CURRICULUM VITAE

Name: Katrina M. Williams

Email: katrinam.williams@gmail.com

Degree and Date to be conferred: Doctor of Philosophy, 2017

Collegiate Institutions Attended:

2005 – 2009 University of Maryland, College Park

Bachelor of Sciences, 2009

Animal Science

2011 – 2017 University of Maryland, Baltimore

Doctor of Philosophy, 2017

Molecular Medicine

Peer-reviewed Publications:

First Author:

Williams K, Mong JA. *Methamphetamine and Ovarian Steroid Responsive Cells in the Posteriodorsal Medial Amygdala are Required for Methamphetamine-enhanced Proceptive Behaviors*. Scientific Reports. 2017 Jan.

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Contributing Author:

Wei X, Li T, Hagen B, Zhang P, Sanchez PG, Williams K, Li S, Bianchi G, Son HS, Wu C, DeFilippi C, Xu K, Lederer WJ, Wu ZJ, Griffith BP. *Short-term mechanical unloading with left ventricular assist devices after acute myocardial infarction conserves calcium cycling and improves heart function*. JACC Cardiovascular Interv., 2013 Apr; 6(4):406-415.

Sanchez PG, Bittle GJ, Williams K, Pasrija C, Xu K, Wei X, Wu ZJ, Griffith BP. *Ex vivo lung evaluation of Prearrest heparinization in donation after cardiac death*. Ann Surg. 2013 Mar; 257(3);534-41.

Professional Society Memberships:

Society for Behavioral Neuroendocrinology (SBN) (2016 – current)
American Association for the Advancement of Science (AAAS) (2015 – current)
Society for Neuroscience (SFN) (2012-current)

Awards and Honors:

College Park Scholars Citation for Life Sciences – Spring 2007
Semester Academic Honors – Spring 2007, Fall 2007
Semester Academic Honors – Fall 2008
GSA Travel Award - 2014
GSA Poster Award - 2015

Abstract

Title of Dissertation: Role of Progesterone Receptor and Src Kinase in Methamphetamine-mediated Proceptive Behavior

Katrina M. Williams, Doctor of Philosophy, 2017

Dissertation Directed by: Dr. Jessica A. Mong, Ph.D.
Associate Professor
Department of Pharmacology

Methamphetamine (Meth) is a psychomotor stimulant strongly associated with increases in sexual drive and impulse in both men and women. These changes in sexual motivation have a greater impact on women due to their likelihood of facing the greater burden of unplanned pregnancies and an increased risk for psychiatric co-morbidities. We have previously established a rodent model of Meth-induced increases in sexual motivation. Using this model, we have demonstrated that in the presence of ovarian steroids, Meth-induced increase in behavior requires activation of the dopamine type-1 receptors and progesterone receptors (PR) in the medial amygdala (MePD). However, the mechanisms through which Meth is interacting with the PR signaling system remain unknown.

The work presented here furthers our understanding of the potential mechanism by which Meth-activated pathways and ovarian steroids interact in the MePD to increase proceptive behaviors. First, using a cell-specific lesion technique, DAUN02 inactivation, we identified a behaviorally relevant cell population in the MePD required for Meth-

induced increases in proceptivity. Furthermore, we demonstrated that within the MePD, Meth increases PR phosphorylation at Ser294 (pSer294-PR). This site is a target of both ERK1/2 and Src kinases, which have both been implicated in the cellular mechanisms underlying female sexual behavior. Indeed, the combined administration of Meth and ovarian hormones increased the phosphorylation of both ERK1/2 and Src kinases suggesting a role for these proteins in Meth-induced increases in PR phosphorylation and proceptivity. To test the possible role of each kinase, pharmacological inhibitors of either Src-family kinases or ERK1/2 were independently infused directly into the MePD. While separately inhibiting both kinases attenuated Meth-induced increases in proceptivity, ERK1/2 inhibition was unable to block the Meth-induced phosphorylation of Ser294-PR. Using shRNA to specifically knockdown the expression of Src in the MePD resulted in an attenuation of the Meth-induced proceptivity and a blockade of Meth-induced pERK1/2 and pSer-294-PR supporting the possibility that Src is a nexus linking Meth induced signaling to PR activation. These findings suggest that in the presence of ovarian steroids, Meth increases Src kinase signaling within the MePD, which subsequently increases pSer294-PR and pERK1/2, which are necessary for Meth-facilitated proceptive behavior.

Role of Progesterone Receptor and Src Kinase in Methamphetamine-mediated Proceptive Behavior

by
Katrina M. Williams

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A PhD is so much more than the diploma.

Science is a way of thinking much more than it is a body of knowledge. –Carl Sagan

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF FIGURES vii

ABBREVIATIONS ix

CHAPTER 1: GENERAL INTRODUCTION 1

Introduction 1
Sexual Behavior: Rats as a Model System 1
Biology of Rodent Sex Behavior 2
Molecular Modulators of Sex Behavior 13
Conclusions on Modulation of Sexual Behavior in the Rat 26
Meth's Effects on Human Sex Behavior 27
Meth's Mechanism of Action 27
Meth's Effects on Female Rodent Sex Behavior 31
Conclusions on Meth and Enhanced Sexual Activity 35

CHAPTER 2: GOALS OF THE DISSERTATION 37

CHAPTER 3: GENERAL METHODS 38

Animals 38
Survival Surgeries 38
Ovariectomies 38
Cannulations 39
Cannula Placement Verification 39
Steroid Hormone Treatment 39
Methamphetamine Treatment 42
Sexual Behavior Testing 42
Western Blotting 42
Immunocytochemistry 43
Statistical Analysis 44

CHAPTER 4: Identification of a Discrete Ensemble of MePD Neurons Involved in Meth-Facilitated Proceptivity 45

Introduction 45
Methods 46
Results 54

Discussion 62

CHAPTER 5: Effects of Meth on Progesterone Receptor Function 66

Introduction 66
Methods 67
Results 68
Discussion 74

CHAPTER 6: ERK1/2 and Src Kinase as Conduits of Progesterone Receptor Rapid Signaling 77

Introduction 77
Methods..77
Results..80
Discussion 94

CHAPTER 7: GENERAL DISCUSSION 99

Summary 99
Implications for Organization of the Medial Amygdala 100
Effects of Dopamine on Gonadal Hormone Signaling 101
Interplay of Signaling Pathways..102
Implications for Human Studies / Human Sexual Dysfunction 105
Conclusion 106

REFERENCES 108

LIST OF FIGURES

Figure 1: The Rodent Estrous Cycle …………….………….………….…………….………...4

Figure 2: Lordosis in the Rat ………….………….………….………….………….………7

Figure 3: Basic Neurocircuitry Underlying Lordosis ………………..…….…………….…..9

Figure 4: Basic Neurocircuitry Underlying Motivated Sex Behaviors ……...….…………12

Figure 5: Comparison Between Ligand-Dependent and Ligand-Independent Hormone Signaling Pathways ….……….………….………….………….………….………..….16

Figure 6: Progesterone Receptor Domains ……….………….………….…………..…….19

Figure 7: ERK1/2 Signaling and Interaction with PR …...…………….………….……...22

Figure 8: Src Kinase ………….………….………….………….………….……………...25

Figure 9: Schematic of Meth Interacting with Components of Dopamine Uptake and Release ………….………….………….………….………….………….……………..29

Figure 10: The Posteriodorsal Medial Amygdala ....…………….………….…………...34

Figure 11: Classical injection schedule ……….….……….……….……….…….…..41

Figure 12: Treatment paradigm for Oil/EB+P and Saline/Meth ………….……………..49

Figure 13: Three-phase treatment paradigm for cFos-LacZ transgenic rats receiving DAUN02 infusions ….……….………….………….………….………….…………..52

Figure 14: Effects of Meth on cFos immunoreactivity (Fos-ir) & Beta-galactosidase immunoreactivity (β-Gal-ir) in the posteriodorsal medial amygdala ……….……...…..55

Figure 15: Effect of Meth on proceptive behaviors (a) and lordosis (b, c) in hormonally primed female rats: a comparison between wild type and cFos-lacZ rats ……………..57

Figure 16: Effects of DAUN02 infused into the MePD on Meth-enhanced female sex behaviors ….……….………….………….………….………….………….…………..59

Figure 17: Effect of DAUN02 infusions on cFos-ir in the MePD ...…….…….………...61

Figure 18: Effects of Meth on progesterone receptor transcription in the MePD one hour after treatment ….……….………….………….………….………….……………...69

Figure 19: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the MePD 15 minutes following the final Meth and EB+P administration ……….………….………….………...………….………71

Figure 20: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the VMN 15 minutes following the final Meth and EB+P administration ……….……….……….……….……….……….……….…….…..72
Figure 21: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the MePD 30 minutes following the final Meth and EB+P administration ……….……….……….……….……….……….……….…….…..73
Figure 22: Effect of Meth and EB+P on ERK1/2 phosphorylation levels in the MePD, 15 minutes after Meth and P4 administration ……….……….……….……….…….…..82
Figure 23: Src is highly phosphorylated in the MePD following Meth and EB+P treatment ……………………………………………………………………………...83
Figure 24: Proceptive Behavior following SCH772984 Infusion ………..…………….…..85
Figure 25: Serine 294 phosphorylation following ERK1/2 inhibition ……….………….86
Figure 26: PP2 inhibits Meth-facilitated increases in proceptivity ……….………...…..88
Figure 27: Src knockdown following shRNA infusions …….…………….…………….90
Figure 28: Src knockdown following shRNA infusions block Meth-enhanced proceptive behaviors …….……….……….……….……….……….……….……….………...91
Figure 29: cSrc is required for Meth and EB+P induced phosphorylation of ERK1/2 and Ser294 on the PR ……….……….……….……….……….……….……….…….…...93
Figure 30: Schematic drawing to illustrate our proposed model …..………….……..…....98

ABBREVIATIONS

5-HT	serotonin
5-HTR	serotonin receptor
AF	activation function domains
aMeA	anterior medial amygdala
AR	androgen receptor
BDNF	brain-derived neurotropic factor
BG	beta galactosidase
BNST	bed nucleus of the stria terminalis
CDK	cell-cycle dependent kinase
CX	castrate
D1R/D2R	dopamine type-1/type-2 receptors
DA	dopamine
DARPP-32	dopamine- and cAMP-regulated neuronal phosphoprotein
DAT	dopamine active transporter
DBD	DNA-binding domain
DHT	dihydrotestosterone
DSM	Diagnostic and Statistical Manual of Mental Disorders
E2	estradiol
EB	17β-estradiol benzoate
EB+P	ovarian hormones; estradiol benzoate and progesterone
ER	estrogen receptor
ERK	extracellular signal-regulated kinase
FDA	Food and Drug Administration
GPCRs	G-protein coupled receptors
GR	glucocorticoid receptor
HSSD	hypoactive sexual desire disorder
IF	inhibition factor
IP	intraperitoneal
ir	immunoreactivity

kPBS	potassium phosphate buffered saline
LBD	ligand-binding domain
LH	luteinizing hormone
LQ	lordosis quotient
LVN	lateral vestibular nucleus
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinase
MeA	medial amygdala
MEK	MAPK/ERK kinase
MePD	posteriodorsal medial amygdala
MePV	posterioventral medial amygdala
Meth	Methamphetamine
mPOA	medial preoptic area
MRF	medullary reticular formation
OVX	ovariectomized
P4	progesterone
PAG	periaqueductal gray
PKA	protein kinase A
PP2	3-(4-chlorophenyl)-1-(1,1-dimethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine; a Src Family kinase inhibitor
PPN	peripeduncular nuclei
PRE	progesterone response element
PR	progesterone receptor
SCH	SCH772984; ERK1/2 specific ATP competitive inhibitor
Ser	serine
SFK	Src Family kinase
SH2/3	Src homology domain 2 or 3
SSRI	selective serotonin reuptake inhibitor
VMAT	vesicular monoamine transporter
VCS	vaginal-cervical stimulation
VMN	ventromedial hypothalamus

Chapter 1. Introduction

Introduction

Sexual behavior is a motivating force for nearly all members of the animal kingdom. Over the last 50 years, the overall knowledge of how "sex hormones" initiate sexual behaviors has exponentially increased. In fact, the underlying neurobiological mechanisms by which steroid hormones such as androgens, estrogens, and progestins lead to basic sex behavior has been well documented in a number of domesticated species as well as humans [1-3]. Even so, there is a paucity of literature describing the neurobiological mechanism underlying female sexual motivation. This is quite surprising, given that recently, female HSDD (hypoactive sexual desire disorder) acquired a pharmacological treatment. This pharmacological treatment, although approved by the Food and Drug Administration (FDA), was under intense scrutiny due to the unclear mechanism of action.

While there has not been much research into the biology of female sexual motivation [4], there have been a number of clinical self-surveys that report Methamphetamine (Meth) use among women as a self-medication libido-enhancing drug, with users often reporting that Meth has made them more confident in their sexual activities. This illicit use of Meth has also been shown to increase sexual motivation in these women [5-7]. What is it about this drug that allows for changes in sexual drive? By modeling Meth-induced increases in female sexual motivation, we can provide valuable insight into the molecular mechanisms underlying female sexual drive.

Sexual Behavior: Rats as a Model System

Human sexual desire is perhaps more easily understood than sexual drive in other species. However, examining what drives or increases the ability to engage in sexual activity is rather similar. For example, the presence of gonadal hormones, such as androgens in humans and estrogens/androgens in the female/male rat respectively, are important for enhancing or

maintaining sexual motivation [8]. Additionally, both species show appetitive behaviors indicative of the desire to copulate [9]. It is also important to note that there are key differences between humans/primates and rats, these being 1) elements of sexual behavior in the rat that are reflexive, while humans/primates do not have reflexive sex behaviors, and 2) primates/humans may experience sexual drive at any time during the hormonal cycle, whereas rats are only receptive at specific time points. As human cellular studies cannot be performed, the rat becomes a dependable analog, and although different hormones influences different behaviors in each species, there is evidence that steroid hormones are involved in similar behaviors in all both species [10-12].

Biology of Female Rodent Sex Behavior

Although key differences exist between primates and rodents, rats have become a widely accepted model of sexual behavior. Rats are especially social and have highly reproducible, hormonally dependent sex behaviors [8, 10]. In female rats, estrogen and progesterone are the ovarian hormones required in order to present both the reflexive and appetitive components of sexual behavior.

<u>Estrous Cycle</u>

Rats and other non-primate mammals have an estrous cycle (Fig. 1), which is comprised of a cyclical rise and fall of reproductive hormones as well as a behavioral component called estrus. Behavioral estrus occurs after ovulation, and allows the animals to become receptive to mating when they are most fertile. In the rat, the estrous cycle occurs over about 4 days, with each day containing a phase of the cycle. During diestrus I, all hormones are low. As diestrus II begins and the ovarian follicles grow and mature, estradiol begins to rise, peaking during the next phase of proestrus. This increase in estradiol is linked to the luteinizing hormone (LH) surge, which causes ovulation to occur and progesterone to rapidly increase. Progesterone peaks at the

beginning of behavioral estrus, thus stimulating the most robust sexual behaviors. This differs from humans and many other primates, which have a menstrual cycle which results in a sloughing of the uterine lining after progesterone levels begin to decline. These species do not have a behavioral estrus and are sexually receptive throughout the cycle. However, there have been some studies which suggest that hormones can still influence sexual motivation, but not control it, in these species [13].

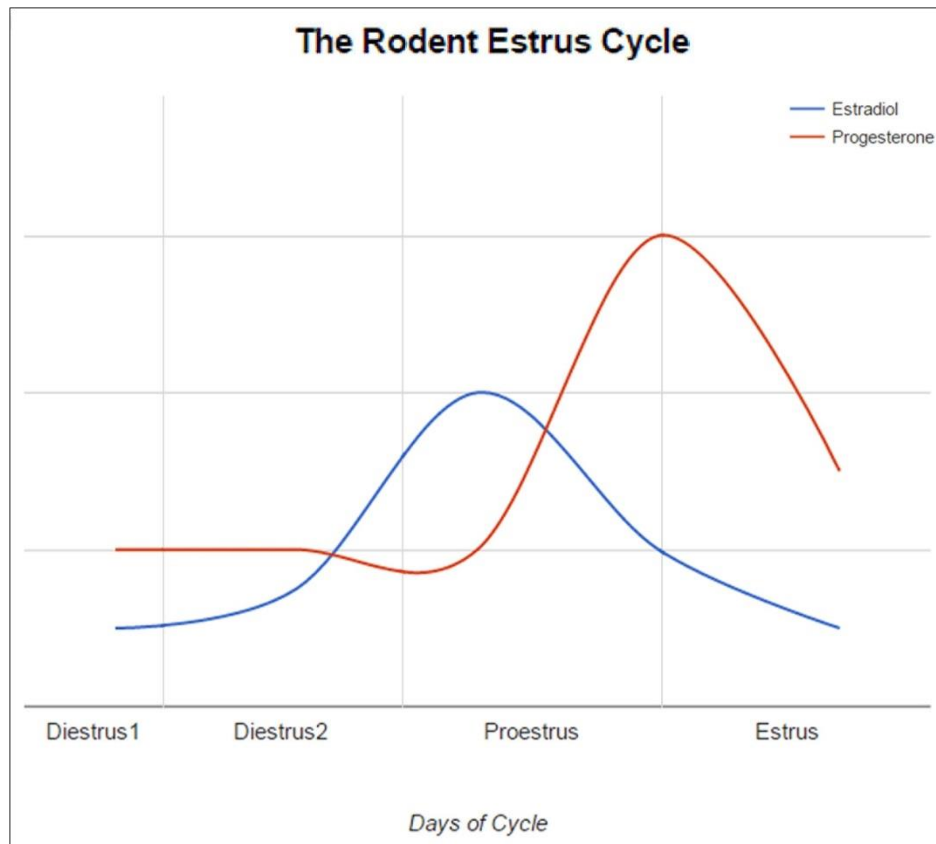


Figure 1: The Rodent Estrous Cycle. Schematic of the rise and fall of estradiol and progesterone over the 3-4 day estrous cycle in the rat. Estradiol begins its steady increase starting in diestrus2, with the peak occurring during proestrus. This leads to the luteinizing hormone surge (not depicted here) and subsequent ovulation. Following the increase in estradiol and ovulation, progesterone peaks signaling the beginning of estrus. Estrus is the portion of the cycle where the female rat (and other species exhibiting 'behavioral estrous') shows behavior indicative of her willingness to mate.

Estradiol

Estradiol is an 18-carbon steroid hormone, synthesized from a cholesterol backbone and downstream from the aromatization of testosterone. Estrogen is synthesized in large quantities in the ovaries, but within the last decade has also has been shown to have *de novo* synthesis within the neural substrate [14]. Estradiol has long been viewed as a "female hormone," and while it is important for female reproductive function and secondary sex characteristics in a number of mammalian species, it is also the hormone responsible for masculinization of the brain following aromatization of testosterone within the neurons [15].

Estradiol has been shown to play a number of roles within the body, but its function within the brain and its control over female sexual behavior has been well described. The reflexive components of feminine sexual behavior can be achieved in ovariectomized (OVX), adrenalectomized female rats following estradiol administration, but the appetitive components will not be fully expressed with estradiol alone [16, 17]. In fact, lordosis (the reflexive component of sexual behavior in the rat), although it can be achieved with estradiol alone, appears more robustly in animals who are treated with progesterone following priming with estradiol.

Progesterone

Progesterone is a 21-carbon steroid hormone, commonly thought of as a predominantly female hormone. Like other steroid hormones, it is synthesized from a cholesterol backbone that is cleaved to form pregnenolone. Pregnenolone is hydrolyzed by 17α-hydroxylase to progesterone (P4). P4 has been long considered a reproductive hormone; it is synthesized in the ovaries, adrenals and placenta, and has an important role in maintaining pregnancy. Within the last decade, P4 has also been found to be synthesized *de novo* within the brain [18-20] which has since opened a number of avenues of study as to its function within the neural substrate.

P4 has long been studied for its influence over female reproductive function. Its role within the uterus and ovaries serve to release the oocyte and provide an appropriate uterine environment for implantation of the zygote [21]. Additionally, P4 plays an important role in lobular-alveolar growth and development within the breast, which, in turn, makes it an important target in breast cancer research [21]. Perhaps the most enigmatic function of P4 has been in its role as a mediator of sexual behavior within the brain. Its local synthesis within the brain further suggests a critical role but not much is known about its function.

Receptive Behaviors

Estrus behaviors are separated into two primary categories in the rat, receptive (reflexive or "copulatory") and proceptive (pre- or paracopulatory)[22]. Receptive sex behaviors are those reflexive behaviors which allow for copulation to occur, as is seen with lordosis. Lordosis a is reflex that occurs following stimulation of the flanks (either by a mounting male, or experimentally) whereby the female exhibits a dorsal curvature of the spine and elevates her head and rump [23] (Fig 2). The somatosensory input is relayed to the spinal cord, and if the female is receptive (i.e. is in the correct hormonal state), the ventromedial nucleus of the hypothalamus (VMN) will transmit excitatory input via the midbrain back to the spinal cord in order to induce muscular contraction [24, 25]. In fact, the VMN is required for the integration of these hormonal cues to drive the reflexive response [26, 27]. A highly receptive female will display lordosis at every mount (LQ) and the intensity of lordosis is determined by the degree of spinal curvature (Fig. 2).

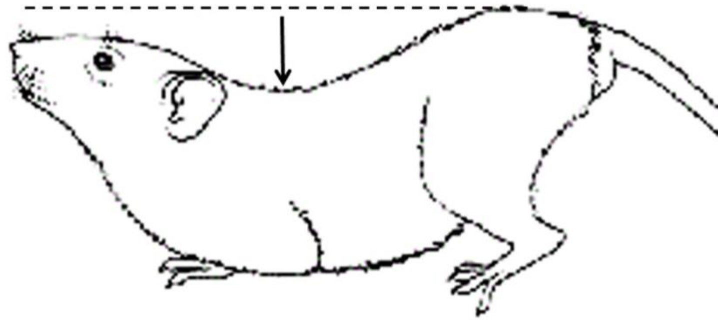


Figure 2: Lordosis in the Rat. Lordosis is a reflexive curvature of the spine adopted by receptive females when the male mounts or the flanks are stimulated. The intensity or depth of the curve, shown here by a dashed line and arrow, is defined as the 'Lordosis Score' or LS. The LS ranges from a '3' or deep curvature of the spine, usually associated with a fixed or frozen posture to a '1' in which the female maintains a straight spine.

Lordosis is primarily driven by the presence of estradiol within the VMN (Fig. 3), but the intensity of its expression is facilitated by progesterone [28, 29]. These behaviors can also be enhanced by release of dopamine via natural causes such as vaginal-cervical stimulation [VCS] or induced by drug administration [9, 30-32]. Although these enhancements of behavior appear to come from differing pathways, dopamine release following VCS is thought to enhance these behaviors via pathways activated by or involving progesterone action [9, 33-35].

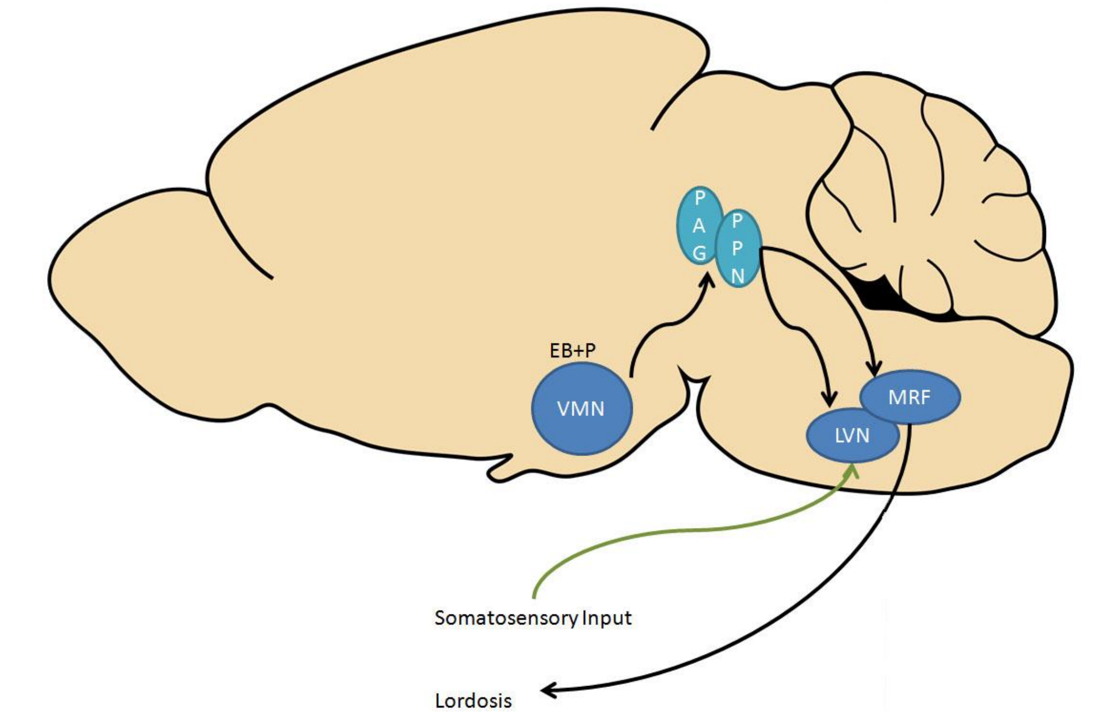


Figure 3: Basic Neurocircuitry Underlying Lordosis. Lordosis is a behavioral reflex following stimulation of the rump and flanks of the female rat associated with mating. Following stimulation of the flanks, the brain integrates somatosensory input to the brain stem, specifically the lateral vestibular nucleus (LVN) and medullary reticular formation (MRF). If the female is in behavioral estrus, estrogens and progestins will bind to their receptors and allow the VMN to signal back to the midbrain, specifically the periaqueductal gray (PAG) and peripeduncular nuclei (PPN), which then relay the signal back to the brain stem to initiate action of the motor neurons controlling the back muscles associated with the lordosis posture.

