviors) was observed (Figures 6B & C). One animal in the saline/oil control group was a high outlier (>2 standard deviations from the mean) for proceptive events and was removed from the analysis.

ERK1/2 is not required for methamphetamine-induced increases in phosphorylation of progesterone receptor at Ser294.

Following ERK1/2 inhibition, phosphorylation levels of progesterone receptor at Ser294 were evaluated to determine if this event occurs downstream from ERK1/2 phosphorylation. While methamphetamine

treatment increased progesterone receptor phosphorylation on Ser294, SCH772984, did not attenuate this effect, suggesting that ERK1/2 is not required for progesterone receptor phosphorylation (Fig 7).

Src phosphorylation is increased in the posteriodorsal medial amygdala but not the VMN following methamphetamine and EB+P treatment.

In some contexts, Src acts upstream of the Raf/MEK/ERK pathway. Furthermore, cSrc has been shown to interact with progesterone receptor, {Lima-Hernandez, 2012 #188} {Boonyaratanakornkit, 2001 #336} {Weigel, 2007 #181}, therefore we wanted to determine if removing activity of Src alone could reduce the combined effect of methamphetamine and EB+P on ERK1/2 and progesterone receptor phosphorylation. We aimed to evaluate rapid changes in Src activation in the posteriodorsal medial amygdala following methamphetamine and/or ovarian hormone administration using Src phosphorylation at tyrosine-416 as a measure of Src activation. In the posteriodorsal medial amygdala, methamphetamine increased pSrc only in the presence of ovarian hormones (Fig. 8B). In the VMN, there was no effect of methamphetamine or ovarian hormones on phosphorylated Src protein (Fig. 8D).

Methamphetamine-facilitated increases in proceptive behavior is blocked by PP2

The aim of this experiment was to investigate the role of Src kinase in methamphetamine-facilitated female sexual motivation in hormonally-primed female Sprague-Dawley rats. As expected, animals that received methamphetamine injections showed a significant increase in proceptive behavior compared to vehicle treated controls ($p < 0.05$). This effect of methamphetamine was completely inhibited by PP2 infusions, which reduced proceptive behaviors to baseline levels when compared to methamphetamine- and vehicle-treated animals (Fig. 9A). In contrast, there were no significant differences among treatment groups in either lordosis score or quotient in these animals (Fig. 9B,C).

cSrc shRNA Knocks down cSrc expression in the Posteriodorsal Medial Amygdala and blocks Methamphetamine-induced proceptive but not receptive behaviors

We used a genetic approach to confirm the PP2 result and establish cSrc specificity, rather than another Src-family kinase. Thus, we infused a cSrc shRNA or scrambled control shRNA into the posteriodorsal medial amygdala. As both control and cSrc shRNA contained a GFP expression vector, each animal had GFP expression verified prior to further analysis (Figure 9D). Any animal not showing GFP expression in the posteriodorsal medial amygdala was excluded from analysis. Western blots validated effective knockdown of cSrc in the cSrc shRNA treated animals when compared to scramble shRNA controls. Samples taken from the posteriodorsal medial amygdala showed that shRNA treated animals expressed only 54% of the active form of Src when compared to the animals treated with nontargeting shRNA. The methamphetamine-induced increase in proceptive behavior was fully blocked by Src shRNA infusion (Figure 9H). In contrast, there was no effect of methamphetamine or cSrc knockdown on receptive behaviors (Figure 9I,J).

cSrc is required for increased ERK1/2 phosphorylation and phosphorylation of the progesterone receptor at Ser294 in the posteriodorsal medial amygdala

Following cSrc knockdown, phosphorylation levels of both ERK1/2 and Ser294-progesterone receptor were evaluated to determine if these events occur downstream from cSrc. As expected, cSrc was required for the methamphetamine-induced phosphorylation of ERK1/2 (Fig 10A,B). Furthermore, cSrc expression was required for phosphorylation of the progesterone receptor on Ser294-progesterone receptor in the posteriodorsal medial amygdala, as knockdown of cSrc fully blocks the effects of methamphetamine on pSer294-progesterone receptor (progesterone receptor (Figure 10C, D). These data establish a fundamental pathway linking methamphetamine to the induction of cSrc, ERK1/2 activation and the phosphorylation of the progesterone receptor at Ser 294. These signaling events in total are required for the increase in proceptive behavior but not reflexive behaviors.

