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**Author**
Christensen, Amy

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# Molecular Aspects of the Regulation of Female Sexual Behavior

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neurobiology

by

Amy Kruger Christensen

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ABSTRACT OF THE DISSERTATION

Molecular Aspects of the Regulation of Female Sexual Behavior

by

Amy Kruger Christensen

Doctor of Philosophy in Neurobiology

University of California, Los Angeles, 2012

Professor Paul Micevych, Chair

The regulation of female sexual receptivity by estradiol is complex and requires both the classical nuclear receptors and membrane estrogen receptors. It is only through the combined actions of both of these types of receptors in the brain and peripheral tissues that the female is ready for sexual behavior. The nuclear receptors dimerize, bind to DNA and regulate transcription, and thereby the translation of new proteins. The membrane estrogen receptors exert their effects through another method. In the arcuate nucleus of the hypothalamus (ARH), hippocampus and striatum, estrogen receptor-α (ERα), the only estrogen receptor shown to be required for sexual receptivity, transactivates metabotropic glutamate receptors (mGluRs) in order to initiate G protein signaling. This leads to the phosphorylation of a diverse array of signaling molecules depending upon which mGluR is activated. In a membrane to nucleus

signaling schema, membrane-initiated estradiol signaling can lead to the activation of cAMP response element binding protein (CREB), which also in turn affects transcription. For the activation of sexual receptivity, the association of ERα with mGluR1a, leading to the phosphorylation of PKCθ, is required in the ARH. In the present set of experiments, I show that three other events within the ARH are necessary for sexual receptivity: caveolin-1 (CAV1) mediated ERα trafficking, spinogenesis, and modulation of activity regulated cytoskeleton associated protein (Arc).

Estradiol regulates levels of membrane ERα modulating its own signaling, and CAV1 is a scaffold protein that traffics receptors to the membrane. In vitro, it has been observed to move ERα and the ERα-mGluR1 complex to the membrane. Without this protein, signaling in these neurons was significantly attenuated. Until now, no work had been done to examine whether CAV1-mediated ERα trafficking is involved in the activation of sexual receptivity in vivo. siRNA directed against CAV1 was used to knock down CAV1 protein in the ARH. This led to a significant reduction in membrane ERα, circuit activation and sexual receptivity.

Estradiol-mediated spinogenesis has been seen in several areas of the brain related to sexual receptivity including the ventromedial hypothalamus (VMH), which is considered the final common output from the limbic-hypothalamic lordosis behavior regulating module. Increases in dendritic spine density in the VMH and ARH have been proposed to regulate this behavior, but there has been no formal test of the idea. I induced the formation of spines in the ARH with estradiol and showed that this was mediated by membrane-initiated signaling requiring mGluR1a and the phosphorylation of cofilin. Deactivation of this natural actin depolymerizing agent allows the formation of new spines. I then used the β-actin polymerization inhibitor, cytochalasin D, to inhibit spinogenesis in the ARH. The loss of estradiol-induced dendritic spines markedly reduced lordosis behavior underlying the importance of morphological changes in the ARH for the estradiol-induction of sexual receptivity.

Mating experience has been shown to upregulate the immediate early genes in areas relevant to lordosis behavior like the VMH. Mating is a rich sensory experience involving tactile, olfactory, and auditory stimulation, which seems likely to modify the circuits that regulate it. I hypothesized that the activation of immediate early genes may modify future sexual behavior. To this end, I examined differences between sexual experienced females that were allowed a mating test after each injection of estradiol and sexually naïve females that were behaviorally tested once, but were given a similar number of estradiol injections as the experienced females. Unexpectedly, experienced rats were less receptive than the naïve females, even though they had more dendritic spines. The immediate early gene Arc was upregulated by the first sexual encounter and may play a role in the reduction of levels of membrane ERα in the ARH in experienced animals. When the Arc upregulation was inhibited with antisense oligodeoxynucleotides, receptivity in experienced females resembled that in naïve rats. These experiments indicate a vital role for Arc in the estradiol-regulation of sexual behavior.