Proceptive Behaviors

The second measure of female rodent sexual behaviors is proceptive sex behaviors. Proceptive sex behaviors have been classically categorized as appetitive behaviors that indicate female sexual motivation or encouragement of the male to mate [23, 36]. Recently, this term has undergone scrutiny as to if there is truly a motivational component to proceptive behavior, and as such these behaviors have also been coined as "paracopulatory" [22]. These behaviors, regardless of terminology, consist of hops, darts, a presentation posture in which the female orients her rump to the male, and ear wiggling [23]. These behaviors have been used as a measure of sexual motivation or goal-directed behaviors by the female with the purpose of achieving copulation [37]. These motivated sex behaviors are driven by the presence of ovarian hormones, and without them, will not occur [37]. Indeed, these behaviors truly do appear to be motivation for a sexually active male as females will exhibit other behaviors, such as lever pressing or nose pokes to gain access to a sexually active male or displaying preference for sexually active males over castrates ([38, 39] , for review [36]). These behaviors also correspond with the amount of time a sexually receptive female will spend with a sexually active male in a paced mating paradigm [36]. In paced mating, the female chooses the intervals she will spend with the male, as she has access to a chamber where she can avoid contact with the male [36]. When female rats are allowed to determine the rate of copulation, dopamine release in anticipation of mating will increase in the nucleus accumbens and striatum similar to other rewarding behaviors such as feeding or drug administration [40, 41]. Indeed these appetitive behaviors do appear to influence the males' behavior by leading the male to chase the female, and often end in copulation [23].

Proceptive behaviors were first noted to only be present in the most receptive females, thus requiring the full complement of ovarian hormones (estradiol and progesterone; EB+P) [36]. These behaviors later were found to require progesterone treatment following priming by estradiol, as increasing doses of estradiol had little effect but changes in progesterone levels

altered the behaviors [17, 36, 42, 43]. While the circuitry for lordosis has been well mapped out, the same cannot be said for proceptive behaviors. Lesion studies have implicated the medial preoptic nucleus, the nucleus accumbens, and the striatum in pacing behavior, whereas cellular activation following mating has implicated roles for the medial amygdala (MeA) and bed nucleus of the stria terminalis (BNST) [44-46]. It appears that proceptivity is controlled by interactions of various nuclei (Fig. 4) that influence motor coordination such as approach behaviors, sex hormone stimulation, and integration of the somatosensory cues to lead to the ultimate output of behavior.

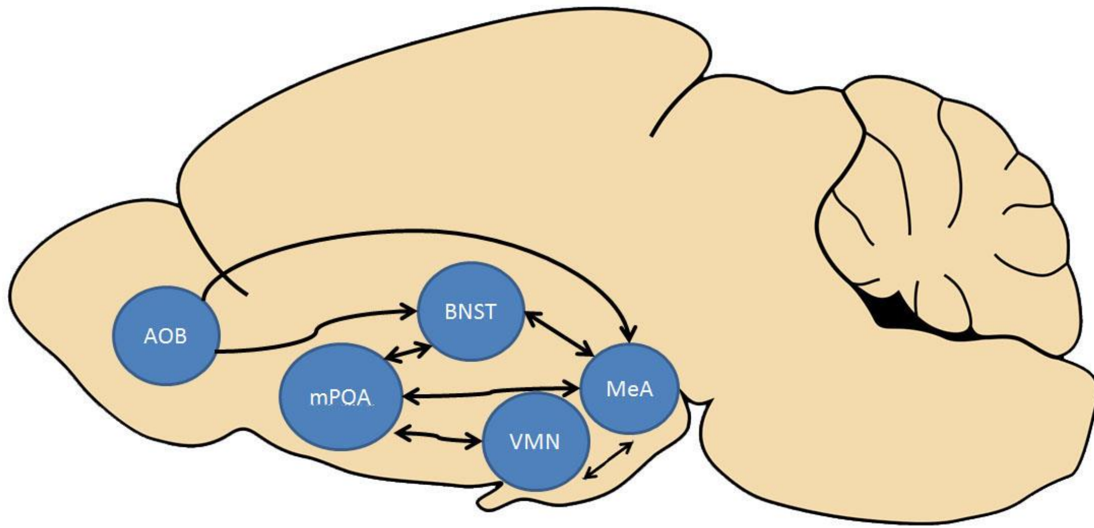


Figure 4: Basic Neurocircuitry of Motivated Sex Behaviors. The accessory olfactory bulb (AOB) projects to the BNST and MeA, thus relaying environmental cues. The MeA has reciprocal projections to the BNST, mPOA, and VMN, which have been shown to mediate sociosexual behaviors.

Molecular Modulators of Sex Behavior

Although ovarian hormones are necessary for the expression of sexual behavior, other signaling effectors, such as neurotransmitters, estrogen and progesterone receptors, and kinases may also play a role in modulating feminine sexual behavior.

Monoamines

Monoamines appear to play a role in modulating behavioral outcomes in both humans and rodents [47-49]. Within this group of compounds, serotonin, norepinephrine, and dopamine can all act on the brain nuclei that have been shown to have a role in sexual behavior.

Serotonin

Serotonin, also called 5-hydroxytryptamine (5-HT) acts at the 5-HT receptors. In the 1960's 5-HT was thought to be inhibitory toward sexual behavior, but since then up to 14 receptor types (5-HTR) have been discovered [50]. Although the varied subtypes do make the picture more complicated, certain subtypes are expressed in nuclei governing expression of female sexual behavior. Serotonergic innervation can be found in the VMN, MeA, and BNST, among other nuclei, and within these regions, the 5-HT1AR, 2AR and CR, and 3R can be found [50]. Within these regions, it appears that in female rats, 5-HT1AR is inhibitory toward sexual behavior, whereas 5-HT2R and 5-HT3R stimulation facilitates sexual behavior [50-52]. It is important to note, however, that function of these receptor can vary between the sexes, and overall, serotonin is associated with sexual refractory periods [47].

Norepinephrine

Norephinephrine (or noradrenaline) is generally considered a neurotransmitter that modulates states of arousal and stress. Within the brain, there are two primary adrenoceptor subtypes, αR and βR, both of which are considered excitatory in nature. These receptors are

expressed at high levels within the medial preoptic area (mPOA), VMN, and amygdala, amongst other regions [53], and have been implicated in rodent sexual behavior [54]. Overall, noradrenergic stimulation appears to facilitate female sexual behavior, although these effects appear limited to lordosis [53]; however, antagonism of these pathways does not appear to have any effect [55].

Dopamine

Although each monoamine has been implicated in modulation of female sexual motivation, dopamine (DA) has been shown to have the most robust effect. DA is a monoamine that is implicated in motivation and reward pathways, and is released from the mesocorticolimbic pathway which includes the MeA and VMN. DA's receptors can be grouped into two main categories, D1-like (D1R) and D2-like (D2R), both of which are present in the MeA and VMN [56]. Overall, it appears that D1R is facilitatory toward female sexual behavior, whereas D2R is inhibitory [57, 58].

As previously mentioned, dopamine is released following VCS, and thus stimulates a more intense lordosis response [59]. In the early 1990's dopamine was shown to lead to nuclear localization of the PR in the absence of ligand [60]. This was further developed into a model of ligand-independent activation, whereby following dopaminergic activation of the D1R, an increase in protein kinase A (PKA) phosphorylation would result in PR phosphorylation and targeting to progesterone response elements (PRE) on the genome in spite of a lack of ligand ([61], for review, [62]). Indeed, this appears to be what is occurring *in vivo* as D1R agonists infused into different brain regions associated with sex behavior have been shown to mimic progesterone-induced enhancements of lordosis, whereas D1R antagonists infused into the mPOA decrease pacing behavior [33, 63, 64].

Although dopamine has been associated with ligand-independent activation of the PR (Fig. 5), there is another non-classical pathway by which D1R actions can affect PR signaling.

Extranuclear PRs have been shown to be involved in rapid and transient signaling, mediated by G protein subunits [65]. Activation of g-protein coupled receptors (GPCRs), of which D1R and other catecholamine receptors are subtypes, leads to activation of downstream MAPK and cSrc, both proteins shown to be involved in non-classical, rapid PR signaling [66-68].

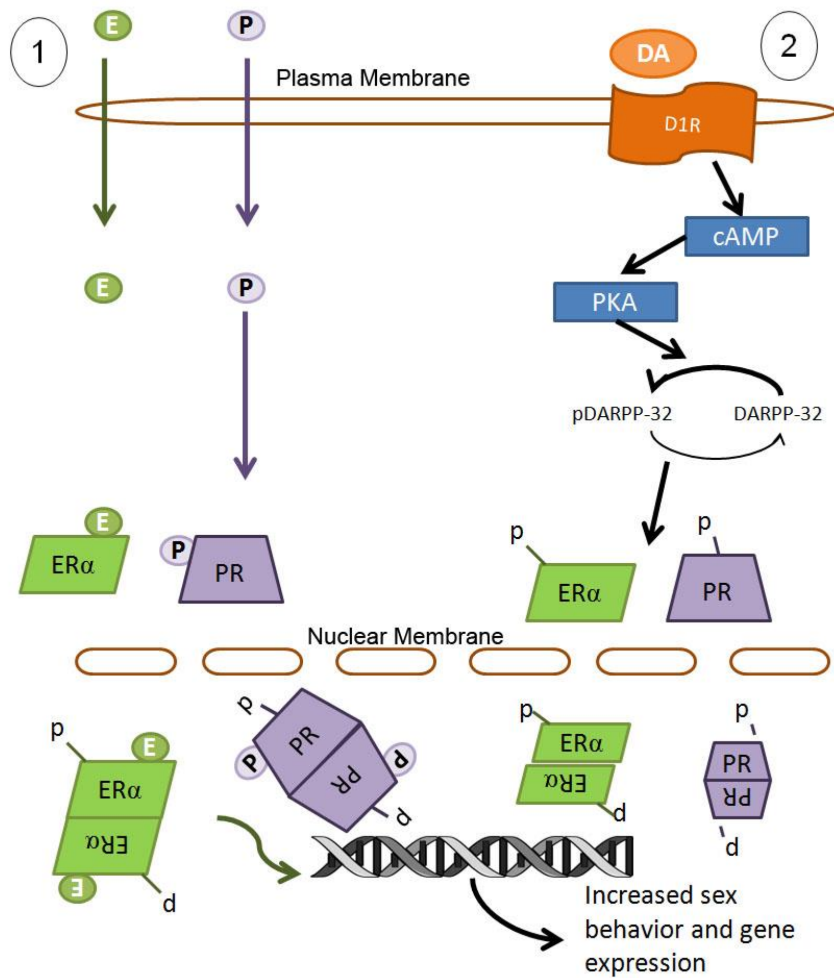


Figure 5: Comparison Between Ligand-Dependent and Ligand-Independent Hormone Signaling Pathways. 1) Ligand-dependent signaling occurs when estradiol binds to the ER or progesterone to the PR. This leads to subsequent nuclear localization, dimerization, and directed gene transcription. 2) In ligand-independent, dopamine-driven signaling, dopamine signals through the D1R, leading to increased cAMP and PKA phosphorylation. PKA leads to increased phosphorylation, and DARPP-32 activity blocks phosphatase activity, thus leading to phosphorylation of ER and PR in the absence of hormone ligand. These phosphorylated receptors then enter the nucleus, dimerize, and lead to gene transcription.

Progesterone Receptors

As previously mentioned, progesterone is required for the full expression of female sexual behaviors. Although progesterone is a necessary component, the involvement of the PR in ligand-independent signaling in addition to its classical, ligand-dependent role, allows for further modulation and enhancement of sexual behaviors.

Nuclear Receptors

The progesterone receptor (PR) is considered a nuclear receptor and has a similar structure to that of the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) and vitamin D receptor. These receptors all have a conserved DNA-binding domain (DBD) within the center of the protein, a C-terminal ligand-binding domain (LBD), and various activation function (AF) domains that occur to either side of the DNA- or ligand-binding domains.

PR has been classically shown to be under transcriptional control by estradiol (E2). Depending on the tissue, signaling via the PR may lead to its down regulation; however, this is not always the case and appears to be tissue specific [21]. Studies by Quadros et. al have shown that the PR exists in the rat brain from early postnatally [69-71], and persists in hormone concentrating regions of the brain, such as the VMN and MeA, through adulthood [72].

PR exists in the cytosol where it binds to heat shock proteins, specifically HSP90, HSP70, and HSP40 [73]. Upon ligand binding, the HSPs dissociate and the PR is able to form dimers which then bind to response elements on the DNA [74, 75]. DNA binding in vivo appears to require a number of cofactors, and these cofactors may actually allow PR to up regulate gene expression without a classical PRE [73].

Structure

When the receptor was first characterized in the 1970's two isoforms were discovered. These two isoforms are transcribed from a single gene with two distinct promoters [76]. The full-length isoform, termed PR_B, is a ~116kD protein consisting of 923 amino acids. At its N-terminus is an AF3 domain, consisting of 164 amino acids that is missing in its ~90kD counterpart PR_A. This AF3 domain allows the recruitment of additional cofactors that enhance its activity [77]. Both proteins have an IF (inhibition factor) domain, followed by an AF1 domain, and the DBD. The C-terminal region of the receptor consists of the LBD which has an AF2 domain [73]. Studies on placenta and T47C breast cancer cells have reported a truncated form of the receptor termed PR_C, which is only 60kD in size and is unable to exert any effects on the DNA, as well as many other small isoforms [78, 79], (Fig.6). These isoforms have yet to be confirmed in the brain, and most of their functions are unknown.

Understanding the function of isoforms B and A in the brain stems primarily from the generation of knockouts in mice. PR_B appears to have a greater role in ligand-activated signaling while PR_A can oppose this action [80]. PR_A does appear to have a greater role in ligand-independent signaling via PKA activation, including that leading to lordosis [81, 82]. This is somewhat contradictory to findings that kinase cascades preferentially phosphorylate the PR_B isoform during sex behavior, but this may be due to the system and study design [83].

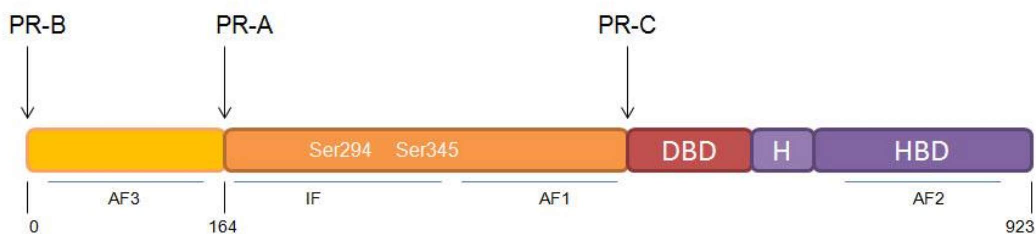


Figure 6: Progesterone Receptor Domains. This schematic outlines the basic structure of the progesterone receptor. The full length version of the receptor, PR-B is 923 amino acids in length. The first 163 amino acids at the N terminus contain and AF3 domain which targets other cofactors to the receptor. Both PR-B and PR-A contain serines 294 and 345, which are named based on their location on the full-length receptor. At the C terminus, there is a DNA binding domain (DBD) and a hormone binding domain (HBD) joined together by a hinge region (H). PR-C is not transcriptionally active and thus far has only been found in placenta and ovarian tissue.

Phosphorylation sites

The PR is a phosphoprotein with up to fourteen serine phosphorylation sites that govern its function [84]. Some of the sites are hormone dependent; however, many of the serines may become phosphorylated based on interaction with kinases, such as CDK (cell-cycle dependent kinase), MAPK, and others [85]. These numerous phosphorylation sites may allow PR to act as a cellular sensor, and keep strict control over its function [86].

Function

PR signaling has proven to be quite complex over the last 20 years. As a nuclear receptor and transcription factor, the classical signaling mechanism in its most simple form states that upon ligand binding, the receptor should go to the nucleus (if not already there) and bind to its response elements in order to lead to gene expression. This classical paradigm has been extended to include not only the traditional ligand-dependent signaling, in which P4 must bind to its receptor to lead to gene transcription, but also ligand-independent receptor activation. It was found that D1R agonists were able to activate PR in the absence of P4 [60, 64]. These agonists did not directly bind to the receptor, but instead lead to activation of kinase cascades, likely through PKA, which lead to phosphorylation of PR [78, 87, 88]. Interestingly, this appears to be the way in which lordosis is stimulated in animals that receive VCS or have been previously mounted by a male, and this effect can be abolished by blocking or down regulating PR [61, 64, 81].

Kinase Involvement in Progesterone Receptor Signaling

Perhaps the most curious of PR's functions is that this "nuclear" receptor has also been found to modulate the cell's function from outside the nucleus. It has long been known that P4 metabolites (which have little to no affinity for PR) modulate membrane receptors for neurotransmitters [89]. However, within the last decade, there has been compelling evidence that the PR is capable of activating kinase cascades without having a nuclear signaling component [67]. In vivo, ERK1/2

inhibitors block the ability of VCS or db-cAMP to enhance receptive and proceptive behaviors [90, 91]. As ERK1/2 can be activated by cSrc, and cSrc activated by GPCRs, Gonzales-Flores and colleagues examined the role of cSrc in the rapid activation of PR. By infusing PP2 (a specific Src-family kinase inhibitor), into the VMN, they were able to block all proceptive and receptive behaviors in the female rat [92, 93].

Extracellular signal-regulated Kinases

Extracellular signal-regulated kinases (ERK), also referred to as mitogen-activated kinases (MAPK), are serine/threonine kinases that are ubiquitously expressed in all tissues [94]. The two most well-known and well-studied of this family are ERK1 and ERK2. According to a review by Roskoski, ERK1 and ERK2 are 84% identical and appear to possess “identical” substrate specificity in vitro[94]. For this reason, both proteins are often referred together as ERK1/2 and will be named such in this thesis.

ERK1/2 (p44/42) is activated by a three-tiered cascade of phosphorylation events (Fig 7). This cascade is typically initiated by the small GTPase Ras. Ras can be activated by a variety of membrane proteins, including G-protein coupled receptors (GPCRs) [94]. Ras phosphorylates cRaf, which then phosphorylates a mitogen activated protein kinase kinase (MEK), which ultimately leads to ERK phosphorylation. ERK1/2 activation requires phosphorylation at two sites – a threonine and a tyrosine [94]. In the striatum, ERK1/2 has been shown to be activated by the combined activity of D1Rs and glutamate receptors (specifically NMDA receptors). Glutamate receptors enhance ERK1/2 upstream activator MEK while dopamine receptors in the striatum are responsible to activating DARPP-32 which decreases phosphatase activity thus prolonging the kinase’s phosphorylation [95].

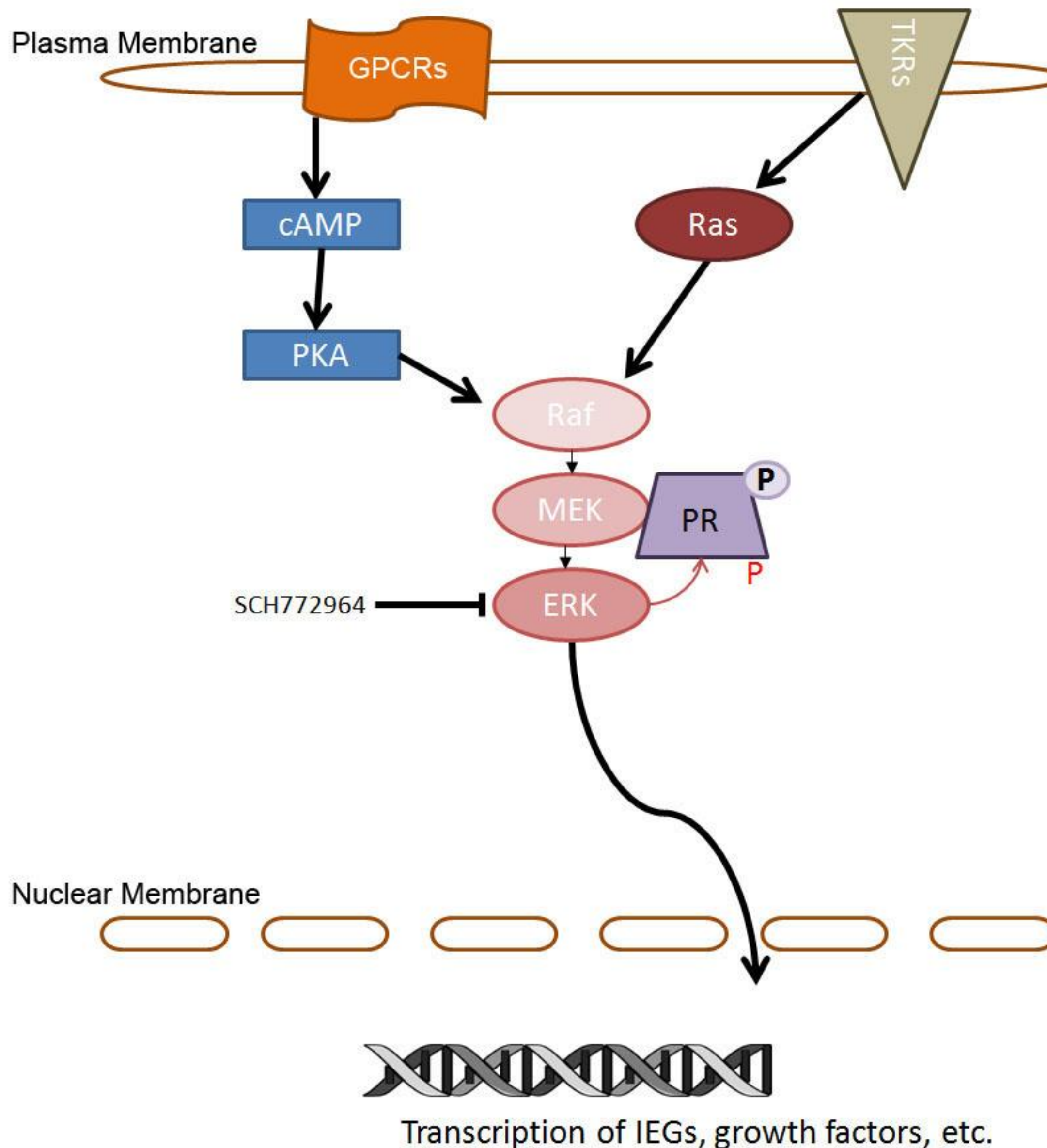


Figure 7: ERK1/2 Signaling and Interaction with PR. ERK1/2 phosphorylation cascade is initiated at Raf, which proceeds to MEK and then ERK1/2. Raf phosphorylation can be achieved via activation of receptor tyrosine kinases (RTKs) or G-protein coupled receptors (GPCRs). PR is able to bind to MEK in a scaffold, thus allowing for phosphorylation by ERK1/2. The ERK1/2 inhibitor SCH772964 is able to block downstream ERK1/2 activity. (cAMP: cyclic adenosine monophosphate, PKA: protein kinase A, IEG: immediate early gene).

ERK1/2 contributes to a variety of processes including cell cycle progression, cell survival, metabolism, and transcription, especially of immediate early gene such as cFos. Within the brain, it has been shown to contribute to cocaine seeking and opiate craving in the striatum [96] and central amygdala [97] respectively, brain-derived neurotropic factor (BDNF) transcription [98] as well as influence ovarian hormone signaling and induction of target genes [83, 99-101]. Recent work in the cancer field has demonstrated that once ERK1/2 is activated, its docking domain becomes open for substrates containing FXF motifs (a docking site for ERK) [102]. In this way, ERK1/2 and other similar MAPKs may be able to bind to PRs thus allowing for a modulation in gene transcription or behavior. It is thought that PR can form a complex with MAPKs, Src, and estrogen receptor to modulate cell growth in cancer [103] or induce signaling in the VMN for baseline sex behaviors [92].

Src Family Kinases

Src kinase or Src, as it will be called in this dissertation, is a 60kD non-receptor protein-tyrosine kinase. It is ubiquitously expressed, but the brain has some of the highest expression levels of any other organ [104]. The Src family kinases (SFKs) play a role in cell differentiation, proliferation, and survival; as such its dysregulation has been implicated in a variety of cancers. The Src family kinases consist of eight members, and they have a well-conserved structure composed of five functional domains (Fig 8). These domains consist of a N-terminal domain which contains signals for lipid modification, Src homology 3 (SH3) and SH2 domains, a kinase domain, and a C-terminal regulatory segment [105].