Discussion

It is well established that progesterone is required for proceptivity in the female rat. Over the last few years, a number of publications {Guarraci, 2014 #324}{Holder, 2015 #195} have shown that the psychostimulant, methamphetamine, is able to enhance proceptivity beyond that seen in the presence of hormones alone. The present work outlines a novel mechanism, cSrc dependent phosphorylation of the progesterone receptor at Ser294 and activation of ERK1/2 in the posteriodorsal medial amygdala, both of which are required for methamphetamine-enhanced proceptive behaviors.

Our lab has previously shown that the D₁R is both necessary and sufficient for methamphetamine's enhancement of hormonally-driven proceptive events {Holder, 2015 #195}. Additionally, the administration of methamphetamine increases the number of progesterone receptor immunoreactive cells in the posteriodorsal medial amygdala, and activation of the progesterone receptor by progesterone is necessary for increased proceptive events {Holder, 2015 #195}. Here, we demonstrate that by overexpressing progesterone receptor in the medial amygdala, *in the absence of methamphetamine*, we can recapitulate the effects of methamphetamine on proceptive behaviors in hormonally primed OVX rats. This further supports previous work and shows the progesterone receptor in the medial amygdala of cycling rats is sufficient to drive proceptive behavior. We hypothesized the mechanism by which progesterone receptors are upregulated in the medial amygdala was through D₁R activation, as ligand-independent activation of hormone receptors has been shown to occur via D₁R in the brain. Surprisingly, although D₁R has been shown to be both necessary and sufficient for the enhancement of behavior, antagonizing the D₁R during methamphetamine administration is not enough to ameliorate the methamphetamine-driven increase in progesterone receptor. However, we found that D₁R antagonism in the presence of methamphetamine decreases progesterone receptor phosphorylation (and in previous literature, proceptive behavior as well) 4 hours following administration.

Progesterone receptors function can be modulated by post-translation modifications, including phosphorylation, sumoylation, ubiquitination, and acetylation, but it is thought that phosphorylation sites determine specificity of action of the progesterone receptor *in vivo* {Bai, 1997 #8;Denner, 1990 #215;Weigel, 1995 #133;Weigel, 2007 #132}{Hagan, 2012 #163}. The progesterone receptor has fourteen

serine phosphorylation sites along its length and each phosphorylation site is thought to be a complex sensory or targeting system for the protein to modulate its functionality within the cell. For example, some of these sites become phosphorylated during hormone binding and receptor dimerization, while others can be phosphorylated by kinases or cyclins during the cell cycle {Hagan, 2012 #163} {Faivre, 2008 #158} {Pierson-Mullany, 2004 #139} {Dressing, 2009 #138}. In order to streamline analysis of the known phosphorylation sites on the receptor, we examined the hormonally responsive phosphorylation sites. Two of the five hormonally responsive sites, Ser294 and Ser345, have also been shown to interact with both hormones and cytosolic kinases, specifically ERK1/2 and cSrc {Daniel, 2007 #131; Faivre, 2008 #158} {Lange, 2000 #260}. Furthermore, ERK1/2 and Src have been shown to be more highly phosphorylated following dopaminergic agonists {Westin, 2007 #400} {Gerfen, 2002 #401}, thus making them attractive upstream targets in methamphetamine-enhanced proceptivity and progesterone receptor phosphorylation.

We found that Ser-294 of the progesterone receptor showed increased phosphorylation in the posteriodorsal medial amygdala 15 minutes following methamphetamine administration, but not the VMN, the primary nucleus driving reflexive sex behaviors in the female rat. Phosphorylation of Ser294 of the progesterone receptor has been demonstrated to enhance the receptor's sensitivity to ligand and lead to more robust progesterone receptor action {Trevino, 2013 #179; Weigel, 1995 #133} {Lange, 2000 #260; Shen, 2001 #335} {Daniel, 2007 #131} {Skildum, 2005 #168}. Similarly, previous work in both breast cancer *in vitro* and brain *in vivo* have shown that progesterone receptor phosphorylation and localization to the membrane via complexes with cytosolic kinases enhances progesterone receptor signaling {Dressing, 2009 #138} {Daniel, 2007 #131}. Some data suggest that pSer294-progesterone receptor may prevent receptor turnover as well {Khan, 2011 #167}, although the opposite result has also been described {Lange, 2000 #260}.