In summary, the regulation of sexual behavior in females is complex and requires the coordination of a multitude of molecular targets. The trafficking of the ERα to the membrane by CAV1 allows for the initiation of estradiol-mediated spinogenesis. During sexual behavior, the circuit that regulates receptivity is further modified by Arc. When all of these molecular and morphological events are positively coordinated, a female will be sexual receptive.

The dissertation of Amy Kruger Christensen is approved.

Arthur P. Arnold

Carolyn R. Houser

Joshua T. Trachtenberg

Paul E Micevych, Committee Chair

University of California, Los Angeles

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# VITA

| | |
|---|---|
| 1983 | Born, San Diego, CA |
| 2005 | B.S., Physiological Science<br>University of California, Los Angeles<br>Los Angeles, California |
| 2005 | B.S., Anthropology<br>University of California, Los Angeles<br>Los Angeles, California |

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## Chapter 1. Introduction

The major sex steroid hormones in females are estradiol and progesterone. Peripherally, the main sites of synthesis are the ovaries with minor contributions from the adrenal cortex and adipose tissue. During childhood, both hormones are produced at very low levels. At puberty, there is a peak of estradiol production, and then levels become cyclical for the remainder of the female's reproductive life (Holland et al., 1998). Hormone receptors are located throughout the body, so once released steroids are able to have a multitude of effects.

There are two classical estrogen receptors, ERα and ERβ. Both are transcription factors and were thought to be synthesized in the cytoplasm and reside exclusively in the cell nucleus. Research over the past several decades has shown that these receptors regulate transcription by directly binding to DNA or stabilizing heterologous transcription factors. Estrogen receptor molecules can also be found on the plasma membrane, where they activate protein signaling cascades. Estradiol regulation of female sexual receptivity in rodents has been developed into a powerful, reproducible and high quantifiable behavioral assay for examining brain circuits involved in regulating lordosis behavior. More recently, it has been demonstrated that ERα in the nucleus and on the membrane is responsible for the regulation of female sexual behavior, and that this behavior can be used as a read-out for studying the signaling pathways used by estradiol. Understanding signaling pathways used by estradiol in reproductive behavior provides valuable insight into estradiol action since estrogen receptors are found throughout the CNS including in the hypothalamus, hippocampus, cortex, cerebellum, and spinal cord (McGuire and Lisk, 1968; Whalen and Massicci, 1975; MacLusky et al., 1986; Smith et al., 1987; Loy et al., 1988) and have important roles in learning and memory, energy balance, neuroprotection, and nociception (Simpkins et al., 1997; Fernandez et al., 2008; Tang et al., 2008; Fan et al., 2010; Park et al., 2011).

Estradiol regulation of female reproduction in the arcuate nucleus of the hypothalamus

Like women, the rat is a spontaneous ovulator. While women have an idealized 28 day menstrual cycle, the female rat has a 4 day estrus cycle. During the beginning of this cycle, estradiol is released from the developing ovarian follicles at very low levels. A few days later, on proestrus, as follicles mature and are ready for ovulation, estradiol levels surge, inducing positive feedback resulting in the surge release of gonadotropin releasing hormone (GnRH) leading to the massive secretion of luteinizing hormone (LH) from the pituitary. The LH induces ovulation and the conversion of the corpus hemorrhagicum into the corpus luteum, which begins to release large amounts of progesterone. The ovarian hormones act on the hypothalamus to activate behavioral circuits. It is only around the time of ovulation that the female rat is reproductively active. This exquisite coordination of ovulation with sexual receptivity is the result of the cyclical release of steroids from the gonads and the priming of multiple brain circuits by these steroids.

Sexual receptivity is regulated by an extensive circuit that receives inputs from the accessory olfactory and tactile sensory systems, the latter induced by the mounting male stimulating the female's flanks and perineum (Kow et al., 1979). In fact, male contact of the flank and pressure onto the rump, tailbase, and perineum is required to elicit lordosis behavior in female rats (Pfaff et al., 1977). Moreover, vaginocervical stimulation that results from mating, or artificial stimulation by an experimenter, is also important for the behavior (Komisaruk and Diakow, 1973; Rodriguez-Sierra et al., 1975). Lordosis behavior requires the combination of sensory input from the male and hormonal priming to activate the limbic-hypothalamic and spinal circuits involved in its regulation (Pfaff et al., 1977). Lordosis is the stereotypic behavior displayed by a sexually receptive female rodent in response to male mounting. It is the reflexive arching of the back and diverting the tail to the side, allowing male intromission.