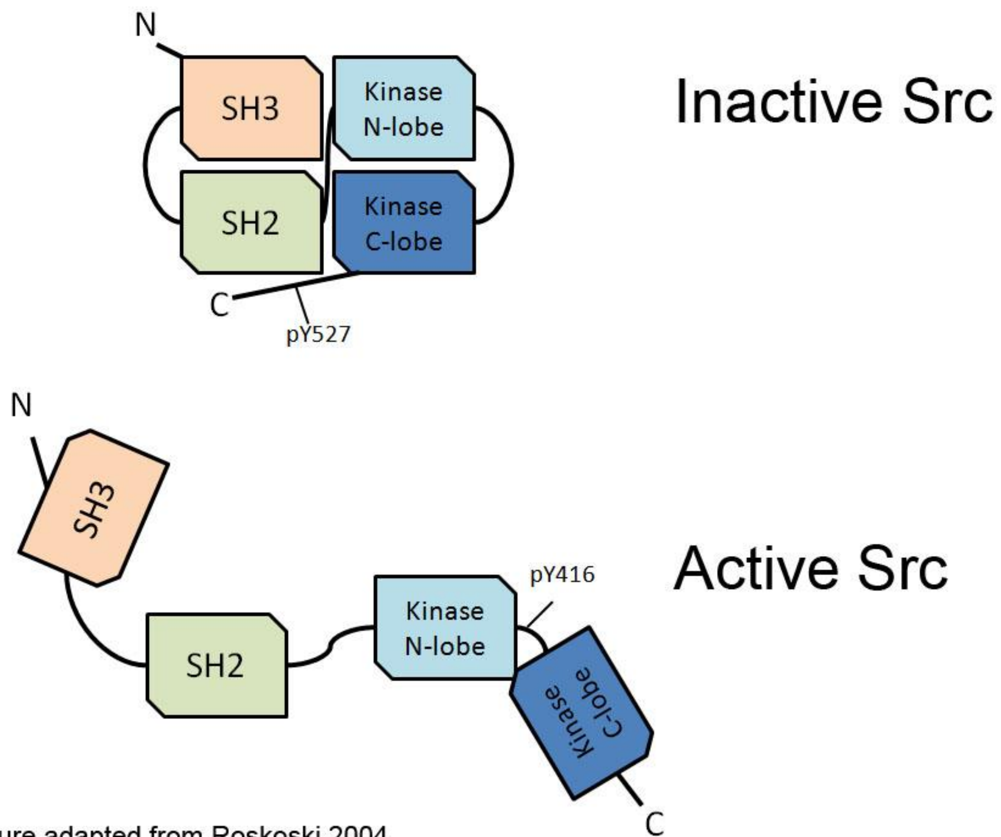


Figure 8: Src Kinase. Inactive Src is phosphorylated at tyrosine 527. Upon dephosphorylation of tyrosine 527, the protein structure unfolds, allowing for access to the SH3 and SH2 binding domains, and subsequent phosphorylation of tyrosine 416 allows for kinase activity.

Of the eight SFKs, only five have been shown to exist in the mammalian brain: Src, Fyn, Yes, Lck and Lyn [106]. Src (p60), the protein for which this kinase family was named has been most thoroughly studied for its roles in cancer and interaction with hormone receptors. Perhaps even more interesting is the existence of a neuronally specific Src (p60+) which consists of six additional amino acids which may contribute to p60+'s extremely high activity in neurons [107, 108]. The Src protein must be dephosphorylated on tyrosine residue 527 (Y527) on its C-terminal tail in order to become activated by phosphorylation of Y416 in the activation loop [109]. Src can become activated by numerous receptors and it is thought that the interaction of individual domains and these receptors are crucial for regulation and specificity of action. Unfortunately, most of the work on Src has been completed in cell lines, and in these cell lines Src can be found throughout the cell, which is not always the case *in vivo* [110]. For example, it has been shown in cell culture that PR activated via an agonist/ligand is capable of activating cSrc kinase/MAPK pathways outside of the nucleus, likely through an interaction of a proline-rich motif within the PR and a SH3 domain in cSrc [66].

Progesterone receptor's ability to interact with these kinase cascades both *in vitro* and *in vivo* has recently become a hot topic in the cancer therapeutic and behavioral neurosciences fields. Prior to the discovery that PR was able to interact with kinases, it was thought to be solely a nuclear receptor and transcription factor. Further understanding of these two seemingly disparate pathways and their ability to influence one another may usher in a new era of molecular study and therapeutic development.

Conclusions on Modulation of Sexual Behavior in the Rat

While overall, sexual behavior is controlled by ovarian steroids (EB+P), it has become increasingly evident that sexual behavior is quite complicated. The reflexive behaviors have been well documented, and the neuromuscular connections elucidated, but the motivational

components of sexual behavior are still not well understood. Multiple brain regions and neuromodulators appear to play a role, and many of the associated regions are connected to one another [50]. As is the case with many diseases or dysfunctions, studying the parts of a "normal" mechanism becomes difficult without disturbing the system in order to determine the role of the individual parts. Likewise, understanding the underlying mechanism for female sexual motivation can be best understood by examining a disturbance to the system, such as that Meth provides.

Meth's Effects on Human Sex Behavior

Meth abuse has been increasing since the mid-1990's, with the 2008 report from the United Nations Office on Drugs and Crime estimating 25 million Meth abusers worldwide [111]. One of the most striking features of Meth use is its penchant for increasing high-risk sex behaviors [5, 7]. These high-risk behaviors are defined as unprotected sex, sex with unknown partners, and increased number of partners – all of which can increase incidence of HIV and transmission of sexually transmitted diseases and unplanned pregnancy. Women appear to also have the added burden of increased psychological stress, including depression, and often use the drug to feel more attractive and to cope with their moods [6]. Treatment for drug abuse has been shown to decrease these behaviors [7] but there is still no preferred method of treatment. This raises the question of why Meth, over alcohol, opiates, and other dopaminergic drugs, increases these high-risk sexual behaviors.

Meth's Mechanism of Action

Meth is a highly addictive psychostimulant that exerts its effects by ultimately purging monoamines from neuron terminals into the intercellular space. These monoamines consist primarily of norepinephrine and dopamine, but also include lesser amounts of serotonin. As Meth enters the brain, it can be taken up by neurons in two ways. First, it is able to diffuse directly into

the neuron, as amphetamine drugs are lipophilic in nature [112], or it may be taken up by the dopamine active transporter (DAT) [112]. Meth and other amphetamines have been shown to be dopamine analogs, thus being recognized by these transporters [112]. Once inside the cell, Meth is able to act on a number of proteins associated with monoamine uptake, storage, and release. Meth can inhibit the vesicular monoamine transporter (VMAT), thus preventing packaging of monoamines into discrete vesicles, as well as block the monoamine oxidase (MAO), which reduces monoamine metabolism. In both cases, cytosolic monoamines begin to increase until the gradient-driven DAT begins pumping monoamines out of the neuron and into the synapse [112, 113]. Meth competes with dopamine and other monoamines for the DAT, which impairs the re-uptake of these neurotransmitters [114].

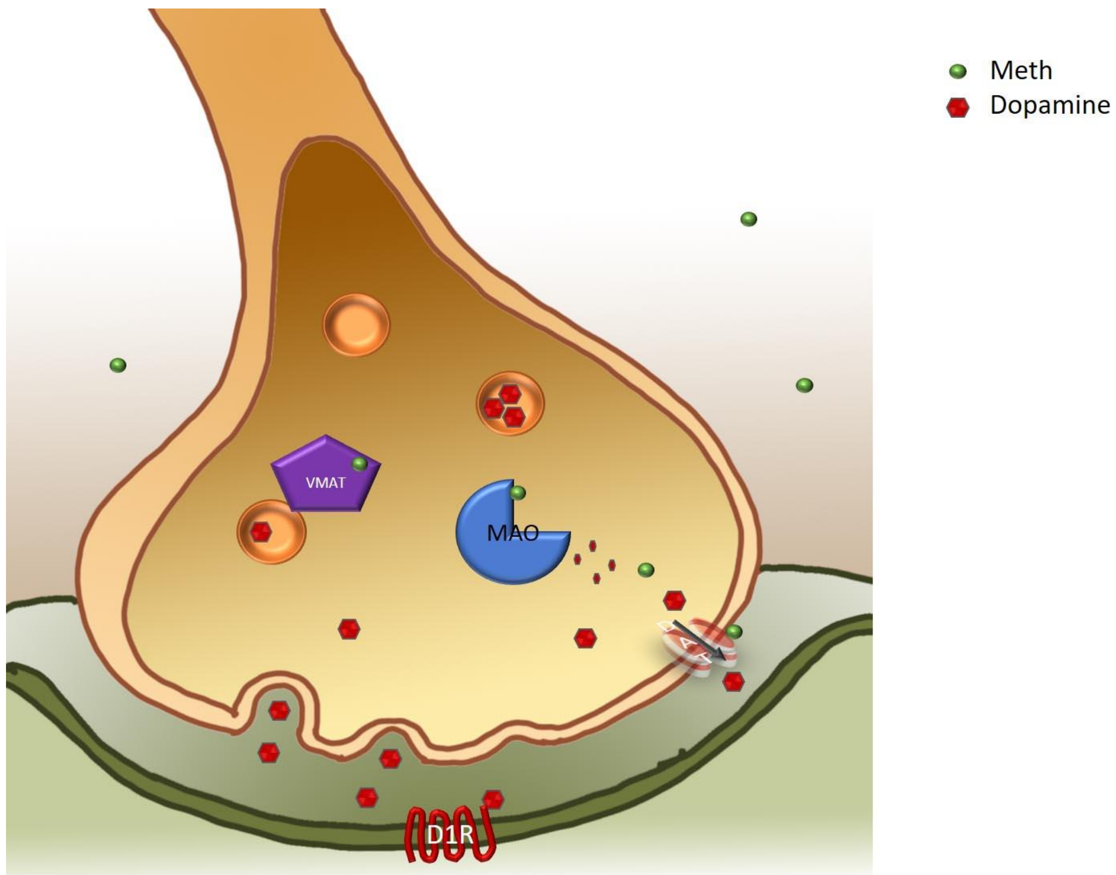


Figure 9: Schematic of Meth Interacting with Components of Dopamine Uptake and Release. Meth enters a neuron via diffusion due to its lipophilic nature or through the DAT. Once in the neuron, Meth inhibits the action of the VMAT and MAO leading to a buildup of dopamine within the cytosol. This increase of intracytosolic dopamine forms a gradient which leads to a reversal of the DAT in efforts to reach equilibrium. Massive amounts of DA are purged from the terminal into the synapse and intercellular space. These released dopamine molecules are not reabsorbed, as the DAT is to longer working as a reuptake transporter, and remain there to signal at the DA receptors.

This uncontrolled release of monoamines both gives salience to the drug for its abusers due to the “feel-good” reinforcing properties of these neurotransmitters, but also results in decreased expression of monoamine receptors. This can lead to tolerance with users trying higher and higher doses just to achieve the same high. Concurrently, Meth has been shown to lead to sensitization, with less drug being required for more damaging effects on the brain and behavior [115, 116].

Meth's Effects on Female Rodent Sex Behavior

A number of studies have been done examining Meth's effects on sex behavior in the rat. Meth is a psychostimulant, and therefore it increases overall movement (hyperactivity) as well as repetitive behaviors (stereotypy) at doses as low as 3mg/kg [117]. These motor effects of amphetamines peak at about 120 minutes following drug administration and are present in the rat up to 150 minutes[118]. In animals treated with Meth, female rats appear effected for a longer timeframe, with these effects lasting as long as 360 minutes [119]. During the development of our model system, these motor effects were taken into account. Stereotypy was evaluated using the scale of Ellinwood and Balster (1974) and open field crossings were recorded to measure changes in locomotor behavior at 15, 150, and 240 minutes after Meth administration. It was determined in this study that by 240 minutes (4h, the time at which we score behavior) the locomotor and stereotypy effects had resolved [120].

In spite of the resolution of these motor effects, Meth is able to increase the number of proceptive events, lordosis quotient (successful mounts) and lordosis score (intensity of lordosis) in hormonally-primed (EB+P) rats [121, 122]. Furthermore, Holder et. al was able to show via increases in cFos and excitotoxic lesions that the posteriodorsal medial amygdala (MePD) was mediating this Meth-induced enhancement of behavior, and when the MeA was lesioned, only the Meth-facilitated increases in behavior were ameliorated with baseline behaviors remaining intact [120, 121, 123].

These behaviors were not just limited to proceptivity and receptivity, but also the time the female rats chose to spend with the males. Paced mating paradigms, where the female controls the amount of interaction she has with the male have been useful for determining the female's interest in copulation. In this test, the female is able to retreat to regions of the cage to which the male has no access. In such trials, Meth reduced the latency for the female to return to the male,

mean percent of exits after mating, and overall rejection behaviors [121]. This interaction does appear to be goal directed as Meth-treated females spend more time with a male partner than a female partner than saline-treated controls [124] and prefer intact or dihydrotestosterone (DHT) – treated castrated (CX) males to untreated-CX males [38].

Since the discovery of these behavioral effects, some components of the molecular signaling pathways behind this behavior have been elucidated. Although Meth causes release of multiple monoamines, dopamine is believed to be the primary effector in this mechanism, as only the dopamine type-1 receptor has been shown to be both necessary and sufficient for the induction of the enhanced proceptivity [123]. Additionally, although it is well established that progesterone is required for baseline proceptive behaviors, the ligand occupied receptor is required in the MePD for Meth's effects on proceptivity, suggesting this is not a ligand-independent or progestin metabolite-induced response [123]. Furthermore, Meth appears to enhance the signaling ability of PR under subthreshold doses of progesterone [125]. Taken together, it appears Meth is altering PR signaling in the MePD to enhance proceptive behaviors, but how these pathways may interact is still unknown.

Medial Amygdala: Nexus of Meth Action

The medial amygdala (MeA) is a subdivision of the amygdaloid nucleus which also consists of a central and basolateral nucleus. The MeA has been shown to be important for sociosexual behaviors, including fear responses, maternal behavior, and sex behavior in both males and females [126, 127]. This nucleus can be further subdivided into three distinct regions based on neuroanatomical differences, the anterior medial amygdala (aMeA), the posterioventral medial amygdala (MePV) and the posteriodorsal medial amygdala (MePD) [127].

Early studies of the MeA (Fig. 10) showed that if the nucleus as a whole was lesioned, sex behavior (in the deermouse) was blocked [128], whereas when the MeA was stimulated,

receptive behaviors, such as lordosis, were increased [129, 130]. In our system, the MeA was discovered to be the drug-sex nexus for proceptive behaviors after Meth administration via lesion studies of the entire nucleus. Baseline proceptive behaviors remained intact after MeA lesion, and only those Meth-induced increases in proceptivity were blocked [121]. This finding suggested a modulatory role for the nucleus in mediation of drug-enhanced sex behaviors. While the medial amygdala as a whole receives projections from the olfactory system and BNST, the MePD receives input from the VMN as well, and is the amygdaloid nucleus primarily involved in reproductive behaviors while the MePV plays a role in fear or defense behaviors [127, 131].

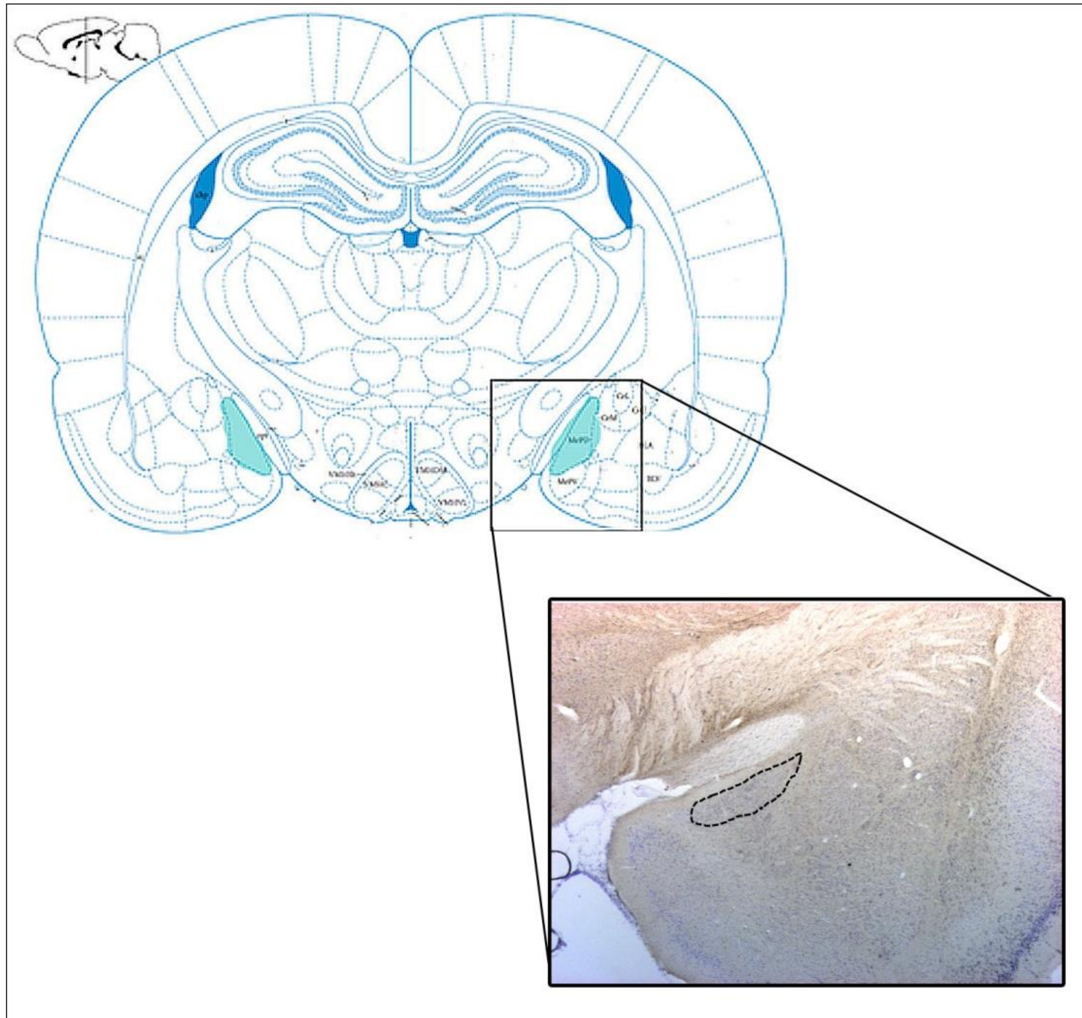


Figure 10: The Posteriodorsal Medial Amygdala. The line drawing is adapted from Paxinos and Watson Brain Atlas and contains a coronal view of the rat brain. The posteriodorsal medial amygdala (MePD) is highlighted in teal. The MePD is a subset of the greater amygdala. The inset image is a photomicrograph of the rat amygdala bordered by the optic tract, with the MePD outlined in black.

The MePD has the highest concentration of steroid hormone receptors of any medial amygdaloid subdivision [126, 131-133]. Additionally, this nucleus contains D1Rs which makes it responsive to dopaminergic drugs such as Meth [56, 134, 135]. The dopaminergic innervation and the presence of estrogen and progesterone receptors in the MePD lead Holder et al. to examine the role of each system in the Meth-induced increase in proceptive behavior. Mary Holder discovered that similar effects on behavior could be found by infusing a D1R agonist into the MePD, but that no other catecholamines alone seemed to give the same response as seen by Meth. Additionally, by giving Meth to estradiol-treated animals, large increases in progesterone receptor immunoreactivity (PR-ir) could be seen in the MePD [123]. Meth's effects on PR protein expression are particularly intriguing given that PR has been historically shown to be upregulated by estrogen receptor (ER) alone.

Since Dr. Holder's discoveries, the MePD has obtained a more robust list of functional significance. It has been found to regulate puberty onset in female rats [136], control erections and plasma LH levels in male rats [137], control "stress eating" [138], and interest in sex specific odors [139]. All these findings point to the MePD as a center modulating motivated behaviors, primarily those that are sexually driven. This nucleus is quite heterogeneous in both its cell populations and functional roles as suggested by the literature. There may indeed be the possibility of further distinct cellular ensembles to govern each of the motivated behaviors linked to this subnucleus.

Conclusions on Meth and Enhanced Sexual Activity

Although humans have a unique social component to sexual motivation, the similarities between the rat and human enhancement of sexual motivation suggests an underlying neurobiological correlate. While not all components of baseline sexual motivation are understood, we know that the MePD integrates signals from D1R and PR to initiate these behavioral changes.

Moving forward, we aim to show that these signals do converge on a common molecular pathway to enhance female sexual motivation.

CHAPTER 2: Goals of the Dissertation

Previous work has suggested that the MePD is the nexus for drug-sex interaction via the D1R and PR pathways. To date, it is still not clear if there is a discrete neuronal population within the MePD that governs Meth's effects on proceptivity, or how these cells incorporate an exogenous signal to enhance motivated sex behavior. Using a model of rodent sex behavior, we propose the following hypothesis:

Hypothesis: Methamphetamine enhances proceptive behaviors via interaction of kinase cascades with the progesterone receptor in a discrete population of neurons in the posteriodorsal medial amygdala.

Aim I: Methamphetamine and hormones activate a discrete population of medial amygdala neurons required for the enhancement of proceptive behavior.

Aim II: Progesterone receptor mRNA and protein phosphorylation is upregulated in the presence of Methamphetamine.

Aim III: ERK1/2 and Src kinase mediate the interaction of progesterone receptor and Methamphetamine signaling.

CHAPTER 3: General Methods

Animals

All animals used were adult female Sprague-Dawley rats (250-300g). Animals were obtained from Charles River Laboratories with the exception of the animals expressing the LacZ gene. The LacZ+ animals were bred in-house and all animals were 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 0900 h) with food and water available ad libitum. Procedures were approved by the University of Maryland, Baltimore, Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Survival Surgeries

Animals were anesthetized with isoflurane and placed on a heating pad to maintain body temperature. All procedures followed aseptic technique. Animals were treated with Carprofen (5mg/kg/mL) subcutaneously as an analgesic postoperatively. Animals were allowed a 7 day recovery period prior to any experimental testing.

Ovariectomies (OVX)

The animals had their flanks shaved and then cleaned with betadine scrub and 70% isopropyl alcohol. An incision was made in the skin and the abdominal muscle wall allowing for removal of the ovary. The muscle wall was sutured closed, and skin was stapled. Closed incisions were treated with topical 3% lidocaine gel and triple antibiotic ointment.

Cannulations

The top of the animal's head and neck was shaved and then cleaned with betadine scrub and 70% isopropyl alcohol. An incision is made in the skin, exposing the skull. Chronic indwelling 25-gauge cannulae (Plastics One, Roanoke, VA) were bilaterally implanted using stereotaxic technique into the MePD of OVX animals (3.0 mm posterior, ±3.55 mm lateral, and 7.0 mm ventral from Bregma). They were affixed to the skull using dental acrylic and dummy stylets were placed into the cannulae in order to keep them clear and free of debris.

Cannula Placement Verification

The animals were transcardially perfused under ketamine/xylazine anesthesia (60 mg/kg, IP) with 0.9% saline containing sodium nitrate, followed by 4% paraformaldehyde in 0.5M kPBS. Brains were stored overnight in a 4% paraformaldehyde solution and then cryoprotected in 30% sucrose in kPBS. After cryoprotection, 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. The sections were rinsed in kPBS and mounted serially on 2% gelatin-coated glass slides. They were rehydrated in dH2O, stained in a 0.5% Cresyl Violet 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 and fell dorsolateral to the optic tract.

Steroid Hormone Treatment

Hormonally primed animals received subcutaneous injections of 17β-estrodiol benzoate (EB) dissolved in sesame oil (100ug/ml); first 5ug followed by 10ug 24 hours later. 48 hours after the first injection of estradiol, the rats received a subcutaneous injection of P4 dissolved in sesame oil

(500 μg, 0.1mL) (Fig. 11). This paradigm mimics the natural rise in hormones across the estrous cycle and has been shown to induce female receptivity [140-143].

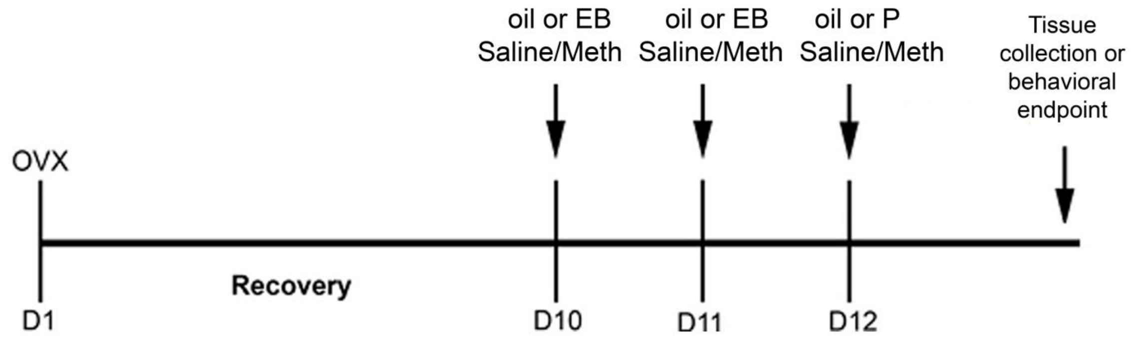


Figure 11: Classical injection schedule. Following ovariectomy (OVX), animals are allowed a week to recover from surgery. Over the course of three days, animals are hormone-replaced to mimic their natural cycle. (EB: estradiol benzoate; P: progesterone).