Both cSrc and ERK1/2 activation have been implicated in progesterone receptor signaling and phosphorylation, namely ERK1/2 can directly phosphorylate Ser294 whereas a phosphorylated progesterone receptor may serve as a docking platform for Src {Dressing, 2009 #138; Hagan, 2012

#163} {Daniel, 2007 #131;Faivre, 2008 #158;Skildum, 2005 #168}. These data suggest that cSrc and ERK1/2 may be involved in the mechanism leading to methamphetamine-facilitated proceptivity as well as the increased phosphorylation of progesterone receptor. These kinases have been shown to be more highly phosphorylated following administration of dopamine agonists; therefore kinase-induced enhancement of progesterone receptor signaling following methamphetamine administration appeared to be the most parsimonious explanation for how methamphetamine increased phosphorylation of this site. In fact, both of these kinases have been implicated in the neural mechanism behind vaginal-cervical stimulation (VCS), whereby dopamine signaling in the VMN enhances lordosis {Lima-Hernandez, 2012 #188}.

Although ERK1/2 activity and phosphorylation of Ser294 were greatest in the methamphetamine and ovarian hormone-treated animals, we were surprised to find that ERK1/2 was not responsible for phosphorylation of Ser294. Others have demonstrated a reduction in proceptive and receptive sex behaviors following PP2 (Src-family kinase inhibitor) infusion directly into the VMN {Gonzalez-Flores, 2010 #193}. However, in our hands, when PP2 is infused in conjunction with ovarian hormones and methamphetamine, we saw a blockade of only methamphetamine-induced increases in proceptivity, without reduction in baseline behaviors. Additionally, this effect was only observed with proceptive behaviors whereas receptive behaviors (lordosis) remain unchanged regardless of treatment. This is in accord with previous findings {Holder, 2010 #24;Holder, 2010 #80;Holder, 2015 #195}, thus strengthening the hypothesis that methamphetamine action in the posteriodorsal medial amygdala serves to enhance motivation for sex.

Previous work on Src in sex behavior was done using SFK inhibitors, which target all Src-family kinase members. Here we used shRNA to demonstrate that cSrc specifically was obligated for this response in the posteriodorsal medial amygdala. Further, we were able to recapitulate the behavioral findings seen with PP2. In aggregate these data support that cSrc, and not another SFK, is responsible for the interaction of methamphetamine and ovarian hormones.

cSrc has been described as upstream of ERK1/2 signaling as well as having the ability to form complexes with both progesterone receptor and ER within the brain {Lima-Hernandez, 2014 #327}. This evidence, taken with the requirement for cSrc in methamphetamine-facilitated proceptive behavior,

suggests that Src is the convergence point for methamphetamine and EB+P interaction, and upstream of ERK1/2 and progesterone receptor phosphorylation. The requirement of ERK1/2 activity for enhanced proceptivity but not pSer294 suggests that progesterone receptor may be acting as a scaffolding protein to enhance kinase signaling required for proceptive behaviors.

Our data demonstrates a novel signaling cascade at work in methamphetamine's enhancement of proceptive behaviors in female rats. We now hypothesize that methamphetamine, and its subsequent dopamine release, activates D₁Rs which initiates phosphorylation of intracellular proteins. The progesterone receptor forms a complex with Src, leading to its phosphorylation at Ser294. This results in both downstream ERK1/2 phosphorylation, progesterone receptor phosphorylation, leading to enhanced sensitivity of the progesterone receptor to progesterone. In parallel, methamphetamine is increasing total levels of progesterone receptor in the medial amygdala. This enhanced sensitivity to ligand combined with increased total progesterone receptor availability ultimately leads to enhanced neural activation of the posteriodorsal medial amygdala {Holder, 2010 #24} {Williams, 2017 #383} and methamphetamine-facilitated increases in proceptive behavior.

Figure 1:

Fig. 1. Antagonizing the D1R does not decrease PR expression. (A) Immunoblotting was performed for total PR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on MePD tissue from female rats treated with Meth and EB+P in the presence or absence of SCH23390. (B) Total PR/GAPDH ratios are shown. ($t_{(10)}=0.853$, $p>0.05$, $d=0.49$). Graph depicts means $\pm$ SEM, $n=6$.