Sensory information is sent to the limbic-hypothalamic part of the lordosis circuit and integrated by a diffuse network that includes the posterodorsal medial amygdaloid nucleus, bed nucleus of the stria terminalis, medial preoptic nucleus (MPN) and the ventromedial nucleus of the hypothalamus (VMH; reviewed in (Micevych and Ulibarri, 1992)) Network information from the limbic-hypothalamic module is integrated in VMH, which sends signals about the readiness of the animal for mating behavior to the periaqueductal grey (PAG), vestibular complex and finally spinal cord motoneurons innervating the trunk musculature that produce the lordotic posture (Pfaff et al., 2008).

The ARH to MPN to VMH microcircuit regulates lordosis

More recently, it was discovered that an important site of estradiol action regulating lordosis behavior was the arcuate nucleus of the hypothalamus (ARH; (Mills et al., 2004)). During the initial time after estradiol administration (up to 20 hrs) in ovariectomized rats, lordosis is inhibited and females are not receptive, but at later time points lordosis is facilitated (~30-56 hrs). The endogenous opioid peptide, β-endorphin (β-END), was shown to modulate this effect of estradiol (Torii et al., 1999). When β-END was infused into the ventricles around the time of estradiol treatment, which was 48 hours before behavior testing, lordosis was facilitated. However, if β-END was administered just prior to the start of the behavior test, sexual receptivity was severely attenuated. β-END cell bodies in the ARH send projections to the medial preoptic nucleus (MPN). Neuropeptide Y (NPY), by activating ARH β-END release, attenuates lordosis behavior (Mills et al., 2004). Eventually, it was demonstrated that estradiol acting in the ARH stimulates the release of NPY onto the Y1 receptor located on MPN projecting β-END neurons. These neurons release β-END into the MPN, where it causes the activation and internalization of μ-opioid receptors (MORs; (Mills et al., 2004)). The activation of MOR initially inhibits reproductive behavior, but is required for the full display of sexual receptivity at later time points. Neurons in the MPN that express MOR in turn project to the VMH to regulate sexual receptivity

(Sinchak et al., 2010). However, their neurotransmitter remains to be elucidated. Thus, when the circuit is activated by estradiol, lordosis behavior is inhibited. This inhibition is relieved by progesterone or by waiting for the MOR inhibition to become attenuated, approximately 48 hrs after estradiol priming (Sinchak and Micevych, 2001).

The activation of this ARH to MPN circuit is rapid, occurring within 30 minutes in ovariectomized females treated with estradiol benzoate (EB). Because of the speed of this activation, membrane estrogen receptors (ERs) were believed to play a role. Indeed, it was shown that the circuit could be activated with a membrane impermeable estradiol, E-biotin (Dewing et al., 2007). E-biotin is estradiol conjugated to a biotin molecule that prevents estradiol from diffusing through the lipid bilayer. This modification permits estradiol to activate receptors on the membrane and not those located in the cytoplasm or nucleus. We showed that ERα on the membrane transactivated the metabotropic glutamate receptor type 1a (mGluR1a) to stimulate this circuit, and that this interaction was required for sexual receptivity (Dewing et al., 2007). Inhibition of mGluR1a signaling with the group I antagonist, LY367,385, resulted in a loss of MOR internalization and attenuation of sexual behavior. Further studies showed that this interaction between ERα and mGluR1a resulted in the activation of PKCθ (Dewing et al., 2008). Inhibition of PKC with 2-[1-(3-dimethylaminopropyl)-1H-indol-3-yl]3-(1H-indol-3-yl) maleimide (BIS) attenuated MOR internalization and female sexual behavior. Pharmacological activation of PKC with the agonist phorbol 12, 13-dibutyrate, even after inhibition of mGluR1a, resulted in the internalization of MORs, suggesting that this was in fact the pathway used by ERα-mGluR1a to regulate receptivity (Dewing et al., 2008).