Methamphetamine Treatment

Experimental animals who received Meth (5mg/mL) received intraperitoneal injections of 0.5mL/kg/day concurrent with hormone or oil injections (Fig 11).

Sexual Behavior Testing

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 Meth injection. On the day of behavioral testing, two sexually naïve Sprague Dawley males were each placed alone in a 50 cm × 38 cm × 25 cm Plexiglas observation chamber and allowed to acclimate over 5 minutes. An experimental female was then placed with each male. Each behavioral test was recorded by a video camera and was completed when 15 min had elapsed. Between behavioral tests, males were allowed 5 minutes to rest before the next encounter, and each female was exposed to both males. Naïve males were chosen for these tests as less vigor encouraged a reduction in rejection behaviors seen. During the tests, the investigator remained at a consistent location approximately 0.5 m away from the observation chambers during all trials.

An experimenter blind to the treatment groups scored the proceptive sexual behaviors. The number of proceptive behaviors (hops, darts, presentations and ear wiggling), receptive behaviors (lordosis score, quotient), rejection behaviors from the females, and male mounts that occurred in 15 min were quantified. To control for any differences in male behavior, female behaviors were averaged between 15 minute sessions. Male mounting was compared between individual animals (male vs. male) as well as to female treatment groups.

Western Blotting

1.5-2mm punches of frozen MePD 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 (3:1000 each; Sigma). Samples were then centrifuged and supernatants collected for analysis. A Coomaasie Blue kit was used to measure protein concentration of each sample (Pierce, Rockford, IL). Samples (10ug) were loaded into the wells of 8-16% Tris-Glycine gels (Invitrogen) and electrophoresed. The proteins were then blotted onto polyvinyl difluoride (PVDF) membranes (Invitrogen), blocked with 1% milk or 5% BSA for 1 hour and incubated with primary antibody overnight. The blots were incubated with an HRP-linked secondary antibody 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/nih-image). The optical densities were measured for each individual band. Band values were normalized to GAPDH.

Immunocytochemistry

Animals were transcardially perfused under ketamine/xylazine anesthesia (80 mg/kg, IP) with 0.9% saline containing sodium nitrate, followed by 4% paraformaldehyde in 0.5MkPBS. Following perfusion, the brains were extracted, post-fixed in 4% paraformaldehyde followed by sucrose embedding (15% sucrose for 24 hours followed by 30% sucrose in potassium phosphate buffered saline and then stored at -80C until being cut on a cryostat. The brains were sectioned at 35um and then cryoprotected (ethylene glycol/glucose in phosphate buffer). The tissue was processed using variations of this basic protocol. Cohorts containing sections from all treatment groups were rinsed in kPBS, then blocked in 0.5% Triton-X, 5% normal goat serum, and 1% hydrogen peroxide in kPBS for 1 hour. Sections were then incubated with the primary antibodies. Sections were then washed and incubated in secondary antiserum, followed by avidin-biotin complex. Sections were developed in ammonia nickel sulfate (25mg/mL, Sigma) and 3,3’

diaminobenzidine tetrachloride (DAB) solution (0.2mg/mL, Polysciences, Warrington, PA). The sections were mounted on 2% gelatin-coated glass slides and cover slipped.

Statistical analysis

All results are expressed as means ± SEM. All statistical tests were conducted using GraphPad Prism software (San Diego, CA USA) on an Intel Duo Core PC. Each chapter will have a detailed description of the statistical tests used for each experiment.

CHAPTER 4: Identification of a Discrete Ensemble of MePD Neurons Involved in Meth-Facilitated Proceptivity

Introduction

Several laboratories, including our own, have demonstrated that Meth facilitates sexual motivation in female rodents [120, 121, 123, 124, 144]. It is well established that proceptive behaviors require priming with estradiol followed by progesterone (EB+P). In our established model, administration of Meth to sexually receptive females (ovariectomized females treated with estradiol and progesterone; EB+P) increases proceptive behaviors over ovarian steroids alone [120, 121, 123]. This meth-induced increase in female sexual motivation is specific to sexually relevant stimuli as meth-treated females display increases in proceptive behavior toward intact males and androgen-treated castrate males but not toward castrate males [38].

Our work suggests a neurobiological correlate to this Meth-facilated female sexual motivation. In sexually naïve females, the combined administration of Meth and EB+P increases MePD neuronal activation as measured by cFos immunopositive cells[121]. Excitotoxic lesion of the MePD eliminates Meth's effects on proceptivity[123]. While whole-nucleus lesions are a valuable tool in determining nucleus function, they are often imprecise and do not allow for the specific targeting of functionally relevant neuronal cell types or ensembles necessary for the out-put behavior.

Recently, Bruce Hope and colleagues have pioneered a technique that allows for determining whether specific neuronal ensembles are required for a specific behavior [145]. Using Sprague Dawley rats expressing a cFos-lacZ transgene, the 'Daun02 inactivation technique' exploits the expression of β- galactosidase (β-gal) under the cFos

promoter to lesion neurons that are activated by a specific stimulus or state while the neighboring non-activated neurons remain intact. The prodrug DAUN02 is converted by β-gal to daunorubicin, which is toxic to the cells resulting in apoptosis[146-148]. As β-gal expression is induced only in neurons with a strong activation of the cFos promoter and not in Fos negative or weakly activated neurons[149, 150], this method is a powerful approach to testing for behaviorally relevant neuronal cell groups or ensembles [145, 149, 150].

To test the hypothesis that EB+P active cells in the MePD are required for enhanced proceptivity, we have adapted this model to test whether the Meth/EB+P responsive cells (as measured by cFos expression) in the MePD are necessary for the associated increase in proceptive behaviors. Here we establish that cFos-lacZ transgenic rats exhibit Meth-induced MePD neuronal activation as well as increases in proceptive behaviors and demonstrate that DAUN02 lesion of Meth/EB+P responsive cells in the MePD constitute a behaviorally relevant neuronal ensemble that is required for the Meth-facilitated increases in proceptive behaviors.

Materials and Methods

1. **Animals:**

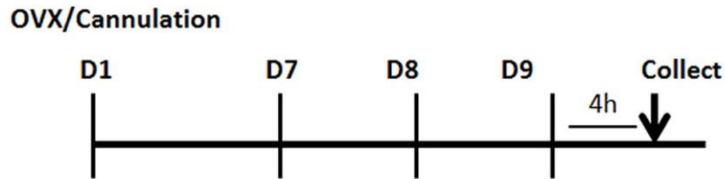
cFos-lacZ transgenic rat[151] (275–300 g; n=45) breeding pairs were obtained by Friedbert Weiss, Ph.D. (The Scripps Research Institute (TSRI)) and were bred in-house and housed in the Laboratory Animal Facility of the Health Sciences Facilities at the University of Maryland, School of Medicine under a reversed 12 h:12 h dark: light cycle (lights off at 0900 h) with food and water available ad libitum. Transgenic pups and their

wild-type littermates were genotyped to verify expression of the c-fos-β-galactosidase fusion protein by taking ear punches or tail segments from pups at the time of weaning. DNA was isolated from samples using isopropanol precipitation. Briefly, tissue was incubated overnight in proteinase K lysis buffer at 55°C, DNA was precipitated with isopropanol from solution and added to a 70% ethanol solution and pelleted. DNA is then amplified using OneTaq kit (New England Biolabs) using 400μg DNA, 10 micromolar forward and reverse primers (IDT, Iowa). Primer sequences were LacZf2538: 5'-GTT GCA GTG CAC GGC AGA TAC ACT TGC TGA-3' and LacZR2926: 5'-GCC ACT GGT GTG GGC CAT AAT TCA ATT CGC -3'. Samples were run on a 2% agarose gel using tris-acetate (TAE) buffer and ethidium bromide. LacZ bands are visualized under UV light at 388bp. All animals were group housed until cannula placement when they were placed in single-housed conditions. All animals were bilaterally ovariectomized under isoflurane anesthesia and allowed a 7–10 day recovery period following surgery.

2. Beta-Galactosidase and Fos immunoreactivity in the MePD following Meth and ovarian steroid treatment in LacZ+ female rats

Previous work has shown that in wild-type Sprague Dawley rats, Fos expression is highest following the combined administration of Meth and EB+P at the time of behavior (4 hours post-treatment) [121]. To verify this trend in our transgenic LacZ+ animals, we examined expression of both Fos and its proxy, β-gal. Four hours after the last Meth and hormone injection (Figure 12), animals were transcardially perfused under ketamine/xylazine anesthesia (98 mg/kg and 18.5mg/kg, respectively; IP) with 0.9% saline containing sodium nitrate, followed by 4% paraformaldehyde in 0.5M kPBS. The brains were extracted, post-fixed, cryoprotected, frozen and processed for immunohistochemistry as described in chapter 3. Briefly, cohorts containing

sections from all treatment groups were rinsed in kPBS, blocked in 0.5% Triton-X, 5% normal goat serum, and 1% hydrogen peroxide in kPBS for 1 hour. Sections were incubated overnight at 4°C with mouse monoclonal antibodies targeted to beta-galactosidase (G8021 Clone GAL-13 Sigma, St. Louis, MO) at a dilution of 1:30000 or 48h at 4°C with a rabbit polyclonal antibody targeted to cFos (Cat No. 18-0172 Calbiochem Invitrogen, Carlsbad, CA) at a dilution of 1:40,000 in 0.05% Triton and 1% normal goat serum in kPBS. Sections were then washed and incubated in a secondary antibody (goat anti-mouse or goat anti-rabbit, respectively; Vector Laboratories, Burlingame, CA) in 0.5% Triton in kPBS for an hour, followed by avidin-biotin complex. Sections were developed in DAB (3-3'-Diaminobenzidine tetrahydrochloride, 04001-5 Polysciences, Inc., Warington, PA). The sections were mounted serially on 2% gelatin-coated glass slides and cover slipped. The number of β-gal- and cFos-immunoreactive cells were counted with the aid of the Neurolucida software (MicroBrightField, Colchester, VT) using laboratory standard methods in accordance with previously defined parameters for the MePD [120, 121]. Briefly, a standardized contour was used to demarcate the MePD. Three brain sections (in series) separated by 140 μm were used. In the event that three sections from the appropriate brain region could not be obtained, the animal was excluded from that region's analysis. Both sides of this bilateral nucleus were included in the analysis, resulting in six counting contours. From these six contours, average β-gal- or cFos- positive cell number per section for each region was derived. Both β-gal and cFos were evaluated in each animal to determine if expression levels followed similar patterns and to make a more direct comparison to cFos expression from previous studies. Slides were anatomically matched and numerically coded so that the investigator conducting analysis was blinded to the experimental group.



Group	Treatments		
Baseline	5ug EB Saline	10ug EB Saline	500ug P4 Saline
EB+P & Meth	5ug EB Meth	10ug EB Meth	500ug P4 Meth

Figure 12: Treatment paradigm for Oil/EB+P and Saline/Meth. Animals are OVX and allowed to recover over a week. Following recovery, animals are either hormonally primed over three days or given oil vehicle. At this time, animals also received either Meth or saline vehicle. Behavior or tissue was collected 4 hours after the final injection.

3. Meth-induced increases in proceptive behavior in Lac Z+ rats

Our previous studies consistently demonstrate that Meth increases proceptive behavior on average 2.5 fold in EB+P treated Sprague Dawley rats [120, 121, 123]. This established behavioral paradigm was applied to the Lac Z+ transgenic rats to verify similar behavioral responses compared to wild-types. The behavioral tests were conducted as described in Chapter 3.

4. DAUN02 Inactivation of Meth- and EB+P-active cells in the MePD

All following phases are shown below in Figure 13.

4.1. Stereotaxic surgeries targeting posteriodorsal medial amygdala

Surgeries were completed as described in Chapter 3. Animals were allowed a 7 day recovery period and then treated as outlined in Figure 6 and the following sections.

4.2. Lesion Phase

All animals received infusions of DAUN02 (HY-13061, MedChemExpress, Princeton, NJ, USA) or vehicle 10 minutes prior to oil or EB+P and saline or Meth injections. During the microinfusions, the dummy stylets were removed and replaced by 33 gauge microneedles that project 1.3 mm below the guide cannulae and were attached via polyethylene tubing to a 25 µl Hamilton syringe (700 series, Hamilton, Reno, NV). Fluid flow rate was controlled by a BASi Bee pump attached to a Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, IN). Bilateral lesions targeting the beta-galactosidase producing cells of the MePD were produced with injections of 0.5 µl of DAUN02 (4.0 µg/µl in 5% DMSO PBS). Infusions occurred over 5 min and the microneedles remained in place for an additional 5 min to ensure diffusion away from the needle tips. Control lesions were performed using the same methods, but using vehicle injections.

Initial pilot studies infused DAUN02 over the first three days of Meth exposure. Optimization of the study led to a reduction of this protocol to a single injection on day 9. Triple infused animals

were compared to singly-treated animals and no statistical difference was found; therefore the groups were collapsed. All animals were given 5-7 days for the DAUN02 to lesion the cells and to be adequately metabolized before entering the next phase.

4.3. Behavioral Phase

All animals were treated with Meth and EB+P as described in Figure 12 and tested for sexual behavior as described above four hours after receiving the combined Meth and P treatment.

4.4. Confirmation Phase

Animals were retreated with oil or EB+P and saline or Meth as they were in the Lesion Phase (Figure 8). Four hours following the final injection, animals were perfused and brains collected for immunohistochemistry and cannula placement verification.

4.5. Cannula Placement verification

Cannula placement was verified as described in Chapter 3.

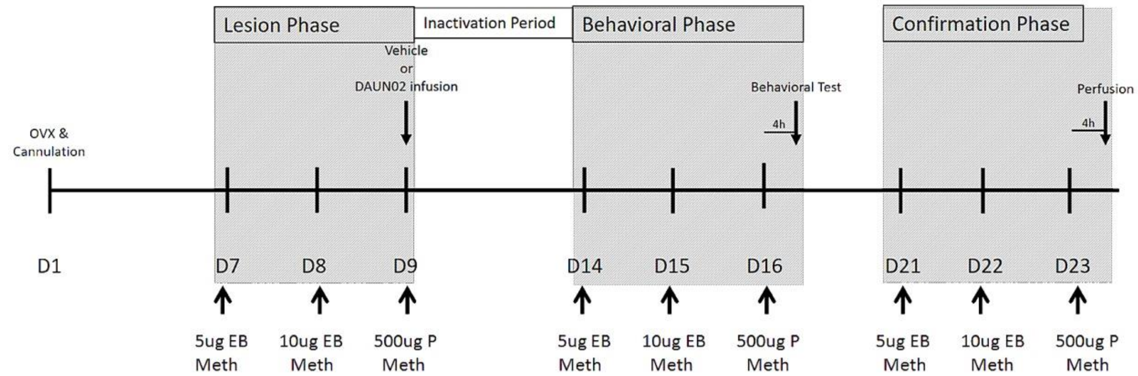


Figure 13: Three-phase treatment paradigm for cFos-LacZ transgenic rats receiving DAUN02 infusions. Animals were OVX/cannulated as described in Chapter 3. Following surgery, animals were assigned to either DAUN02 or Vehicle treatment groups. All animals received the combined treatment of Meth and EB+P to induce the highest levels of behavior. A single volume of DAUN02 or DMSO-vehicle was infused on Day 9, animals experienced hormonal priming and Meth administration over 3 cycles. The first cycle induced the lesion, an inactivation period to allow for clearance of the drug and effected cells followed, the second cycle was followed by behavioral assay as described in Chapter 3, and the final cycle resulted in tissue collection 4 hours after the final injection of Meth/P4.

5. DAUN02 inactivation of cFos+ cells of the MePD in hormonally primed rats

Brains collected for cannula placement verification also underwent cFos IHC. Briefly, during the confirmation phase as seen in Figure 8, animals were retreated with both Meth and hormones and collected four hours after progesterone treatment on day 24. cFos IHC was completed as described above to determine if fewer cells expressed cFos, during the time of behavior, after DAUN02 infusions.

Statistics

All results are expressed as means ± SEM. The β-gal- and Fos-positive cell counts used for verification of the model were analyzed using a student's t-test with drug as the independent variable. Behavioral tests conducted to compare wild-type and cFos-lacZ transgenic rats were analyzed using a two-way ANOVA with strain (wild-type and cFos-lacZ) and treatment (EB+P & saline vs. EB+P & Meth) as independent variables. DAUN02 inactivation effects on proceptive behavior were analyzed using a one-way ANOVA, and the receptivity effects were evaluated using a student's t-test comparing vehicle and DAUN02 infusions. cFos immunohistochemistry used for verification of lesions was analyzed using a student's t-test comparing vehicle and DAUN02 infusions. All analyses except the t-tests were followed by a Tukey's post-hoc test.

Administration of Meth increased Fos- and Beta Galactosidase-immunoreactivity in the MePD of hormonally-primed cFos-lacZ transgenic rats.

Similar to our previously published data on Sprague-Dawley wild-type females[121], the number of Fos-immunoreactive (-ir) cells increased in the MePD following Meth administration in the cFos-lacZ transgenic females (Fig 14a). This is quantified in Fig 14b, which shows approximately 2-fold increase in Fos-ir after the administration of Meth/EB+P, compared to saline/EB+P-treated controls ($t_{(4)}=2.28$, $p<0.05$; Fig 14b). Similarly, the number of β-gal-ir cells in the MePD of the cFos-lacZ rats increased following Meth administration to hormonally primed females (Fig. 14c). This was quantified to be a 3-fold increase after the administration of Meth/EB+P ($t_{(4)}=2.09$, $p=0.05$; Fig. 14d); however, the number of β-gal -ir cells in the MePD was notably fewer than the Fos-ir cells in both treatment groups.

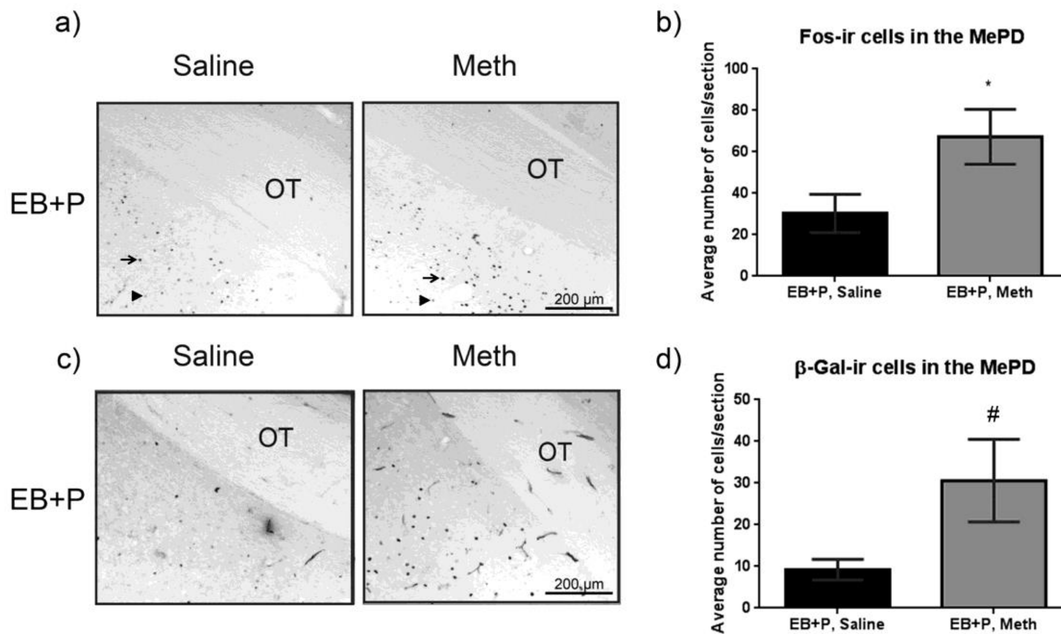


Figure 14: Effects of Meth on cFos immunoreactivity (Fos-ir) & Beta-galactosidase immunoreactivity (β-Gal-ir) in the posteriodorsal medial amygdala. Adult OVX cFos-lacZ transgenic rats were primed with EB+P and received either saline or Meth (5mg/kg). Four hours after the final injection, tissue was collected for cell counts. (a) The photomicrographs represent the Fos-ir in the MePD. Arrows identify highly expressed cFos-ir and arrow heads identify low expressing cFos-ir. Both expression types were included in quantification. (b) Quantification of cFos-ir in the MePD. A t test revealed a significant difference between groups ($t_{(4)}=2.29$, *$p<0.05$, n=3). (c) The photomicrographs represent the β-Gal-ir in the MePD. (d) Quantification of β-Gal-ir in the MePD. A t-test shows a strong trend for increase in the presence of Meth ($t_{(4)}=2.09$, #$p=0.05$, n=3). OT = optic tract.

Meth administration increases proceptive behaviors in hormonally-primed cFos-lacZ transgenic rats.

Meth significantly increased proceptive behaviors in hormonally primed wild type and cFos-lacZ transgenic rats compared to those treated with EB+P alone. A two-way ANOVA with drug and genetic strain as factors revealed a significant main effect of Meth ($F_{(1,12)}=61.99$, $p<0.0001$; Fig. 15a). A Tukey's *post hoc* test indicated that Meth increased proceptive behaviors in both WT (**$p<0.005$) and cFos-lacZ transgenic rats (***$p<0.0005$) compared to their respective saline controls. The two-way ANOVA additionally revealed a significant main effect of strain (WT v. LacZ+) ($F_{(1,12)}=5.75$, $p<0.05$; Fig. 15a). A Tukey's *post hoc* test indicated that the difference between strain resulted from each saline control being significantly different from the other strain's Meth-treated group ($^{a}p<0.01$, $^{b}p<0.0001$). There was no effect of drug or strain for either lordosis quotient ($F_{(1,13)}=1.46$, $F_{(1,13)}=1.46$, Fig. 15b) or lordosis score ($F_{(1,14)}=0.001$, $F_{(1,14)}=2.91$, Fig. 15c).

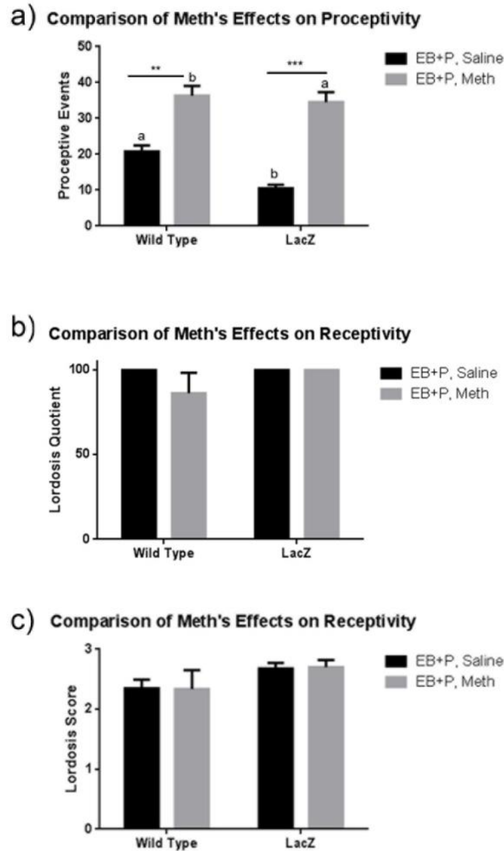


Figure 15: Effect of Meth on proceptive behaviors (a) and lordosis (b, c) in hormonally primed female rats: a comparison between wild type and cFos-lacZ rats. Adult OVX WT Sprague-Dawley and cFos-lacZ transgenic rats were primed with EB+P and received either saline or Meth (5mg/kg). Behavioral tests were performed four hours after the final injection. A two-way ANOVA demonstrates there is a main effect of Meth ($F_{(1,12)}$=61.99, p<0.0001, n=3-5; Fig 15a) and Strain ($F_{(1,12)}$=5.75, p<0.05, n=3-5; Fig.15a) on proceptive behavior. A Tukey's *post-hoc* multiple comparison test reveals that the saline treated animals are significantly different from their respective Meth treated group (WT: **p<0.005, LacZ: ***P<0.0005) and the saline and hormone treated animals are significantly different from the other strain's Meth treated group (^{a}p<0.01, ^{b}p<0.0001), but each saline treated or meth-treated pairs are not different from each other. There was no effect of drug or strain for either lordosis quotient ($F_{(1,13)}$=1.46, $F_{(1,13)}$=1.46, Figure 15b) or lordosis score ($F_{(1,14)}$=0.001, $F_{(1,14)}$=2.91, Figure 15c).

Meth induction of cFos-ir cells in the MePD decreased following a single DAUN02 infusion

Following DAUN02 infusion and behavioral testing, tissue was evaluated for the number of cFos positive cells four hours following the final administration of Meth and EB+P. A student's t-test revealed that DAUN02 infusion resulted in a significant decrease in cFos-ir cells in the MePD compared to Vehicle-infusion ($t_{(15)}=2.163$, *$p<0.05$; Fig. 16c).