Figure 2:

Fig. 2. PR Overexpression in the MeA, in the absence of Meth, increases proceptivity in female rats. (A) Total proceptive behaviors (hops, darts, solicitations, and ear wiggles) occurring over 15 minute exposure to sexually active male rat. ($t_{(17)}=3.742$, $p=0.0016$, $d=1.70$). (B) Hops and darts subset of behaviors from panel A occurring over 15 minute exposure to sexually active male rat. ($t_{(17)}=3.830$, $p=0.0013$, $d=1.75$). (C) Female rats injected with the virus showed significantly increased lordosis intensity ($t_{(17)}=2.366$, $p=0.03$, $d=1.08$). (D) There were no significant changes to rejection behaviors ($t_{(17)}=0.752$, $p>0.05$, $d=0.343$). Control group contains both GFP only viral transfection and those animals whose injection missed the MeA. Graph depicts means $\pm$ SEM, $n=8$. *** $p<0.005$, * $p<0.05$ compared to control by t-test.

Figure 3:

Fig 3. Progesterone receptor shows increased p Ser294-PR, but not pSer345-PR in the MePD, but not the VMN, 15 minutes following Meth and EB+P treatment. (A) Immunoblotting was performed for pSer294-PR, pSer345-PR, total PR and GAPDH on MePD tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (B) pSer294-PR/GAPDH ratios are shown. ($F_{(1,38)}=6.04$, $p=0.0187$, $n=8-14$). (C) pSer345-PR/GAPDH ratios are shown. ($F_{(1,33)}=0.1646$, not significant, $n=8-14$). (D) tPR/GAPDH ratios are shown. ($F_{(1,44)}=7.107$, $p=0.0107$, $n=7-17$). (E) Immunoblotting was performed for pSer294-PR, pSer345-PR, total PR and GAPDH on VMN tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (F) pSer294-PR/GAPDH ratios are shown. (ns, $n=8-14$). (G) pSer345-PR/GAPDH ratios are shown. (ns, $n=8-14$). (H) tPR/GAPDH ratios are shown. (ns, $n=7-17$). Graph depicts means $\pm$ SEM. * $p<0.05$, main effect compared to saline controls by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 4:

Fig. 4. Antagonizing the D1R decreases phosphorylation of the progesterone receptor at serine 294. (A) Immunoblotting was performed for pSer294-PR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on MePD tissue from female rats 4 hours after treatment with Meth and EB+P in the presence or absence of SCH23390. (B) pSer294-PR/GAPDH ratios are shown. ($t_{(10)}=1.97$, $p=0.032$, $d=1.14$). Graph depicts means $\pm$ SEM. * $p<0.05$ compared to control by t-test.

Figure 5:

Fig. 5. ERK1/2 shows increased phosphorylation 15 minutes following the combined administration of Meth and EB+P in the MePD but not the VMN. (A) Immunoblotting was performed for pERK1/2 (Thr202/Tyr204), total ERK1/2 (tERK) and GAPDH on MePD tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (B) pERK/tERK ratios are shown. Interaction of EB+P and Meth ($F_{(1,20)}=16.99$, $p=0.0005$, $n=6$) and main effect of EB+P ($F_{(1,20)}=4.587$, $p=0.0447$, $n=6$). (C) Immunoblotting was performed for pERK1/2 (Thr202/Tyr204), total ERK1/2 (tERK) and GAPDH on VMN tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (E) pERK/tERK ratios are shown (ns, $n=6$). Graph depicts means $\pm$ SEM. * $p<0.05$, compared to saline treated control, ** $p<0.005$ compared to oil controls by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 6:

Fig. 6. Meth-facilitated increases in proceptive behavior is blocked by SCH772984. (A) Total proceptive behaviors (hops, darts, solicitations, and ear wiggles) in hormonally-primed female rats occurring over 15 minute exposure to sexually active male rat. (Interaction of Meth and SCH772984: $F_{(1,19)}=13.9$, $p=0.0014$, $n=6$). (B) Lordosis quotient (lordosis/mount) was significantly reduced by SCH772984. ($F_{(1,19)}=4.556$, $p=0.0461$, $n=6$). (C) Female rats injected with SCH772984 showed no effect on lordosis intensity (ns, $n=6$). * $p<0.05$ compared to saline control, ** $p<0.005$ compared to vehicle control by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 7:

Fig. 7. ERK1/2 is not required for Meth-induced increases in phosphorylation of PR at Ser294. (A) Immunoblotting was performed for pSer294-PR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on MePD tissue from female rats 15 minutes after treatment with Meth and EB+P in the presence or absence of SCH23390. pSer294-PR/GAPDH ratios are shown. ($F_{(1,17)}=9.109$, $p=0.0077$, $n=6$). Graph depicts means $\pm$ SEM. * $p<0.05$ compared to saline control by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 8:

Fig. 8. Src phosphorylation is increased in the MePD but not the VMN following Meth and EB+P treatment. (A) Immunoblotting was performed for pSrc (Tyr416), total Src, and GAPDH on MePD tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (B) pSrc/Src ratios are shown. Interaction of EB+P and Meth ($F_{(1,18)}=5.117$, $p=0.0363$, $n=6$). (C) Immunoblotting was performed for pSrc, total Src and GAPDH on VMN tissue from female rats treated with Meth or saline and EB+P or oil vehicle. (D) pSrc/Src ratios are shown (ns, $n=6$). Graphs depict means $\pm$ SEM. * $p<0.05$, compared to saline treated control by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 9:

Fig. 9. Phosphorylated Src in the MePD is required for Meth-facilitated increases in proceptivity.

(A) Total proceptive behaviors (hops, darts, solicitations, and ear wiggles) in hormonally-primed female rats occurring over 15 minute exposure to sexually active male rat. (Interaction of Meth and PP2: $F_{(1,14)}=13.9$, $p=0.0014$, $n=5$; Main effect of Meth: $F_{(1,14)}=5.139$, $p=0.0398$, $n=5$; Main effect of PP2: $F_{(1,14)}=6.962$, $p=0.0195$, $n=5$). (B) Lordosis quotient (lordosis/mount) was unaffected by Meth or PP2 (ns, $n=5$). (C) Female rats infused with PP2 showed no effect on lordosis score (ns, $n=5$). * $p<0.05$ compared to saline controls, ** $p<0.01$ compared to vehicle control by two-way ANOVA followed by Tukey's multiple comparisons test. (D) Fluorescent image of representative shRNA infusion into the MePD. OT = optic tract, cell nuclei are labeled with DAPI to determine anatomical landmarks, virally transfected cells express GFP. (E) Immunoblotting was performed for pSrc, total Src, and GAPDH on MePD tissue from female rats treated with shRNA targeted to Src or scrambled shRNA. (F) pSrc/Src ratios are shown. ($t_{(22)}=2.379$, $p=0.0137$, $n=11-13$). (G) Src/GAPDH ratios are shown. $t_{(20)}=2.101$,

p=0.0243, n=11-13). (H) Total proceptive behaviors in hormonally-primed female rats occurring over 15 minute exposure to sexually active male rat. (Interaction of Meth and shRNA: $F_{(1,18)}=7.907$, p=0.0115, n=6; Main effect of shRNA: $F_{(1,18)}=8.581$, p=0.0090, n=6). (I) Lordosis quotient (lordosis/mount) was unaffected by Meth or shRNA (ns, n=6). (J) Female rats infused with shRNA showed no effect on lordosis score (ns, n=6). **p<0.01 compared to scramble control by two-way ANOVA followed by Tukey's multiple comparisons test.

Figure 10:

Fig.10. cSrc is required for increased ERK1/2 phosphorylation and phosphorylation of the PR at Ser294 in the MePD. (A) Immunoblotting was performed for pERK, tERK, and GAPDH on MePD tissue from female rats treated with shRNA targeted to Src or scrambled shRNA. (B) pERK/ERK ratios are shown. (Interaction of Meth and shRNA: $F_{(1,19)}=5.095$, p=0.0360; Main effect of Meth: $F_{(1,19)}=11.65$, p=0.0029, Main effect of shRNA: $F_{(1,19)}=11.77$, p=0.0028, n=6). ^ap<0.005, ^bp<0.001, ^cp<0.005 by two-way ANOVA followed by Tukey's multiple comparisons test. (C) Immunoblotting was performed for pSer294 and GAPDH on MePD tissue from female rats treated with shRNA targeted to Src or scrambled shRNA. (D) pSer294-PR/GAPDH ratios are shown. (Interaction of Meth and shRNA: $F_{(1,14)}=5.93$, p=0.0289, n=6). ^ap<0.05, ^bp<0.05 by two-way ANOVA followed by Tukey's multiple comparisons test.