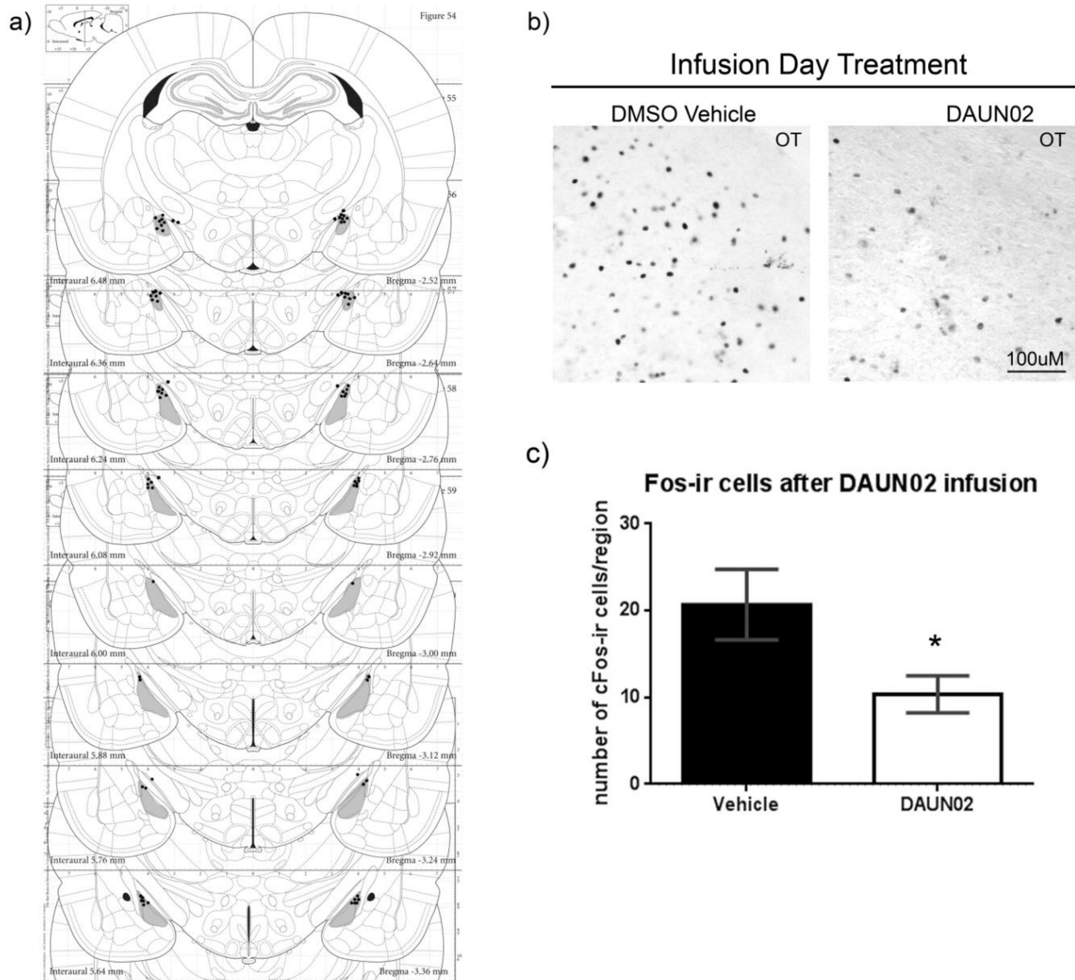
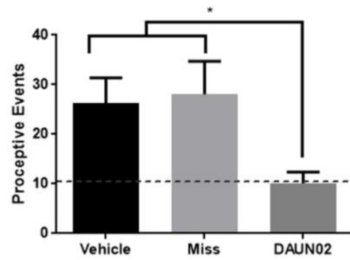
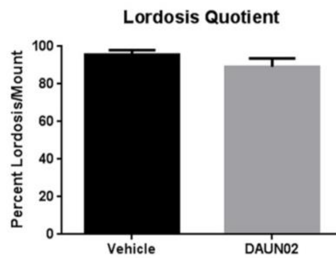


Figure 16: Effect of DAUN02 infusions on cFos-ir in the MePD. (a) Schematic reconstruction of the cannula placements in the MePD (cannula ends are marked in black, any black circle outside of the nucleus as defined in grey is considered a "miss"). Coronal sections through the medial amygdala, adapted from Paxinos and Watson (2005). Animals with cannula placement outside of the MePD were not included in behavioral analysis. (b) Photomicrographs represent cFos in the MePD after Meth and hormone administration following DAUN02 or vehicle infusions. (c) A student's t-test showed DAUN02 significantly reduced Fos-ir cells in the MePD compared to the vehicle-treated cohort ($t_{(15)}$=2.163, *$p<0.05$, n=9). OT = optic tract

DAUN02-inactivation of Meth/EB+P-responsive cFos+ cells reduced Meth's effects on proceptivity

A one-way ANOVA revealed that DAUN02 infusions directly into the MePD of cFos-lacZ transgenic rats treated with Meth and EB+P reduced Meth-facilitated increases in proceptivity ($F_{(2,25)}=1.176$, $p<0.05$; Fig. 17a). A Tukey's multiple comparison test indicated that DAUN02 treated rats exhibited significantly fewer proceptive behaviors, about 1.5-fold fewer, compared to the Vehicle infused controls (*$p<0.05$; Fig. 17a). Moreover, proceptive behaviors in DAUN02-infused animals that had improperly placed cannulae (i.e. not targeting the MePD) were not significantly different from the vehicle infused controls but did display significantly more proceptive behaviors than the properly targeted cohort (*$p<0.05$, Fig. 17a). Significant differences were not noted in the lordosis quotient or lordosis score ($t_{(14)}=1.34$, $p=0.20$, Fig. 17b; $t_{(14)}=1.25$, $p=0.23$, Fig. 17c).

a)


b)


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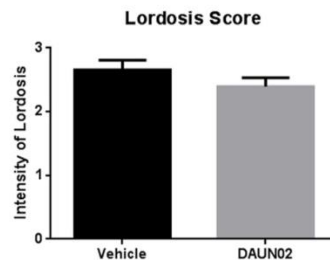

Figure 17: Effects of DAUN02 infused into the MePD on Meth-enhanced female sex behaviors. Following DAUN02 inactivation, animals were hormonally primed and treated with Meth (5mg/kg) over three days. Behavioral assays were completed four hours following the final treatment. (a) A one-way ANOVA revealed DAUN02 significantly reduced proceptive sex behaviors in Meth and hormone treated animals ($F_{(2, 25)}$=4.381, $p<0.05$, n=7-11). Tukey's post-hoc comparison reveals that both vehicle-treated (n=11), and animals with inappropriate cannula placement (miss, n=7) show significantly greater numbers of proceptive behaviors than animals who had DAUN02 infused into the MePD (n=10) (*$p<0.05$). Dashed line shows EB+P-only baseline behavioral mean from Figure 4a for comparison. (b), (c) Receptive Sex behaviors after DAUN02 infusion showed no significant differences between groups for Lordosis Quotient or Lordosis Score.

Discussion

The findings presented here demonstrate that the Fos-lacZ transgenic rats expressing β-gal under the cFos promoter exhibited similar functional neuroanatomical and sexual behavioral responses to Meth as previously reported [121] and that targeted DAUN02-inactivation of EB+P/ Meth responsive cells in the MePD attenuated Meth-facilitated increases in proceptive behaviors.

We and others have reported that Meth increases proceptive (or solicitational) sex behaviors in female rats [120, 121]. In addition to the increases seen in proceptive behavior, Meth leads to increased neuronal activation of the MePD as shown by increased levels of cFos immunoreactivity [121]. We recently demonstrated via excitotoxic lesion studies that the MePD is necessary for Meth-induced increases in proceptive behaviors. However, until now it was not clear whether the MePD neurons activated (i.e. Fos-ir) by the combination of Meth and ovarian hormones (EB+P) played a significant role in mediating the Meth-induced proceptivity. We hypothesized that these Meth/EB+P responsive MePD neurons may represent a discrete neuronal ensemble responsible for the modulation of proceptivity in the presence of Meth.

In a series of reports, Hope and colleagues have recently established that the c-Fos-LacZ rats in combination with the DAUN02 inactivation technique is a suitable model for selectively eliminating specific neuronal ensembles and testing for their causal role in specific behavioral outputs [146-150]. The DAUN02 lesions are ideal for determining the role of specific cells, as they do not cause a global disruption of the parenchyma [150]. Instead, only those cells expressing high levels of Fos (and thus β-

Gal) are removed via apoptosis [148]. Similar to what others have found in other regions, in sexually naïve animals, we observed fewer β-Gal-ir cells than Fos-ir cells within the MePD. While the potential exists that this is due to incomplete transmission of the transgene (perhaps in heterozygous animals), it has been shown that β-gal expression is induced only in neurons with strong expression of Fos [149, 150]. Curiously, fewer cFos-ir cells were observed in the vehicle-treated controls for the DAUN02 inactivation phase (Fig 16c; vehicle group) compared to the sexually naïve cohort treated with Meth and EB+P (Fig 14b). This reduction in c-Fos-ir cells between experiments may be due to the repeated Meth exposure. In other brain regions, repeated or chronic Meth administration reduces the number of Fos-ir cells [152, 153]. In the DAUN02 experiments, animals received nine Meth injections compared to three received by the sexually naïve animals.

Nevertheless, in the current study, the number of Meth /EB+P responsive MePD cells (as measured by cFos-ir) were significantly reduced in the DAUN 02 treated animals compared to the vehicle controls treated with Meth/EB+P, suggesting that the DAUN 02 treatment was effective in removing a significant portion of the neuronal population responsive to the combined treatment of Meth and ovarian steroids. Moreover, the Meth-induced increase in proceptivity was absent in the DAUN 02 –treated animals further suggesting that this ensemble of MePD cells responsive to the combination of Meth and ovarian steroids in necessary for the increase in behavior. While some cFos-ir cells in the DAUN02 treated cohort remained, they were not sufficient to allow Meth-induced increases in proceptivity. These remaining cFos-ir cells may have escaped the cell-specific lesion due to inactivation but not cell death following DAUN02

administration [148]. Alternatively, as repeated Meth exposure results in sensitization [115, 116, 154], it is possible that the remaining cFos-ir cells following DAUN02 exposure were initially the low-expressing cFos cells that did not produce enough β-gal to become lesioned in the first phase of the study. However, following three weeks of Meth exposure, these cells may have become high responders as a result of repeated Meth exposure. Finally, animals who received DAUN02, but who had improper cannulae placement outside of the MePD (i.e. "misses") exhibited levels of proceptive behavior similar to that of the vehicle treated controls; further supporting the specificity of our cell-targeted lesion. Thus taken together these findings support the hypothesis that Meth's effects on proceptivity are dependent on a discrete population of neurons within the MePD, and that this ensemble is relevant specifically to the behavioral effects of Meth on proceptivity alone.

Previous studies implicate the MePD as a key site for the modulation and enhancement of female sexual motivation [36, 124, 129, 130, 155-157]. The MePD processes and integrates sexually relevant signals such as chemosensory [158] and somatosensory signals [45, 159-162], suggesting that the MePD may act as a nexus in the expression of motivated sexual behaviors. Lesions of the MeA lead to a decrease in lordotic responses [130], proceptive behaviors [129, 155], and conditioned place preference[156], whereas stimulation of the MeA increases receptive behaviors such as lordosis [129, 130]. Our previous [120, 121, 123] and current findings that a specific ensemble of MePD neurons responsive to Meth and EB+P is necessary for the enhancement of proceptive behaviors, but not for the display of baseline sexual behaviors suggests that a greater than baseline activation of the MePD is necessary in the

enhancement of sexual motivation. Our findings are in agreement with those of Afonso et al, who have identified a naturally occurring variant in Long–Evans rats in which females exhibit “super-solicitational” behaviors [155, 163]. These super- solicitational rats have a 2.5-fold increase in Fos-positive cells in the MePD compared to normal rats [155], and MePD lesions abolish the expression of the solicitation behaviors [155]. The mechanisms mediating the increase in the activation of the MePD neurons in the Long-Evans variant are not clear. However, in our model of Meth-facilitated proceptive behaviors, signaling via the dopamine D1 receptor and progesterone receptor in the MePD are both required for the Meth-induced increase in female sexual behaviors [123]. Future work using the cFos-lacZ rats will examine whether the dopamine and progesterone signaling component requires separate MePD cell populations.

In summary, our current findings in a rodent model suggest that Meth enhances female sexual motivation via actions on a discrete neuronal population in the MePD. Taken together with our previous work, these findings suggest that this specific neuronal ensemble may mediate dopamine and progesterone signaling, both of which are necessary for the Meth-induced facilitation of proceptive behaviors. Moreover, the discovery that Meth-induced increases in proceptivity requires a specific neuronal ensemble, which is highly responsive to Meth and ovarian steroids, sets the foundation for a better understanding of the neurobiology that links sexual motivation and Meth use.

CHAPTER 5: Effects of Meth on Progesterone Receptor Function

Introduction

Female sexual behavior in the rat has been linked to a few small, hormone-concentrating regions of the brain, including but not limited to the VMN and the MePD. Within these regions, changing concentrations of estradiol and progesterone allow for fluctuations in sexual behavior along the estrus cycle [16]. 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 entirely on high levels of progesterone following priming with estradiol to manifest. As described in Chapter 4, Meth's effects on proceptive behavior are linked to a discrete population of cells within the MePD which respond not only to EB+P, but also Meth.

Meth results in marked catecholamine release into the synapse [113], and likewise increases proceptive behaviors in OVX, hormone-primed rats with physiologic levels of circulating progesterone [121]. This is important to note, as ligand-bound PR does appear to be necessary for this effect as Meth-induced enhancement of proceptive behaviors is prevented by blocking PR signaling in the MePD [123]. Similarly, Meth appears to sensitize PR to its cognate ligand, allowing for normal levels of behavior following subthreshold doses of P4 [125]. Could the release of catecholamines in the MePD result in an enhancement of PR signaling? These data do suggest that Meth may enhance PR signaling, likely via the neuromodulatory role of DA [123], thus resulting in increased proceptive behaviors.

Although there is abundant literature on the role of ovarian hormones in rodent sexual behavior, little work has been done on the intracellular signaling mechanisms of PR in the MePD which lead to enhanced proceptive behavior. PR signaling can be enhanced in two ways:

increased receptor density (via reduced turnover or enhanced transcription) or increased action of individual receptors. Current findings do not clearly elucidate if either (or both) of these options is more likely to occur within our model. However, it has been shown that Meth and other dopamine type-1 receptor agonists can increase the number of PR immunoreactive cells [123, 164, 165]. Likewise, in cancer models, it has been shown that phosphorylation of specific serine sites may stabilize the receptor to reduce turn over, as well as increase the likelihood of receptor response to ligand [166, 167]. The aim of this chapter is to determine which of these is most likely to occur in the MePD after Meth administration.

Materials & Methods

Animals

All animals were obtained from Charles River and housed as described in Chapter 3. Adult female Sprague Dawley rats were OVX and treated with oil or EB+P and saline or Meth according to our standard protocol (Figure 1).

RNA extraction, cDNA synthesis, and qPCR

Brain tissue was collected on dry ice 1 hour after the final injections. Tissue was cut at 300μM and 3 serial sections containing the MePD and 4 serial sections containing the VMN were placed onto a glass slide. Sections were then punched using a 1.5mm or 2mm micropunch (Fine Science Tools, Foster City, CA) for the MePD and VMN respectively. Brains were stored at -80C until ready for RNA extraction.

RNA was extracted using TRIzol (Invitrogen) and 1-bromo-3 chloropropane, and cDNA was synthesized using the iScript cDNA synthesis kit (Biorad) and 1ug of RNA. 3uL of cDNA reaction was used as a template for qPCR with the primer sequences 5’-ACT GTC CAG CAT GTC GTC TG -3’ (NCBIPR2F) and 5’-AGT GGC GGA ACC AGT TGA AT -3’ (NCBIPR2R). Each sample was run in triplicate and normalized to 18S rRNA .

Western Blotting

Brain tissue was collected on dry ice 15 minutes after the final injections. Tissue was processed as described in Chapter 3. Briefly, immunoblots were incubated with anti-phosphoroserine 294-PR, anti-phosphoserine 345-PR (Cell Signaling), and anti-total PR antibodies (2APR, Santa Cruz) at 1:1000 overnight at 4C. Blots were then exposed to anti-rabbit (phospho-blots) or anti-mouse (total PR) HRP-linked secondary antibody (Cell Signaling) at 1:2000 for 1 hour. Blots were analyzed as described in Chapter 3, and normalized to GAPDH.

Statistics

All results are expressed as means ± SEM. PR mRNA analysis was completed using a t-test with treatment as the independent variable. PR phosphorylation and ERK1/2 phosphorylation experiments were analyzed using a two-way ANOVA with drug (saline or meth) and hormone (oil or EB+P) as independent variables. Behavioral tests were analyzed with two-way ANOVAs with Meth and Infusion as independent variables. All statistical tests were conducted using GraphPad Prism software (San Diego, CA USA).

Results

Meth increases progesterone receptor mRNA. Previous work has shown that dopaminergic agonists, including Meth, increase PR protein. Here, we aimed to determine if this effect in our system was due to increased gene expression. Our positive control, estradiol benzoate, increases PR mRNA by 2-fold over saline/oil control in a Mann Whitney t-test (*$p<0.05$; Fig. 18a). Similarly, a Mann-Whitney t-test shows that by 1 hour after injection, Meth increases PR mRNA over that of saline/oil control by 1.8-fold (*$p<0.05$; Fig. 18b), and the combination of EB+P and Meth increases PR mRNA over that of saline/oil control by 2-fold (**$p<0.01$; Fig. 18c).

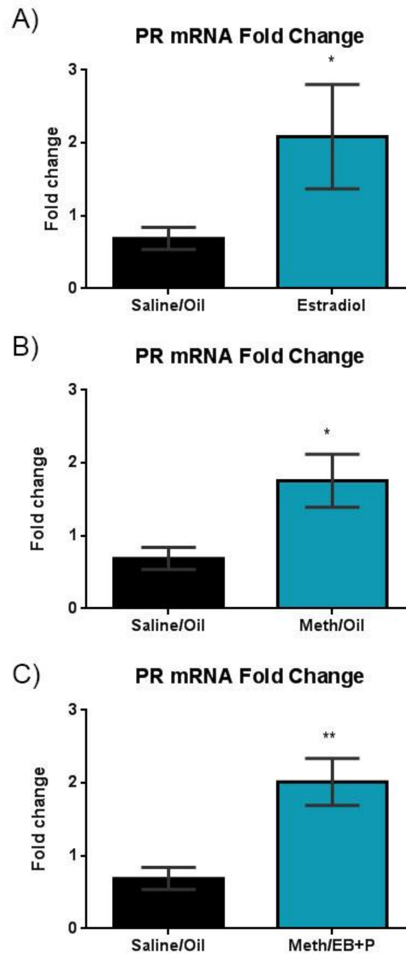

Figure 18: Effects of Meth on progesterone receptor transcription in the MePD one hour after treatment. Adult OVX rats were treated over 3 days with either (i) saline and oil, (ii) saline and Estradiol, (iii) Meth and oil, or (iv) the full combination of Meth and EB+P. A) A Mann Whitney t-test shows that Estradiol increases PR mRNA over a saline/oil baseline (*$p<0.05$, n=5, Fig. 18a). B) Similarly, a t-test shows that Meth increases PR mRNA over a saline/oil baseline by about 2-fold (*$p<0.05$, n=5-7, Fig. 18b). C) The combination of Meth and Estradiol increases PR mRNA expression 2-fold greater than saline/oil control (**$p<0.01$, n=5, Fig. 18c). Data represented as mean ±SEM.

The combined treatment of methamphetamine and ovarian hormones alters progesterone receptor phosphorylation in the MePD. A two-way ANOVA reveals a main effect of Meth on phosphorylation of serine 294 in the MEPD 15 minutes after progesterone and Meth administration ($F_{(1, 38)}=6.03$, $*p<0.05$; Fig. 19a). There is no significant difference between groups for phosphorylation of serine 345 in the MePD 15 minutes after progesterone and Meth administration (Fig. 18b). Total PR in the MePD shows a main effect of Meth administration, with Meth significantly increasing PR protein levels in the MePD by 15 minutes post-injection ($F_{(1, 44)}=7.11$, $*p<0.05$; Fig 18c).

There is no effect in the VMN on phosphorylation of Serines 294 or 345. Two-way ANOVAs revealed that there were no significant differences between groups in the VMN for phosphorylation of Ser294 ($F_{(1,26)}=0.19$; Fig 20a), or total PR ($F_{(1,20)}=0.13$; Fig 20c). Two-way ANOVA revealed Ser345 showed a main effect of hormone treatment ($F_{(1,14)}=4.89$, $p<0.05$; Fig 19b), but there were no further differences found in a Tukey's multiple comparison test.

Phosphorylation decreases by 30 minutes after treatment. Phosphorylation levels were also evaluated in the MePD 30 minutes after treatment. Thirty minutes following Meth and/or hormone administration, phosphorylation at serine 345 is barely detectible, with a two-way ANOVA showing no differences between groups ($F_{(1,8)}=0.23$; Fig 21a). Likewise, phosphorylation of serine 294 is not able to be resolved via western blot at this time point (data not shown). Total PR no longer shows a significant difference between groups at this time ($F_{(1,8)}=3.3$; Fig 21b).

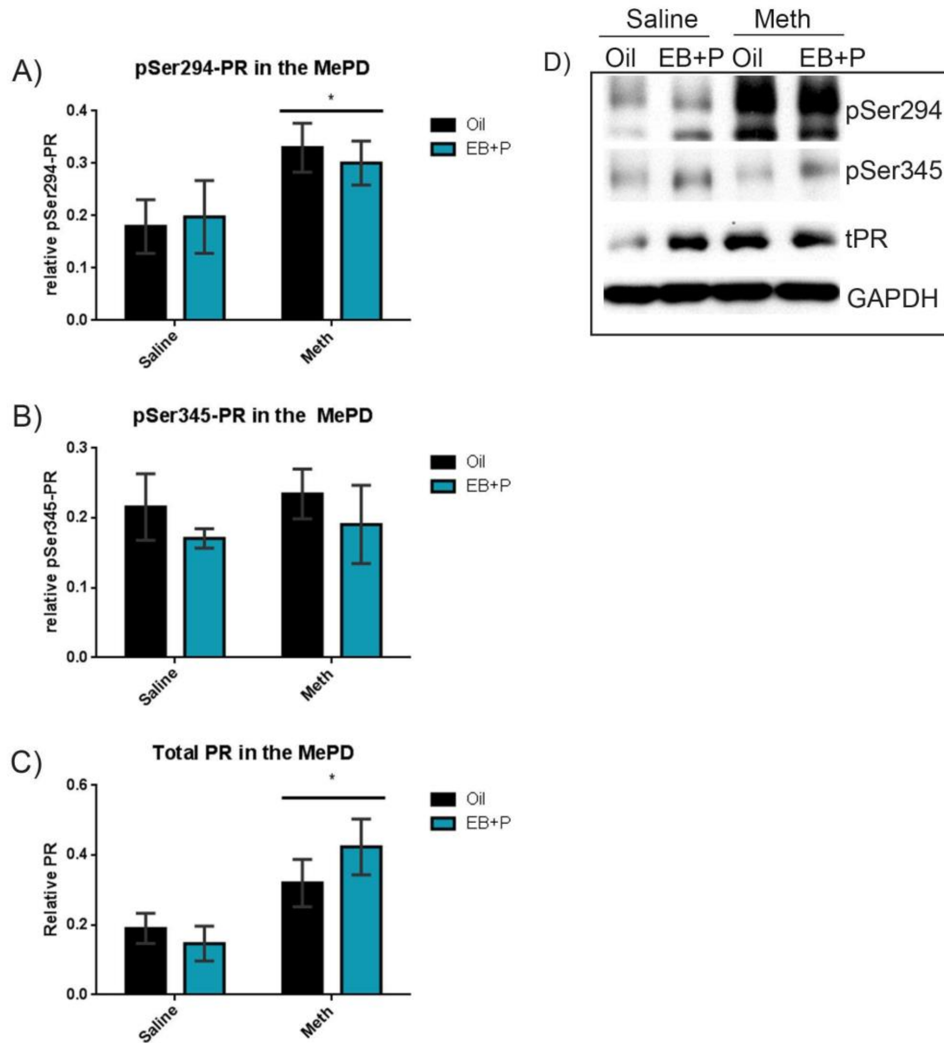

Figure 19: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the MePD 15 minutes following the final Meth and EB+P administration. Adult OVX rats were treated over 3 days with our standard Meth and steroid hormone paradigm, and tissue was collected 15 minutes following the final administration of Meth and EB+P. A) A two-way ANOVA shows a main effect of Meth on phosphorylation of serine 294, 15 minutes following the final injection of Meth and EB+P ($F_{(1, 38)}$=6.03, *p<0.05, n=8-10). B) A two-way ANOVA shows there were no significant effects on serine 345, 15 minutes following the final injection of Meth and EB+P. C) A two-way ANOVA shows a main effect of Meth on total PR expression in the MePD ($F_{(1, 44)}$=7.11, *p<0.05; n=7-17). D) Representative blots from each treatment group; pSer294 blots are spliced. Data represented as mean ±SEM.

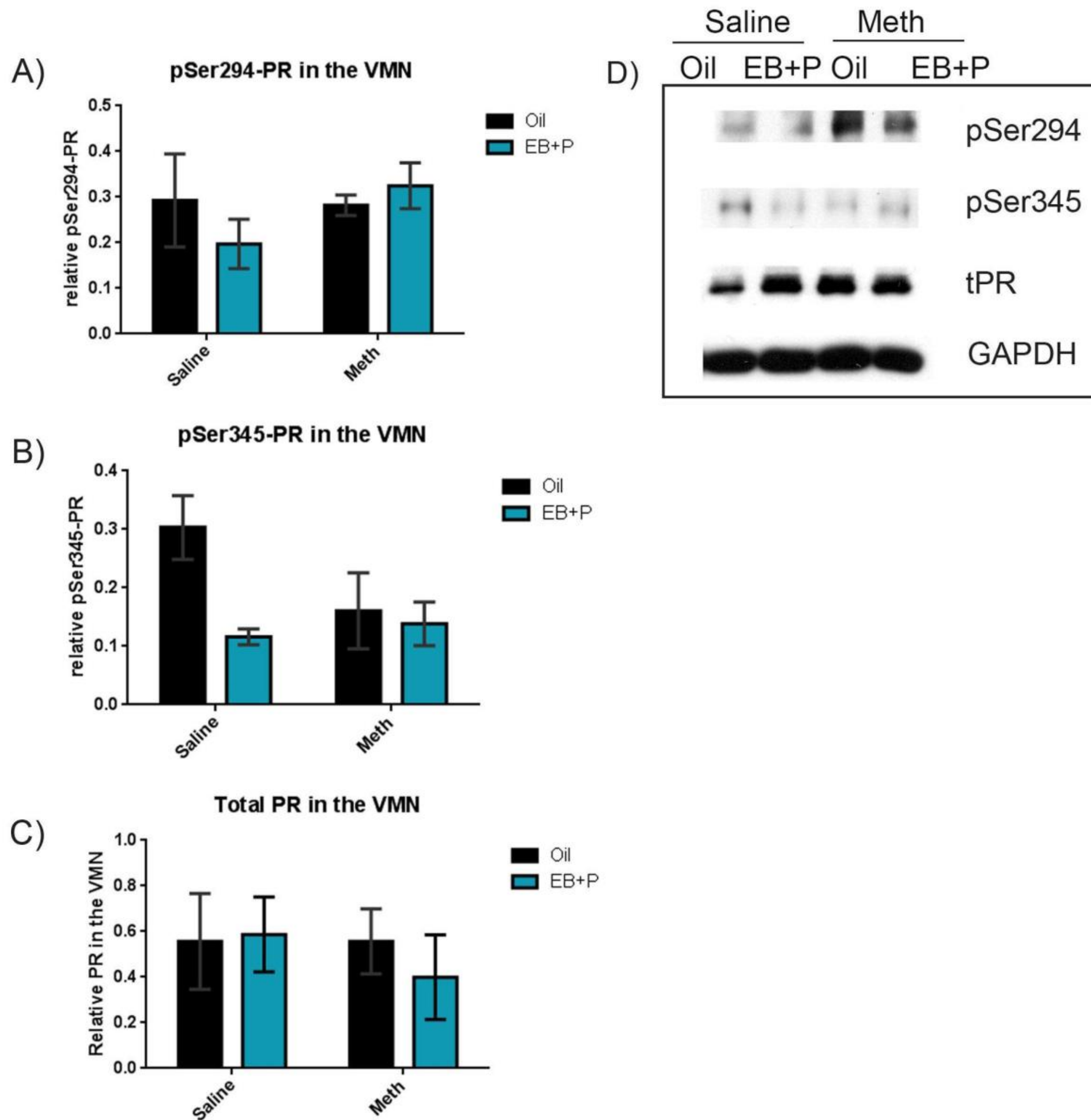

Figure 20: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the VMN 15 minutes following the final Meth and EB+P administration. Adult OVX rats were treated over 3 days with our standard Meth and steroid hormone paradigm, and tissue was collected 15 minutes following the final administration of Meth and EB+P. A-C) There were no significant differences between groups for serines 294 or 345 or total PR expression. D) Representative blots from each treatment group. Data represented as mean ±SEM.

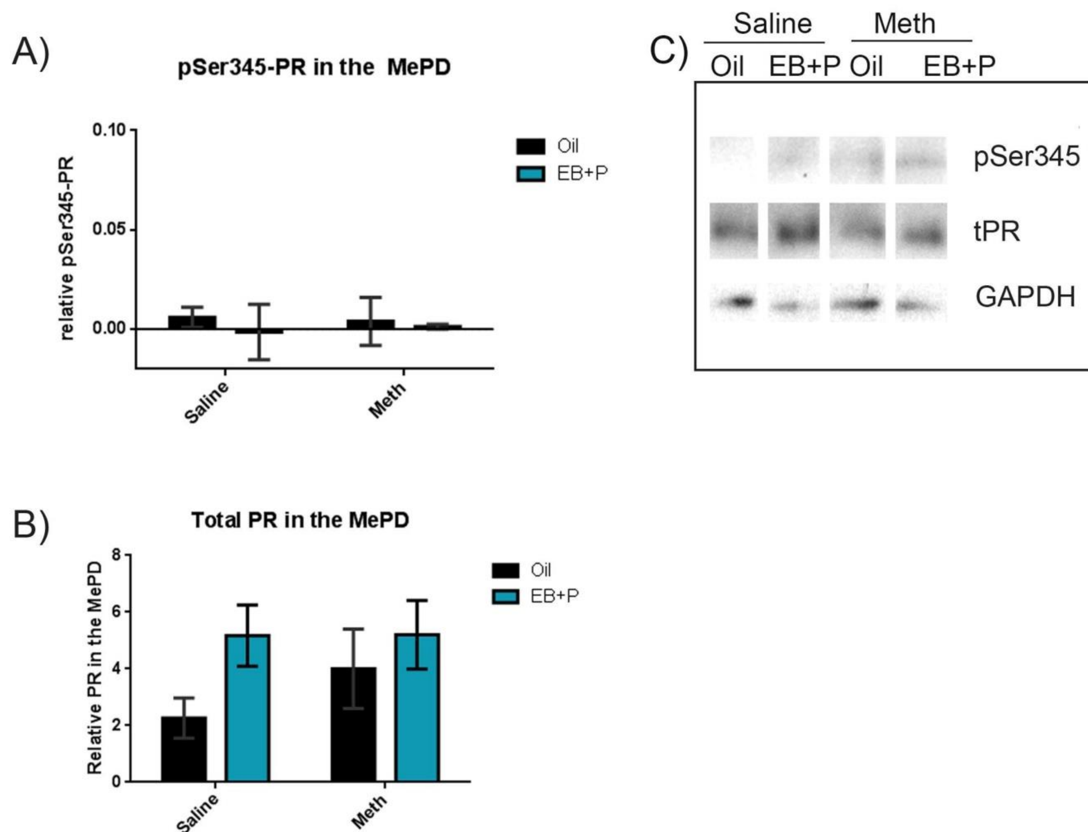

Figure 21: Effects of Meth and Ovarian hormones on PR phosphorylation at Serines 294 & 345 and total PR expression in the MePD 30 minutes following the final Meth and EB+P administration. Adult OVX rats were treated over 3 days with our standard Meth and steroid hormone paradigm, and tissue was collected 30 minutes following the final administration of Meth and EB+P. A) Phospho-serine345-PR was barely detectible by Western Blotting 30 minutes after the final injections, and no significant differences were observed between treatment groups (n=3). B) A two-way ANOVA did not reveal any significant differences in total PR expression at this time point (n=3). C) Representative blots from each treatment group. Data represented as mean ±SEM.

Discussion

Proceptive behavior has been shown to be strongly linked to progesterone signaling [42, 168]. Within the MePD, we have shown that both progesterone and its cognate receptor are required for Meth induced increases in proceptivity [123]. Additionally, Meth is able to increase PR protein and increases proceptive behaviors even under conditions of subthreshold doses of P [125]. It is unknown if it is the increase in PR or another signaling mechanism that leads to the enhancement of proceptivity. Here we have shown that while Meth increases PR from a transcriptional level, it also enhances PR's phosphorylation.

It is striking that Meth, a dopaminergic drug, can increase PR protein levels in the absence of estradiol [123]. Estradiol has long been known to be the primary transcriptional regulator of PR expression [72]. In fact, the rising levels of estradiol in proestrus (Fig. 1) serve to increase PR, thereby facilitating the onset of behavioral estrous once progesterone levels too begin to rise. Previously, we have shown that the combination of Meth and EB+P increase PR protein levels 4 hours following treatment [123]. Likewise, we show a Meth induced increase in PR protein levels 15 minutes following Meth/EB+P treatment. To verify that the increases seen in PR protein levels are due to increased gene transcription, we first showed that one hour following the second dose of EB (Fig. 17a), PR mRNA levels are increased. We were able to demonstrate that animals who received only Meth showed a similar increase from saline/oil baseline (Fig. 17b). Additionally, the full complement of treatments (3 day paradigm as seen in Fig. 11) also increases PR mRNA. It is of note that each treatment only increased PR mRNA by about 2-fold over the saline/oil baseline. This may be due to ligand-independent activation of estrogen receptor alpha [164, 165, 169, 170], as this has been shown in the presence of dopaminergic agonists, but this work is beyond the scope of this thesis. While this data is novel in that it is the first time, to our knowledge, that Meth has been shown to increase PR mRNA, this increase does not appear to

be of a large enough magnitude to explain the Meth-enhanced proceptivity over EB+P alone, or the increased sensitivity to subthreshold doses of progesterone in the presence of Meth.

Previous work has suggested that Meth also serves to enhance PR sensitivity to ligand [84, 171]. The progesterone receptor is able to be modulated by post-translation modifications, including phosphorylation, sumoylation, ubiquitination, and acetylation [85]. While the receptor can be modified in all these ways, it is thought that the phosphorylation sites are what direct activity of the PR *in vivo* [84, 85, 87, 88, 172]. The PR has fourteen serine phosphorylation sites along its length, but only five of these sites are hormone dependent [85]. 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 phosphorylation sites are important for hormone binding and receptor dimerization, but others can be phosphorylated by kinases or cyclins during the cell cycle [85, 103, 173, 174]. Two of these five sites, Ser294 and Ser345, have been shown to interact with both hormones and cytosolic kinases, which may be upregulated following dopaminergic agonists, thus making them attractive targets in Meth-enhanced proceptivity [166, 173, 175].

Although both serine phosphorylation sites are considered both hormone- and kinase-responsive, they have very different roles. Our first site, Ser294, has been described as becoming phosphorylated in response to growth factor signaling, which primes the receptor for enhanced sensitivity to ligand and removal of repressive sumoylation [85]. This site becomes phosphorylated in response to mitogen-activated protein kinase (MAPK) activity [103, 176]. Once phosphorylated, pSer294 has been shown to increase the receptor's sensitivity to ligand [85]. Ser345 too can become activated by kinase signaling; however it is reported to require PR to be bound by ligand in order for phosphorylation to occur at this site, thus leading to the ability of PR to interact in a scaffold complex with Src kinase or SP1 transcription factors [85]. As kinase

action is a rapid event, occurring over minutes [177], we looked at the phosphorylation status as early as 15 minutes after the final Meth treatment and progesterone injection.

In our model, Meth enhances PR phosphorylation at Ser294 only in the MePD but not the VMN (Fig. 18, 19). We are able to observe this 15 minutes following the final treatment; however, by 30 minutes following treatment phosphorylation events at Ser294 and 345 appear to have resolved (Fig. 16). There is some published data suggesting that pSer294 may be preventing turnover of the receptor [176], although findings are contradictory [166]. If receptor turnover is increased, the increase in PR protein/message may negate this effect. We also see an increase in total PR protein levels 15 minutes after P when combined with Meth administration as well (Fig. 18c), but this again appears to be in a state of transition as these differences are no longer seen 15 minutes later (Fig 20b).

As previously mentioned, these phosphorylation sites are known to interact with kinases, specifically ERK1/2 and Src [103, 166, 173, 175, 178]. In fact, both of these kinases have recently been implicated in VCS (and thus dopaminergic) modulation of female sex behavior in the VMN [93]. Additionally, as Src and ERK1/2 have been shown to be activated by dopaminergic drugs, this suggests that ERK1/2 or Src signaling may be involved in the mechanism leading to Meth-facilitated proceptivity as well as the increased phosphorylation of PR.

CHAPTER 6: ERK1/2 and Src Kinase as Conduits of Progesterone Receptor Rapid Signaling

Introduction

Progesterone receptors have proven to be highly interactive with a number of other signaling molecules within the cell. Within recent years, both PR 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 [91-93]. Anne Etgen and colleagues have shown a robust effect of ERK1/2 and Src family kinase inhibition on hormonally-driven female sexual behaviors [90-93, 179]. As with most research focusing on female sexual behaviors, these experiments were only completed within the VMN or using systemic administration of antagonists, thus the possibility of this mechanism occurring within the MePD remains unexplored.

ERK1/2 activation via PR has been shown in some cases to require the involvement of the non-receptor tyrosine kinase, cSrc *in vitro* [180]. cSrc may be involved in PR activation either through direct interaction of the PR with the SH3 region of the kinase [181] or via interaction of other signaling molecules [180]. Similar to ERK1/2 activation by progesterone, Src activation has not only been shown to be enhanced by the presence of progesterone, but also to lead to downstream ERK1/2 activation [67, 85, 173, 175]. Although Src family kinase members appear to have a role in baseline hormonally driven behavior in the VMN, it is still unclear if 1) it is cSrc specifically responsible for the behavior, 2) this mechanism exists in other brain regions, and 3) cSrc is the convergence point for Meth and PR signaling.

Materials & Methods

Animals

Animals were housed and treated as described in Chapter III. Briefly, adult Sprague Dawley rats were OVX and allowed a week to recover. Animals were then treated with saline or Meth and oil or EB+P as described in Chapter 3.

Western Blot

Brains were collected on dry ice 15 minutes after the final injection for Western Blot. Tissue was processed as described in Chapter 3. Briefly, immunoblots were incubated with anti-Src, anti-p-Src, anti-ERK, anti-p-ERK and anti-pSer294-PR antibodies (Cell Signaling) at 1:1000 overnight at 4C. Blots were then exposed to anti-rabbit HRP-linked secondary antibodies (Cell Signaling) at 1:2000 for 1 hour. Blots were analyzed as described in Chapter 3, and normalized to GAPDH.

Cannulation

Animals receiving PP2 or SCH772964 infusions and shRNA injections were cannulated as described in Chapter 3.

ERK Inhibition

All animals received hormone replacement (EB+P) as defined in our standard protocol. Ten minutes prior to the final saline or Meth injections, animals received bilateral infusions of either 2.5ug SCH772984 or equivalent volume 3% DMSO vehicle. During the microinfusions, the dummy stylets were removed and replaced by 33 gauge microneedles that project 1.3 mm below the guide cannulae and were attached via polyethylene tubing to a 25 μl Hamilton syringe (700 series, Hamilton, Reno, NV). Fluid flow rate was controlled by a BASi Bee pump attached to a Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, IN). Infusions occurred over 5 min and the microneedles remained in place for an additional 5 min to ensure diffusion away from the needle tips. Behavioral tests were run 4 hours later as described in Chapter 3.

Src Family Kinase Inhibition

All animals received infusions of PP2 (3-(4-chlorophenyl)-1-(1,1-dimethyl)-1H-pyrazolo[3,4-d] pyrimidin-4-amine, 13198, Cayman Chemical, Ann Arbor, MI, USA) or DMSO vehicle 10 minutes prior to the final oil or progesterone and saline or Meth injections. During the microinfusions, the dummy stylets were removed and replaced by 33 gauge microneedles that project 1.3 mm below the guide cannulae and were attached via polyethylene tubing to a 25 µl Hamilton syringe (700 series, Hamilton, Reno, NV). Fluid flow rate was controlled by a BASi Bee pump attached to a Bee Hive controller (Bioanalytical Systems, Inc., West Lafayette, IN). Bilateral infusions of 0.5 µl/70ng occurred over 5 min and the microneedles remained in place for an additional 5 min to ensure diffusion away from the needle tips.

shRNA Injections

All animals received bilateral injections (2uL/hemisphere) of Src shRNA (10^8 TU/mL SMARTvector Lentiviral Rat Src UBC-TurboGFP, Clone V3SVRN04_18913577, Dharmacon, Lafayette, CO) or scramble control (10^7 TU/mL SMARTvector Non-targeting UBC-TurboGFP Control Particles 2 Dharmacon, Lafayette, CO) at time of OVX. The top of the animal's head and neck was shaved and then cleaned with betadine scrub and 70% isopropyl alcohol and an incision is made in the skin, exposing the skull. Holes were drilled into the skull using stereotaxic technique (3.0 mm posterior, ±3.55 mm lateral from Bregma) and a needle was dropped into the MePD (8.8 mm ventral from skull surface). Infusions occurred over 10 minutes and the needle was left in place an additional 5 minutes to allow for diffusion away from needle tip.

shRNA verification

Each animal had three 35uM slices taken 140uM apart which were mounted onto a slide and GFP was visualized to determine injection placement. Transfection efficiency was determined and knockdown verified using fresh-frozen punches of the MePD which were homogenized and analyzed using Western Blot for pSrc.

Behavior

Sex behavior was recorded as described in Chapter 3.

Statistics

All results are expressed as means ± SEM. pSrc and pPR experiments were analyzed using a two-way ANOVA with drug (saline or meth) and hormone (oil or EB+P) as independent variables. All behavior was analyzed using a two-way ANOVA with drug (saline or Meth) and infusion (PP2 or shRNA) as independent variables. shRNA knockdown was confirmed using a t-test. All tests were followed with a Tukey's multiple comparisons test unless otherwise noted. All statistical tests were conducted using GraphPad Prism software (San Diego, CA USA).

Results

ERK1/2 shows increased phosphorylation at 15 minutes after the combined treatment of Meth and Ovarian hormones in the MePD but not the VMN. ERK1/2 phosphorylation levels were evaluated as a marker of ERK1/2 activity. A two-way ANOVA found a main effect of hormone and drug interaction ($F_{(1,20)}$=16.99, $p<0.001$) as well as a main effect of hormone treatment ($F_{(1,20)}$=4.587, $p<0.05$) on ERK 1/2 phosphorylation in the MePD (Fig. 21B). There was no effect of Meth or EB+P on total ERK1/2 in the MePD ($F_{(1, 15)}$=0.56; Fig 22C). In the VMN, there was no significant effect of drug or hormones on ERK1/2 phosphorylation, although there was a trend for increased phosphorylation following Meth ($F_{(1, 18)}$=0.72, p=0.059; Fig. 22E). Similar to the MePD, the VMN showed no effects of Meth or EB+P on total ERK1/2 expression ($F_{(1, 19)}$=2.33; Fig. 22F).

Src phosphorylation is increased in the MePD but not the VMN following Meth and EB+P treatment. The aim of this experiment was to evaluate rapid changes in Src activation in the MePD following Meth and/or EB+P administration using Src phosphorylation at tyrosine-416

as a measure of Src activation. In the MePD, a two-way ANOVA shows an interaction of Meth and hormones ($F_{(1, 18)}=5.12$, $p<0.05$; Fig. 23B), with meth increasing pSrc in the presence of EB+P. In the VMN, another hormone-responsive brain region, there was no effect of Meth or hormones on phosphorylated Src protein ($F_{(1, 20)}=3.66$; Fig. 23E). In both regions, total Src protein remains unchanged between treatment groups ($F_{(1, 19)}=3.15$, Fig. 23C; $F_{(1, 20)}=3.22$, Fig. 23F).

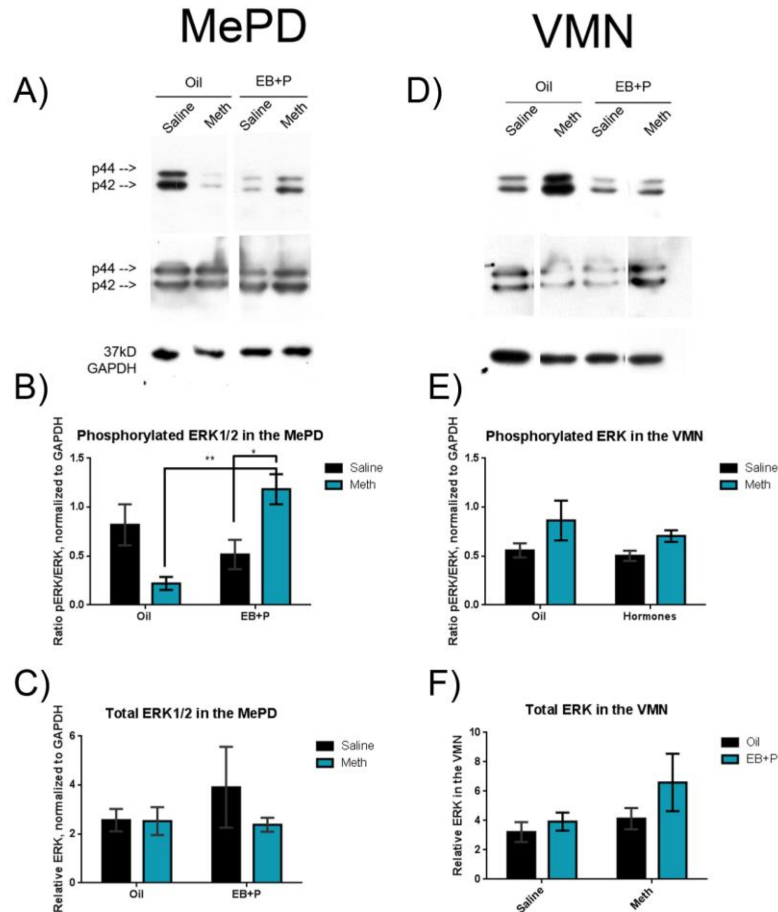

Figure 22: Effect of Meth and EB+P on ERK1/2 (p44/42) phosphorylation levels in the MePD, 15 minutes after Meth and P4 administration. Adult OVX rats were treated over 3 days with our standard Meth and steroid hormone paradigm, and tissue was collected 15 minutes following the final administration of Meth and EB+P. A) Representative western blot for the MePD; p42/44: ERK1/2 B) A two-way ANOVA shows a significant main effect of EB+P and Meth interaction ($F_{(1,20)}=16.99$, $p<0.001$) as well as a main effect of hormone treatment ($F_{(1,20)}=4.587$, $p<0.05$) on ERK 1/2 phosphorylation in the MePD. A Tukey's multiple comparison test revealed these differences to be driven by the high phosphorylation in the Meth and Hormones group over Meth alone (**$p<0.005$) or hormones alone (*$p<0.05$) (n=6). C) Two-way ANOVA revealed no differences in total ERK expression in the MePD (n=6). D) Representative western blot for the VMN. E,F) Two-way ANOVAs revealed no significant differences between groups in pERK1/2 or total ERK in the VMN 15 minutes following Meth and EB+P administration (n=6). pERK1/2 data is representative of pERK1/2 over total ERK1/2. All data normalized to GAPDH control. Gel images spliced from representative bands. Data represented as mean ±SEM.

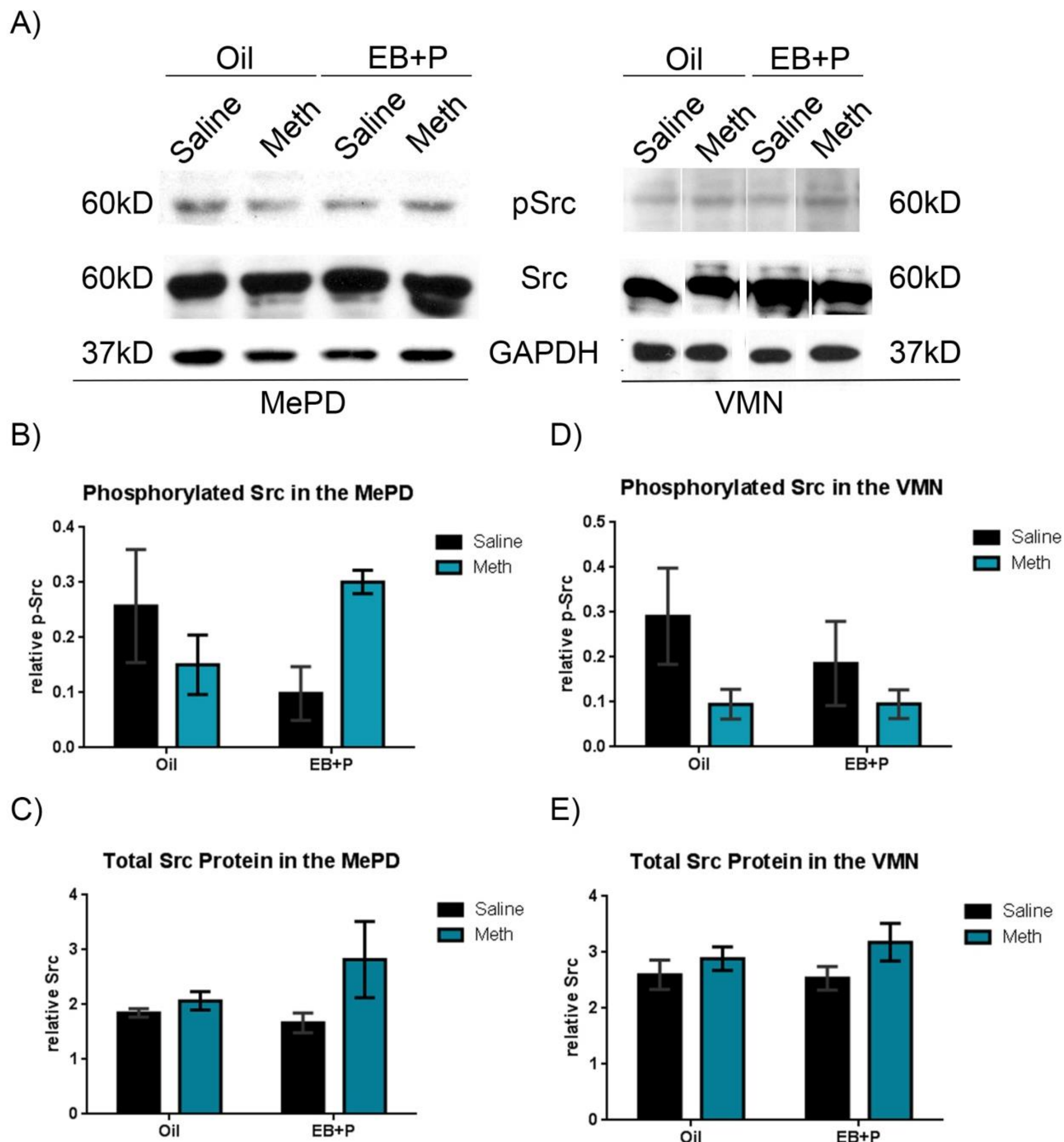

Figure 23: Src is highly phosphorylated in the MePD following Meth and EB+P treatment. Animals were treated with saline or Meth and oil or EB+P over three days. Fifteen minutes following the final injection, MePD was collected and analyzed for pSrc. A) Representative blots from the MePD and VMN. B) A two-way ANOVA shows there is an interaction of meth and hormones ($F_{(1, 18)}$=5.12, $p<0.05$, n=6) in the MePD, with meth increasing pSrc in the presence of EB+P. C) There were no significant differences in total Src protein in the MePD following Meth or EB+P treatment. D, E) There were no significant differences between groups in the VMN for pSrc or total Src protein. All samples were normalized to GAPDH. VMN gel images spliced from representative bands. Data represented as mean ±SEM.

ERK1/2 is required for Meth-induced increases in proceptivity. To determine if ERK1/2 was required for increases in proceptive behavior following Meth administration to hormone-primed rats, we infused a specific, ATP-competitive inhibitor, SCH772984. There was a main effect of inhibitor by Meth interaction ($F_{(1,19)}$=13.90, $p<0.005$, Fig. 24A). A Tukey's multiple comparisons test revealed a significant increase in proceptive behavior in Vehicle treated rats in the presence of Meth (*$p<0.05$), as well as decrease in proceptive behavior in Meth-treated, inhibitor infused animals in comparison to Meth-treated, Vehicle-infused controls (**$p<0.01$). A two-way ANOVA revealed a main effect of Inhibitor infusion on lordosis quotient $F_{(1,19)}$=4.56, $p<0.05$, Fig. 24B), but no effect on lordosis score was observed (Fig. 24C). 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 Meth-induced increases in phosphorylation of PR at Ser294. Following ERK1/2 inhibition, phosphorylation levels of PR at serine 294 were evaluated to determine if this event occurs downstream from ERK1/2 phosphorylation. A two-way ANOVA revealed a main effect of Meth on pSer294-PR ($F_{(1,17)}$=9.11, $p<0.01$, Fig. 25B). A Tukey's multiple comparison test revealed that there is a significant increase in pSer294-PR levels in Meth-treated, SCH772984-infused animals over their saline-treated controls (*$p<0.05$).

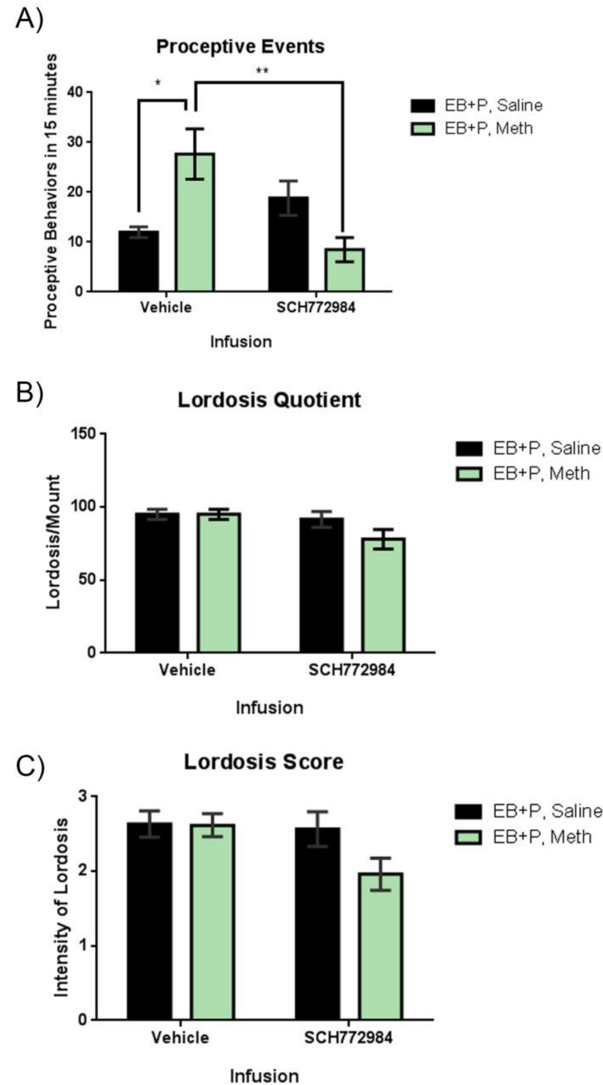

Figure 24: Proceptive Behavior following SCH772984 Infusion. Adult rats were OVX and bilaterally cannulated targeting the MePD one week prior to treatment. All animals were hormonally primed, and a single dose of SCH772984 (SCH, 0.25ug/hemisphere) was infused 5 minutes prior to the final Meth or Saline injection. Behavior was observed 4 hours later. A) A two-way ANOVA reveals a significant interaction of Meth and SCH on proceptive behavior ($F_{(1,19)}$=13.9, p<0.005, n=5-6). A Tukey's multiple comparisons test revealed a significant increase in proceptivity in Meth, Vehicle-treated animals over Vehicle, Saline treated controls (*p<0.05), as well as a significant decrease in proceptivity in Meth, SCH treated animals in comparison to Meth, Vehicle-treated controls (**p<0.005). B) A two-way ANOVA shows a main effect of Inhibitor on Lordosis Quotient ($F_{(1,19)}$=4.56, p<0.05). C) No effect was seen on Lordosis Score. Data represented as mean ±SEM.

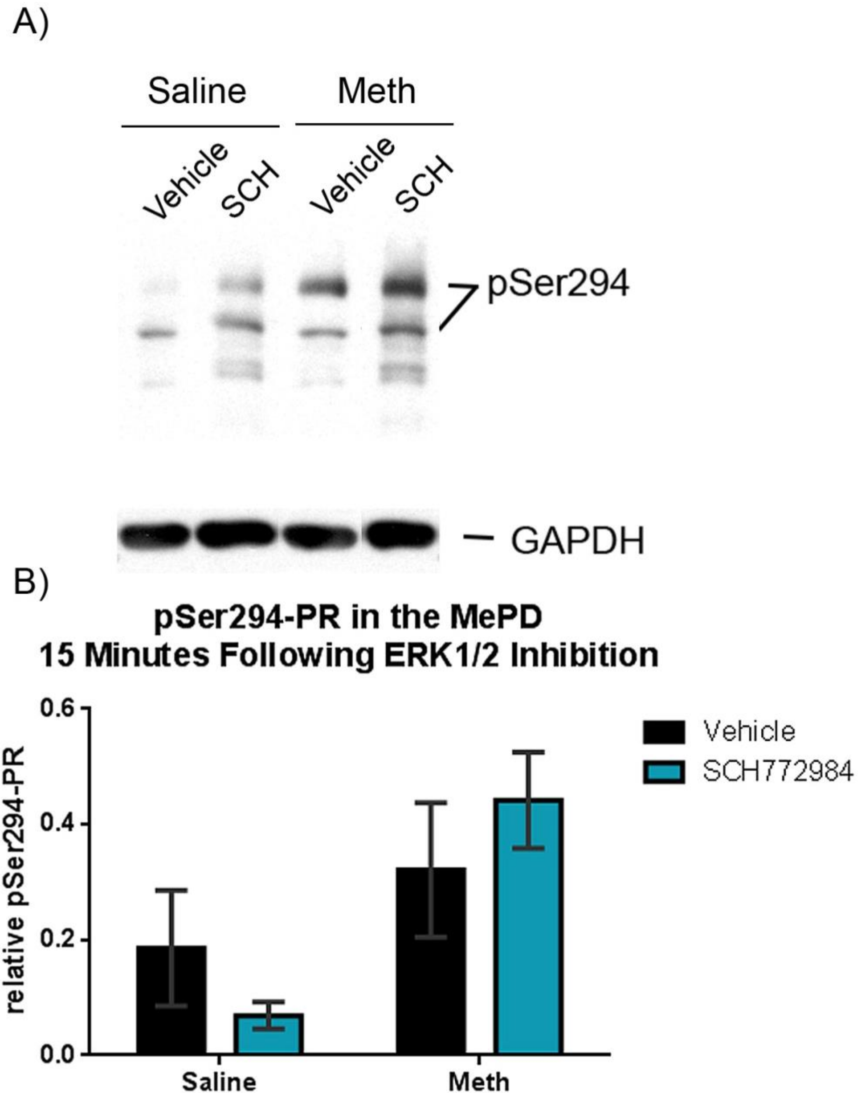

Figure 25: Serine 294 phosphorylation following ERK1/2 inhibition. Following behavioral testing, animals received a second round of Meth and SCH treatment, and were collected 15 minutes following the infusions to determine if ERK1/2 activity was the cause of increased pSer294-PR. A) Representative Western Blots. B) A two-way ANOVA showed a main effect of Meth of pSer294-PR .($F_{(1,17)}$=9.11, $p<0.01$, n=4-6). Data represented as mean ±SEM.

Meth-facilitated increases in proceptive behavior is blocked following PP2 infusions

The aim of this experiment was to investigate the role of Src kinase in meth-facilitated female sexual motivation in hormonally-primed female Sprague-Dawley rats. A two-way ANOVA revealed a main effect of drug ($F_{(1, 14)} = 5.14$, $p<0.05$), infusion ($F_{(1, 14)} = 6.96$, $p<0.05$, Fig. 26A), as well as an interaction of treatment and infusion ($F_{(1, 14)} = 7.62$, $p<0.05$, Fig. 26A). Similar to our previous findings, a Tukey's *post-hoc* test reveals animals that received meth injections showed a significant increase in proceptive behavior compared to vehicle treated controls ($p<0.05$). In the *post-hoc* test we also found that PP2 infusions reduced proceptive behaviors to baseline levels when compared to Meth- and vehicle-treated animals, keeping them at baseline behavior (vehicle/meth vs PP2/saline: $p<0.05$, vehicle/meth vs PP2/Meth: $p<0.01$). There were no significant differences among treatment groups in either lordosis score or quotient in these animals.

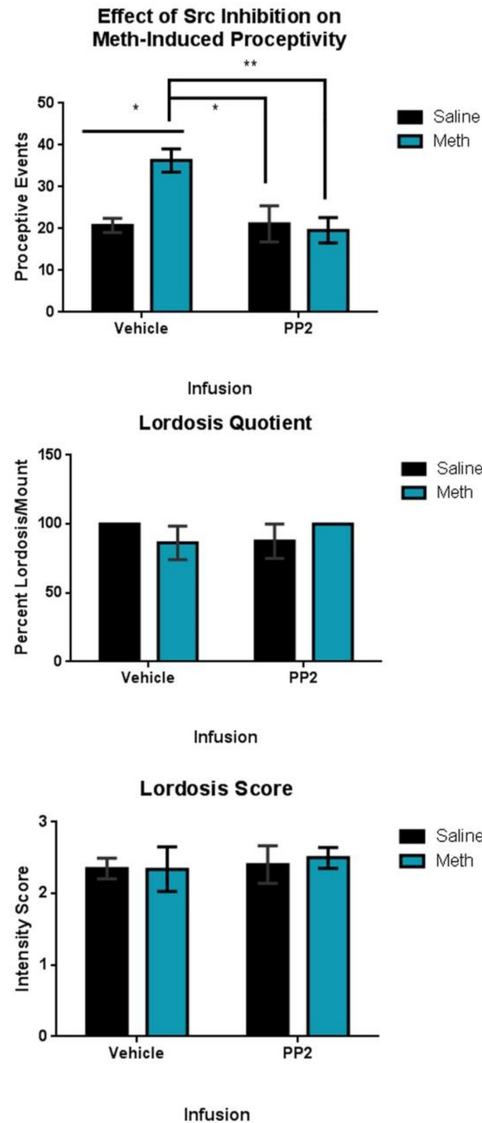

Figure 26: PP2 inhibits Meth-facilitated increases in proceptivity. A) A two-way ANOVA revealed a main effect of drug ($F_{(1, 14)} = 5.14$, $p<0.05$, n=4-5), infusion ($F_{(1, 14)} = 6.96$, $p<0.05$, n=4-5), as well as an interaction of treatment and infusion ($F_{(1, 14)} = 7.62$, $p<0.05$, n=4-5). A Tukey's *post-hoc* test reveals Meth- and vehicle-treated animals showed a significant greater number of proceptive events compared to saline- and vehicle-treated controls (*p<0.05), saline and PP2 treated animal (*p<0.05), and Meth- and PP2 treated animals (**p<0.01). Data represented as mean ±SEM.

Verification of cSrc shRNA-induced knockdown

Each animal had GFP expression verified prior to further analysis (Fig. 27A). Any animal not showing GFP expression in the MePD was excluded from analysis. A t-test showed a significant decrease in pSrc and Src in shRNA treated animals when compared to non-coding RNA controls ($t_{(22)}=2.68$, $p<0.01$, Fig. 27C, D).

Proceptive behavior is reduced following Src shRNA infusions

A two-way ANOVA showed a main effect of shRNA infusion ($F_{(1,18)}=8.58$, $p<0.01$) and an interaction of shRNA and Meth ($F_{(1,18)}=7.91$, $p<0.05$, Fig. 28A) on proceptive behavior. A Tukey's *post-hoc* test determined that the proceptive behaviors of the Meth-treated and shRNA-infused animals was significantly reduced from the Meth-treated scrambled control group ($p<0.01$).

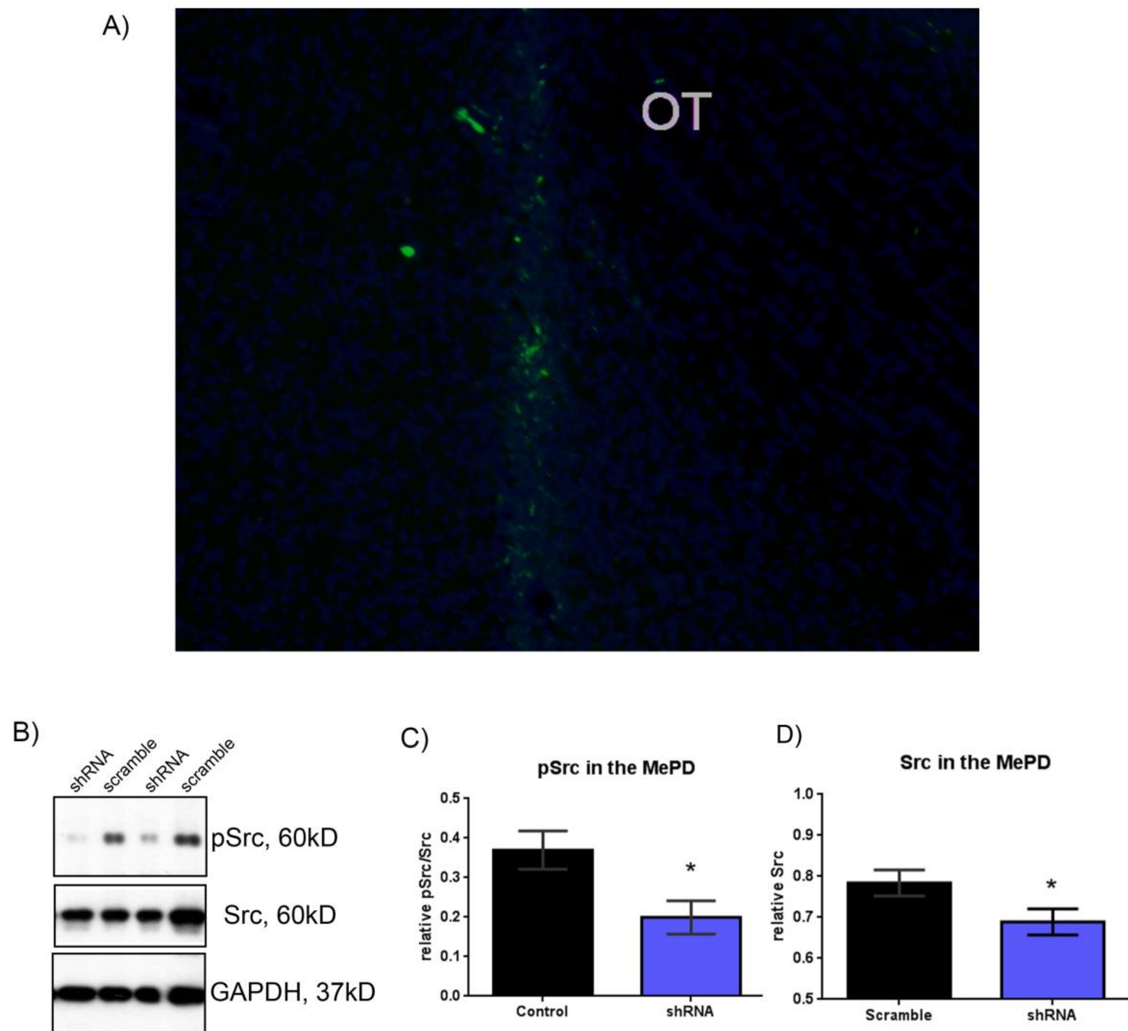

Figure 27: Src knockdown following shRNA infusions. A) Representative image of GFP-positive injection tract. B) Representative pSrc and total Src Western Blot following shRNA or scramble control infusions. C) A t-test reveals a significant reduction in pSrc following cSrc-shRNA infusion ($t_{(22)}=2.68$, $*p<0.01$, n=11-13). D) A t-test reveals a significant reduction in Src following cSrc-shRNA infusion ($t_{(20)}=2.10$, $*p<0.05$, n=10-12). Data represented as mean ±SEM.

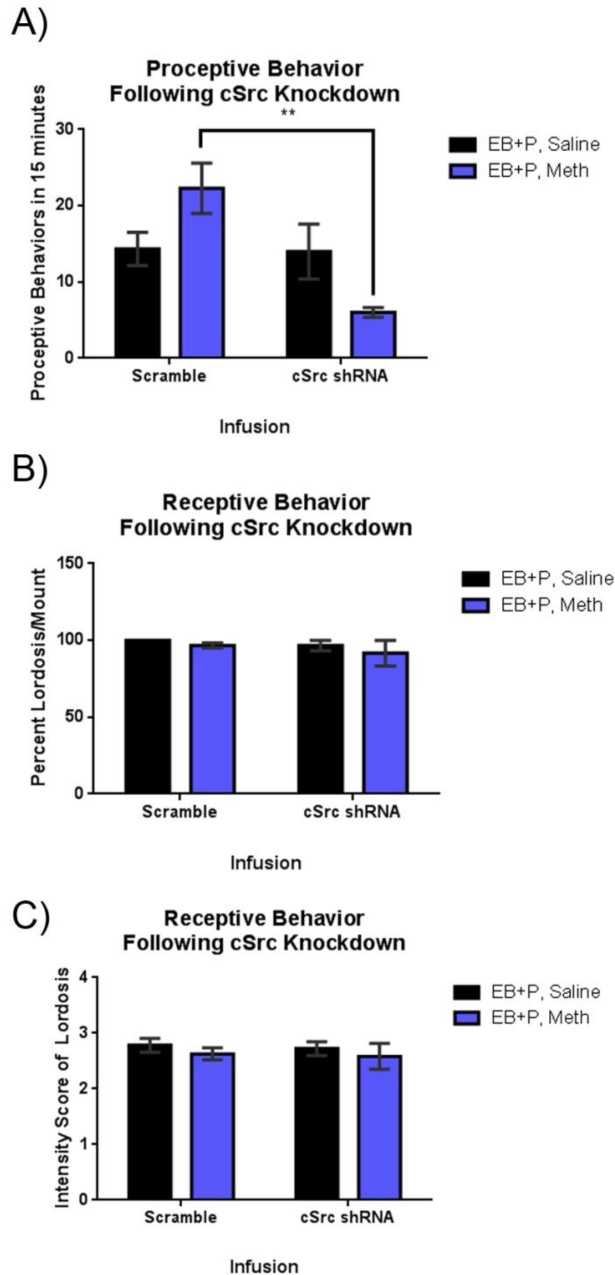

Figure 28: Src knockdown following shRNA infusions block Meth-enhanced proceptive behaviors. A) A two-way ANOVA reveals a main effect of shRNA infusion ($F_{(1,18)}=8.58$, $p<0.01$, n=4-7), and an interaction of shRNA and Meth ministration ($F_{(1,18)}=7.91$, $p<0.05$, n=4-7). A Tukey's multiple comparison's test revealed a significant difference between scramble infused, Meth-treated animals and shRNA infused, Meth-treated animals (**$p<0.01$). E, F) There were no significant difference between groups for either measures of receptivity.

cSrc is required for increased ERK1/2 phosphorylation and phosphorylation of the PR at serine 294 in the MePD

Following cSrc knockdown, phosphorylation levels of both ERK1/2 and PR at serine 294 were evaluated to determine if these events occur downstream from cSrc. A two-way ANOVA shows a main effect of both Meth ($F_{(1,19)}$=11.65, $p<0.01$) and shRNA ($F_{(1,19)}$=11.77, $p<0.01$ Fig. 29B) on pERK1/2 in the MePD with an interaction of Meth and shRNA ($F_{(1,19)}$=5.10, $p<0.05$, Fig. 29B). A Tukey's multiple comparison's test revealed that both the Meth- and scramble-treated animals have significantly increased pERK1/2 over both saline/scramble ([a]$p<0.005$) and saline/shRNA ([b]$p<0.001$) treated animals. Additionally, this test revealed that cSrc shRNA reduces phosphorylation of ERK1/2 in Meth treated animals ([c]$p<0.01$).

A two-way ANOVA shows an interaction of both Meth and shRNA on pSer294-PR in the MePD ($F_{(1,14)}$=5.93, $p<0.05$, Fig. 29D). A Tukey's multiple comparison's test revealed that in the Meth- and scramble-treated animals have significantly increased pSer294-PR over both saline/scramble controls ([a]$p<0.05$) and Meth/cSrc shRNA treated animals ([b]$p<0.05$, Fig. 29D).

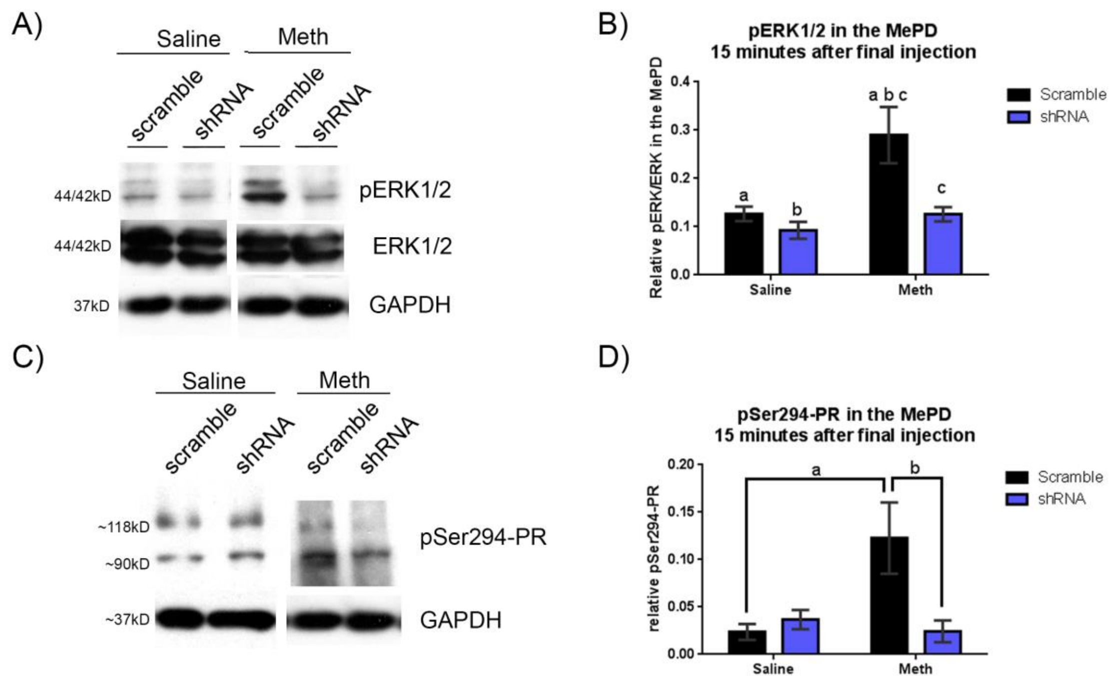

Figure 29: cSrc is required for Meth and EB+P induced phosphorylation of ERK1/2 and Ser294 on the PR. A) Representative Western Blot of pERK1/2 following shRNA infusions. B) A two-way ANOVA shows a main effect of both Meth ($F_{(1,19)}$=11.65, p<0.01, n=2-5, Fig. 28B) and shRNA ($F_{(1,19)}$=11.77, p<0.01, n=5-6, Fig. 28B) on pERK1/2 in the MePD with an interaction of Meth and shRNA ($F_{(1,19)}$=5.10, p<0.05, n=5-6, Fig 28B). A Tukey's multiple comparison's test revealed that in the Meth- and scramble-treated animals have significantly increased pERK1/2 over both saline/scramble ([a]p<0.005) and saline/shRNA ([b]p<0.001) treated animals. Additionally, this test revealed that cSrc shRNA reduces phosphorylation of ERK1/2 in Meth treated animals ([c]p<0.05). C) Representative Western Blot of pSer294-PR in the MePD. D) A two-way ANOVA shows an interaction of both Meth and shRNA on pSer294-PR in the MePD ($F_{(1,14)}$=5.93, p<0.05, n=5, Fig. 28D). A Tukey's multiple comparison's test revealed that in the Meth- and scramble-treated animals have significantly increased pSer294-PR over both saline/scramble controls ([a]p<0.05) and Meth/cSrc shRNA treated animals ([b]p<0.05). Data represented as mean ±SEM.

Discussion

The present work outlines a mechanism by which Meth and PR interact, centering on Src kinase, thus leading to a facilitation of the signaling events that we believe increase proceptivity. First, we showed that ERK1/2 and Src's enhanced activation occurs as early as 15 minutes after the combined treatment of Meth and hormones. Second, we have demonstrated that the Src and ERK1/2 kinase cascades were required for this progesterone-dependent behavior as by blocking the ability of either kinase to phosphorylate downstream proteins we blocked Meth's effects on proceptivity alone. Finally, we were able to show that Src activation is upstream of ERK1/2 phosphorylation and phosphorylation of Ser294 on the PR.

While evidence implicated kinase enhancement of PR signaling following Meth administration, the mechanism and pathways remained unknown. In Chapter 5, we demonstrated an increased phosphorylation of the PR at serine 294, a site known to be important in enhanced sensitivity to ligand and a more robust PR action [84, 101, 166, 171, 175, 178], in a region-dependent manner following Meth administration. This phosphorylation site can interact with MAPKs, specifically ERK1/2 [85, 103] suggesting that ERK1/2 signaling may be involved in the mechanism leading to Meth-facilitated proceptivity as well as the increased phosphorylation of PR. Additionally, Src has been implicated in progestin-driven behaviors [93], and may signal upstream of ERK1/2, and thus another likely target for Meth's mechanism of action.

Indeed, we found that both ERK1/2 and Src showed increased phosphorylation in the Meth and EB+P groups than either treatment alone (Fig. 21 & 22). It is important to note that there is a high level of phosphorylation in the absence of Meth and EB+P as well. This is likely due to estradiol regulation of phosphatase function, and in the absence of E2, phosphorylation may increase in certain cell types [182, 183]. Even so, it is important to keep in mind that without the presence of hormones, we do not see any expression of sexual behaviors. As ERK1/2 has

been shown to directly phosphorylate PR at Ser294, we aimed to determine if ERK1/2 activity was required for Meth-facilitated proceptivity.

The activity of ERK1/2 is necessary for Meth-enhanced proceptive behavior as when SCH772984 (SCH), an ERK1/2 specific ATP competitive inhibitor, was infused bilaterally into the MePD, Meth-induced increases in proceptivity were blocked, but baseline behaviors remain intact (Fig. 23a). It is important to note that SCH is a potent inhibitor. The doses used here, although targeted to the MePD, likely reduced signaling from cells other than those contained in our behaviorally relevant neuronal ensemble (see Chapter 4). As the MePD does project to the other nuclei implicated in sexual behaviors (Fig. 4), it is possible that some signaling to the VMN may have been inhibited as well, thus leading to the decrease we see in LQ in this experiment alone.

Although ERK1/2 activity and phosphorylation of Ser294 were greatest in the Meth and EB+P treated animals, and ERK1/2 was required for the Meth-induced increases in proceptive behavior, we were surprised to find that ERK1/2 was not responsible for phosphorylation of Ser294 (Fig. 24). This suggests that ERK1/2 is likely acting in a functionally parallel pathway or downstream to that of PR action. There is evidence that ERK1/2 acts as a coactivator with PR in order to induce transcription of genes that do not contain a classical PRE [101]. Although the question remains as to which gene(s) within the MePD may be responsible for changes in proceptive behavior, the colocalization of ERK1/2 and PR on the chromatin may provide valuable clues in the future for how or if these two proteins interact in this mechanism.

As cSrc has been described as upstream of ERK1/2 signaling as well as having the ability to form complexes with both PR and ER within the brain [179], we posit that cSrc, and not ERK1/2 may be the intersection of Meth and hormone signaling in the MePD. Previous work by others has demonstrated a reduction in proceptive and receptive sex behaviors following PP2

infusion directly into the VMN [92]. However, upon infusing PP2 into the MePD in hormone-primed OVX rats, there is no effect in overall behavior. Instead, when infused in conjunction with hormones and Meth, we saw a blockade of Meth-induced increases in proceptivity alone without a 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 [120, 121, 123], thus strengthening the hypothesis that the MePD serves to enhance motivation for sex.

As all previous work on Src in sex behavior was done using SFK inhibitors, we wanted to confirm that the function was due to cSrc specifically, and not another kinase within the family. Using a shRNA targeted to cSrc, we were able to recapitulate the behavioral findings seen with PP2. Therefore, these behaviors were confirmed to be due to Src, and not another SFK with a shRNA knockdown of cSrc.

The animals treated with the shRNA were checked for transfection efficiency via Western Blot of the activated, or phosphorylated, form of Src. Samples taken from the MePD showed that shRNA-treated animals expressed only 63% of the active form of Src when compared to the animals treated with non-targeting shRNA. It is important to note that our behaviorally relevant neuronal ensemble, while contained within the MePD, is not represented by all MePD neurons. Similarly, our shRNA remained within a discrete region following injection. Thus, our MePD samples contain some cells which will respond to Meth and EB+P effects and those that remain unaffected. Therefore, we felt that the overall Src activation was the more appropriate measure for the region instead of total Src, which may be washed out by the untransformed cell population. This may also be why we didn't see a more robust knockdown of pSrc within our region of interest.

Src has been shown to be upstream of the Raf/MEK/ERK pathway [180] as well as interacting with PR, [93, 167, 181], therefore we wanted to see if by removing the activity of Src alone, we were able to reduce the combined effect of Meth and EB+P on ERK1/2 and PR phosphorylation. Similar to findings in Figs.18 & 21, Meth and EB+P treatment results in a high level of ERK1/2 phosphorylation and PR phosphorylation at Ser294. Knockdown of Src blocked phosphorylation of both these proteins following Meth administration. This evidence, taken with the requirement for cSrc in Meth-facilitated proceptive behavior, suggests that Src is the convergence point for Meth and EB+P interaction, and upstream of ERK1/2 and PR phosphorylation (Fig. 30).

Overall, we have shown that Src is the convergence point for Meth and PR signaling. It is likely that Meth, and subsequent dopamine release, activates D1Rs which initiates phosphorylation of intracellular proteins. The PR forms a complex with Src, leading to its phosphorylation at Ser294. This results in 1), downstream ERK1/2 phosphorylation and 2), enhanced sensitivity of the PR to progesterone. This enhanced sensitivity to ligand, and potentially activation of a cofactor in ERK1/2, ultimately leads to enhanced activity of the MePD and Meth-facilitated increases in proceptive behavior.

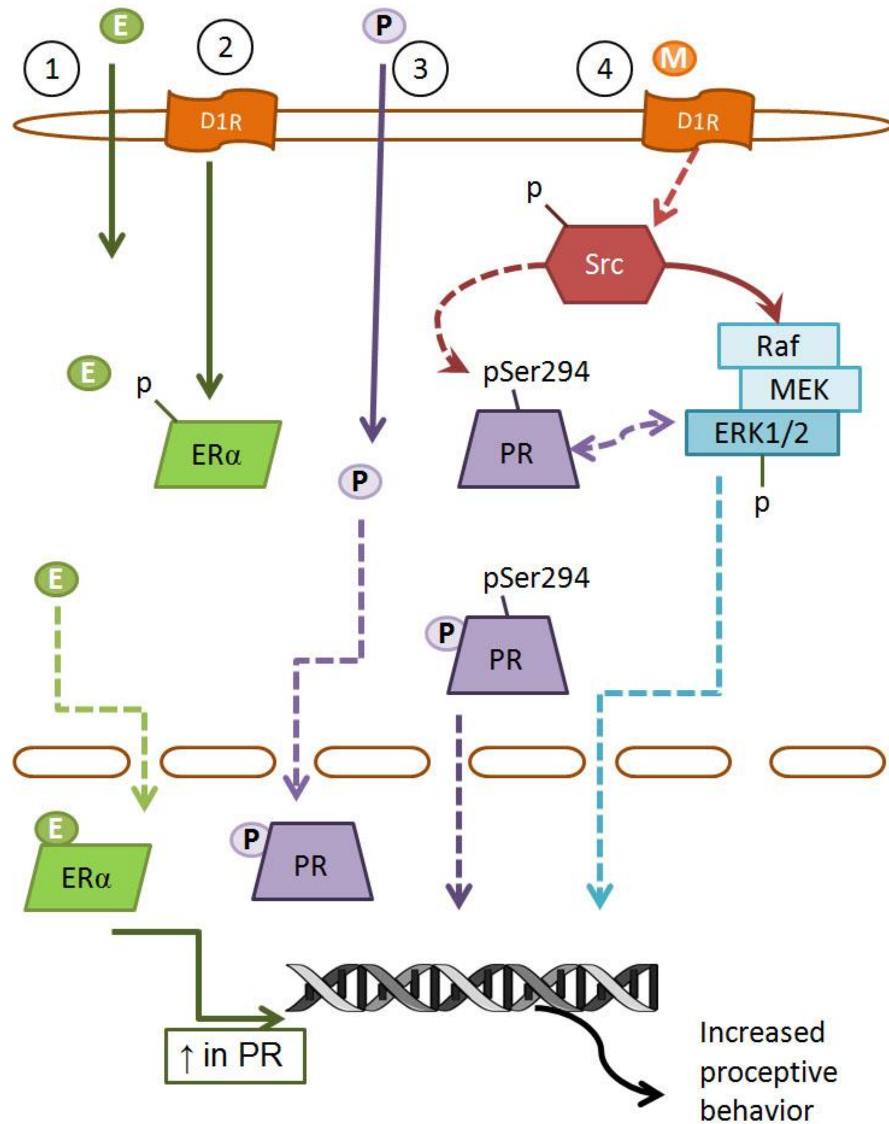

Figure 30: Schematic drawing to illustrate our proposed model. 1) Estradiol enters the cell and binds to ER, leading to ligand-dependent gene transcription. 2) Following Meth-induced dopamine release, signaling via the D1R also leads to ER translocation to the nucleus and ligand-independent transcription of the PR. These are the priming stages. 3) Similarly, progesterone can enter the cell and lead to ligand-dependent signaling. 4) Following activation of the D1R, Src interacts with the PR, leading to phosphorylation of Src and PR at Ser294. This results in downstream ERK1/2 phosphorylation and increases proceptive behavior.

CHAPTER 7: GENERAL DISCUSSION

Summary

Female sexual motivation, especially the neural mechanisms behind it, remains poorly understood despite years of studies on gonadal steroids and neural circuitry. In humans, work is limited to self-surveys to determine desire and arousal [184, 185] and fMRI scans [186] to detail brain activity that corresponds to given stimuli. Some human surveys have looked at Meth abuse and subsequent increases in risky sex behavior to unravel the questions of what drives such behavior. Although Meth abuse has been shown to highly correlate with increased risky sex behaviors in women [5, 6] and this is able to be replicated in a rodent model [120-123], until now the intracellular mechanisms resulting in this behavior have remained unknown. Here we sought to examine the mechanisms behind Meth-facilitated increases in proceptivity in the female brain.

First, we found that the Meth-facilitated increases in proceptive behavior are linked to a specific neuronal ensemble within the MePD. This neuronal ensemble shows an increased level of activation (as determined via cFos-ir) following the administration of Meth and EB+P. Our second finding was that Meth increases PR density and activation in the MePD. Meth alone had been shown to increase PR protein, but we have shown here that it does so via increased transcription. Similarly, in the presence of EB+P and without, Meth enhances site-specific phosphorylation of the PR at serine 294. Lastly, we explored pathways by which Meth could interact with the PR, finding the Src/ERK1/2 signaling cascade to be directly involved in the Meth induced increases in proceptivity. Src appears to be the convergence point of these two pathways, as it is required not only for the Meth-induced increases in proceptive behavior, but also the increased ERK1/2 and PR phosphorylation. This data begins to address the gaps in our knowledge regarding intracellular mechanisms underlying sexual motivation as well as PR function within the brain.

Implications for Organization of the Medial Amygdala

The MeA is heterogeneous nucleus in both structure and function [187]. Overall, the amygdaloid complex is thought to be responsible for emotionally driven behaviors, such as fear, anxiety and sexual drive, and the MeA is no exception. This nucleus can be functionally divided into its anterior region, and two posterior regions, with each subnucleus having its own functional role [131], and as we've shown here, the MePD has been studied for its modulation of sex behavior[44, 120, 121, 123, 124, 144]. Although the majority of behavioral studies have been conducted in rats and mice, the amygdala has also been implicated in human sexuality using fMRI studies [186, 188], but even this work does not shed any light on the activation of specific cells leading to the initiation of behavior.

On a cellular level, it has been shown that the MePD receives projections from the olfactory bulb and sends projections to hypothalamic nuclei [131, 187, 189]. The MePD is also sensitive to gonadal steroids in both sexes [126, 190]. It is not a unique conclusion then, that the MePD uses environmental cues to modulate outcomes of sex behavior [189, 190]. To our knowledge it has not been shown how these cues, especially more global cues like Meth administration, can lead to specific behavioral outcomes.

In effort to identify the nucleus responsible for Meth-facilitated increases in sex behavior, Holder et al. lesioned the MePD using ibotenic acid, and thus was able to block only the Meth-induced increases in proceptivity [123]. Lesion studies done by others [128-130] have shown differing roles for the nucleus, but it is likely that these results are due to lesions that either extend beyond one subnucleus or that are in different subnuclei all together. However, we have recently shown using cellular activation studies, that four hours following Meth and EB+P administration that the MePD shows increased neuronal activation over either treatment alone [121]. Aside from

correlation, there was nothing directly linking these activated cells to both the treatments and the subsequent increase in proceptive behavior.

In order to circumvent these difficulties, we utilized a model pioneered by Bruce Hope, termed DAUN02 inactivation. By using this technique (described in Ch. 4), we were able to successfully eliminate only those cells in the MePD that were activated by the combination of Meth and EB+P, and by doing so blocked the Meth-facilitated increases in proceptivity. These studies have functionally connected these neuronally active cells to the increases in proceptivity seen with the combined administration of Meth and ovarian hormones.

This finding is pertinent to how we look at the MePD as a whole. As seen in Figure 14, the cFos-ir cells, although greater in the Meth/EB+P treated animals, are not an overly large or dense population, and thus leave the function of the overwhelmingly larger percentage of cells within the region unknown. This is likely true for other brain regions as work done by Bruce Hope and colleagues also showed a discrete population of activated neurons in the accumbens required for cocaine craving [145, 149, 150]. While these cellular activity studies do not elaborate on the function of all cells within the region, it does provide a useful tool in describing these cellular populations. Labeling these behaviorally relevant neurons with β-Gal will allow for examination of morphological and functional differences between cells. For example, although the D1Rs have been implicated in previous work as being necessary for Meth-facilitated proceptivity, we still do not know whether the D1Rs exist on the hormone concentrating cells or if they colocalize with PR. Similarly, we do not know if the behaviorally relevant neuronal population is GABAergic or glutamatergic. Future work will be able to utilize these techniques to further elucidate the roles of individual cell types.

Effects of Dopamine on Gonadal Hormone Signaling

Previous work by Holder et al. has shown that the D1R is the only catecholamine receptor both necessary and sufficient for Meth-induced increases in proceptive behavior. Although other catecholamines may have an impact on female sexual motivation, dopamine has long been shown to interact with ovarian steroids to induce this behavior. D1R agonists have been shown to increase lordosis when infused into the VMN in a PR dependent manner [33, 64, 191]. This interaction is believed to be entirely ligand-independent, as mutations of the C-terminal tail of the PR prevents D1R-enhancement of sexual behavior, but does not prevent normal ligand binding and receptor activation [88]. Similarly, dopamine has been shown to increase ER-dependent transcription in a ligand-independent manner [192], including upregulating PR in multiple neonatal brain regions [165].

These data support our finding that Meth (likely via D1R activity) increases PR mRNA, and that this may be occurring via ligand-independent activation of ERα within the MePD. It is thought that D1R increases downstream cAMP, thus allowing for ligand-independent phosphorylation of the receptor [170] (Fig. 5). While we believe that this interaction between ERα and the D1R is likely occurring within the MePD, we do not believe that ligand-independent interaction is occurring with the PR following D1R activation. By infusing the PR antagonist, RU486, directly into the MePD, we are able to block only the Meth-induced enhancement of proceptive behavior [123]. Additionally, in estradiol-primed animals that do not receive progesterone, regardless of Meth administration, proceptive behaviors do not increase [125]. However, when these animals are given a subthreshold dose of progesterone (a dose that should not initiate proceptive behaviors) Meth restores the full complement of sex behaviors [125]. Thus, we believe that in order for Meth-facilitated proceptivity to occur, progesterone and its cognate receptor must both be present, and that Meth enhances the response of the receptor to ligand.

These hormone-drug interactions are likely occurring in numerous instances, many of which go unnoticed. Meth, along with other amphetamine drugs, is still prescribed for the

treatment of ADHD in adolescents [193]. It is not outside of the realm of possibility that these drugs might influence the changing levels of hormone receptors in the brain across puberty. Similarly, serotonergic drugs, such as selective serotonin reuptake inhibitors (SSRIs) have been shown to interact with ERs in both an estrogenic and antiestrogenic fashion dependent upon dose [194, 195], while estrogen replacement has been shown to upregulate serotonin-related genes in marmosets [196]. It is becoming increasingly obvious that our drugs need to be evaluated in both sexes as interaction with gonadal steroids can have a profound effect on both the function of the drug as well as the patient's overall well-being.

Interplay of Signaling Pathways

Ligand-dependent and ligand-independent nuclear receptor pathways have been accepted as the canonical mechanism of signaling; however, recent work is beginning to tease apart signaling mechanisms that appear to alter the function of the receptors. In classical PR signaling, progesterone diffuses into the cell, where it is able to bind to the PR. These ligand-bound receptors will then enter the nucleus, dimerize and become phosphorylated, and act with co-activators at PREs. If dopamine is released simultaneously, at least in the VMN, there will be an increase in cAMP activity and PKA phosphorylation. PKA activity results in numerous downstream phosphorylation events, including that of the PR, co-activators important for PRE-associated gene transcription, and DARPP-32 activity ([191], for review, [62]). Overall, the dopamine release causes an increase in phosphorylation and in particular ERK1/2 activity [197-199]; however, this phenomenon has not been examined within the MePD.

The importance of this interplay of pathways has become clear within the cancer literature. In hormone-responsive cancers, such as breast cancer, the PR interacts with growth factor signaling cascades [175], thus leading to hormone-dependent tumor growth. In fact, it was the association of PR with the ERK1/2 signaling pathways that appeared to be most important for

this effect [176, 178]. More recently it has been shown that this pathway is initiated at the level of Src kinase [103], and that the interaction of Src and PR in this noncanonical pathway allows for targeted specificity of action dependent upon the phosphorylation status of the PR [103, 172, 173].

These kinase/PR interactions have been shown to occur in the VMN as well, with ERK1/2 being required for the dopamine-stimulated increases in lordosis following VCS [90]. This model was further developed when kinases upstream from ERK1/2, specifically the cSrc/Raf cascade was required for this effect as well [92]. As PR has been shown to be able to complex with Src and other proteins [103], it is hypothesized that baseline sex behavior results from canonical ligand-dependent hormone signaling within the VMN, and that this behavior can become enhanced during a bout of mating behavior via dopamine release following successful mounts and intromissions. This enhancement is driven via activation of cell-surface receptors leading cSrc to bind with both PR and ER, thus upregulating ERK1/2 activity, and likely PR/ERK1/2 driven gene expression [101, 179].

Work in the lab has previously established a role for D1R and PR signaling within the MePD in Meth-facilitated increases in female sexual motivation. Although progesterone is required for proceptive behavior in the rat, Meth is able to enhance progesterone-driven behaviors when sub-physiologic doses of progesterone is administered [125]. Prior to the work here, it was unknown how these two signaling pathways were interacting to lead to this increase in proceptivity. Phosphorylation of serine 294 on the PR has been shown to increase sensitivity of the receptor to ligand [175, 178], suggesting that this may be the mechanism by which Meth enhances PR-driven behaviors. Indeed, we saw that Meth does increase phosphorylation of the PR at Ser294 (Fig. 18). Given data that both ERK1/2 and Src are able to interact with PR both in culture and in the brain [103], as well as increase activity following Meth administration [95, 200, 201], it seemed a likely target for how the Meth induced kinase pathways lead to phosphorylation

of the PR and its hyper reactivity to ligand. Thus, Figure 28 demonstrates the likely model for Meth-facilitated increases in proceptive behavior. First, canonical signaling of the ER leads to increases in PR. Second, Meth administration leads to activation of the D1R's, which subsequently lead to phosphorylation of cSrc. Activated cSrc leads to phosphorylation of PR and subsequently ERK1/2. PR and ERK1/2 may then be acting as cofactors, leading to gene transcription. Regardless of genomic outcome, these events lead to an enhancement of proceptive behavior, most likely due to the MePD's integration of the signaling events altering the output to the VMN.

Implications for Human Studies / Human Sexual Dysfunction

Human sexuality has long enraptured our attention. Starting as early at the mid-1940's, research into human sexuality began to take shape, although it took until the mid-1960's for the human sexual response cycle (Masters and Johnson 1966) to be mapped out. Yet another 20 years passed before "sexual disorders" became diagnosable as described in the Diagnostic and Statistical Manual of Mental Disorders (DSM) [202]. At the time, these sexual disorders were classified as physical dysfunctions, but more recent work has shown most patient complaints, especially in women, are not due to mechanical dysfunction but rather a loss of interest in sex [202]. With the advent of drugs like Viagra, there became an interest in a drug that would have an impact on female libido. Efforts have been met with difficulty, perhaps due to the variability in what affects sexual desire in women [184, 203].

Paradoxically, testosterone treatments (along with E2) have been the preferred treatment for decreased libido in women [203]. These doses are at a much lower level than would be seen in men, but in women testosterone at low levels appears to have an important role in sexual drive. Recently, the interest in nonhormonal libido-enhancing drugs has been on the rise. In 2015, a female libido enhancing drug, Flibanserin, was released under intense scrutiny as the effects were

quite modest in a mixed pre- and post-menopausal group [204]. Furthermore, in pre-menopausal women the drug was more effective, but also contained a host of side effects from sedation to severe hypotension. Due to these side effects, the inability for patients to safely drink any alcohol, and the required dosage to be taken daily, the drug never took off. It also received some criticism due to the inability to separate social factors from human sex drive. In fact, the understanding of female human sexual motivation and libido is quite shallow, and aside from work done by the Kinsey Institute, has remained relatively unexplored [186].

In humans, the work that can be done to decipher the neural mechanisms underlying female sexual motivation is limited. There have been fMRI studies which have linked the human amygdala to sexual arousal [186], but the majority of sexual studies are completed using self-surveys. These types of survey have been frequently used with Meth abusers. A high percentage of female Meth abusers report taking the drug to increase their self-confidence and feeling of "sexiness," or to make their sexual experiences more pleasurable [6, 205]. This is in contrast to other drugs of abuse where this functional use of the drug is not reported. As the sexual side effects appear to motivate the drug taking behavior, it becomes important to understand the interplay of drugs of abuse and female sexuality.

Conclusion

The data presented here emphasize the need to consider interactions between rapid signaling events following drug administration and the hormonal milieu within the brain. The National Institutes of Health has recently required the use of both sexes in basic research, and this is especially important as these drug-hormone interactions within the brain can potentially impact patient well-being. Furthermore, we have begun to address mechanisms leading to desire or motivated behaviors on an intracellular level. The brain, admittedly, is likely the most complex organ; however, the parallels between a neuron's signaling cascades and those seen *in vitro* are

remarkably similar. It is going to become increasingly more important as the field moves forward to take on a multidisciplinary approach in how we address behaviors and the pathways that drive them.

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