Membrane estrogen receptors in reproduction

There are several lines of evidence to suggest that so-called nuclear ERα is also a membrane receptor even though it does not have a typical membrane channel receptor or 7-transmembrane protein motif of a G protein-coupled receptor. First, it can be activated by

membrane impermeable estradiol constructs (Dewing et al., 2007). Second, it can be biotinylated suggesting that it has an extracellular region (Bondar et al., 2009; Dominguez and Micevych, 2010). Thirdly, it can be found associated with mGluR1a, a membrane protein, by co-immunoprecipitation (Dewing et al., 2007; Dewing et al., 2008). Fourth, activating membrane ERα stimulates G protein signaling cascades (Lagrange et al., 1997; Abraham and Herbison, 2005). The membrane impermeable estradiol conjugated to bovine serum albumin, E-BSA, can be given prior to a low dose of estradiol to stimulate sexual receptivity. The second subthreshold estradiol treatment is needed to prime the circuit that extends from the limbic system to the spinal cord. Although nuclear estrogen receptors are required to activate the estradiol mediated gene transcription in estrogen responsive cells (Parsons et al., 1982), treating with a membrane-constrained estradiol and free estradiol is as efficacious as two doses of free estradiol (Kow and Pfaff, 2004). This suggests that membrane ERs initiate signaling that is required for sexual receptivity, but transcription and translation stimulated by the classical ERα in the nucleus is essential.

Besides activation with membrane impermeable constructs, these compounds have been used to visualize estradiol binding on the membrane. E-BSA conjugated with fluorescein isothiocyanate reveals an initial membrane localization followed by a rapid internalization, reminiscent of ligand-induced internalization (Moats and Ramirez, 1998, 2000; Benten et al., 2001; Sinchak and Micevych, 2003; Dominguez et al., 2009). To demonstrate that these binding sites are ERα, surface biotinylation was used to label membrane proteins. In these experiments, a membrane impermeant biotin construct was washed onto cells, the cells lysed and membrane proteins identified by western blotting. ERα has been found on the membrane of hypothalamic astrocytes, embryonic hypothalamic neurons, and an immortalized hypothalamic cell line (N-38) (Gorosito et al., 2008; Bondar et al., 2009; Dominguez and Micevych, 2010; Dominguez et al., Submitted). In fact, estradiol has been shown by this method to regulate the membrane levels of its own receptor. Within 5 minutes of estradiol

treatment, cells increased levels of ERα on the membrane. By 2 hours, these levels returned to baseline and at later time points actually continued to decrease suggesting that ERα was being downregulated with continuous stimulation, decreasing its signaling. These studies indicate that membrane levels of ERα are regulated like other membrane receptors via ligand-binding induced internalization. The endocytosis of estradiol bound ER requires the phosphorylation of the ER and binding of β-arrestin, as has been described for other membrane receptors (Dominguez et al., 2009). Importantly, mGluR1a is trafficked and internalized in parallel with the ERα when neurons and astrocytes are stimulated by estradiol, and blocking either ERα or mGluR1a with the appropriate antagonist abrogates internalization (Dominguez et al., Submitted).

ERα is not itself a G protein coupled receptor and cannot initiate signaling cascades described for estradiol, such as PKA, PKC, MAP kinase, and Akt, without a molecular partner ((Lagrange et al., 1997; Boulware et al., 2005; Szego et al., 2006; Dewing et al., 2008; Quesada et al., 2008); reviewed in (Vasudevan et al., 2005)). The metabotropic glutamate receptors were proposed as signaling partners of ERα in cultured hippocampal cells (Boulware et al., 2005). The ERα-mGluR1a complex was shown to increase the phosphorylation of cAMP response element binding protein (CREB). The interaction between mGluR1a and ERα has been shown in ARH tissue and in cultured hippocampal cells by co-immunoprecipitation (Boulware et al., 2005; Dewing et al., 2008). CREB has also been shown to be phosphorylated in the ARH after estradiol treatment, so it was likely that ERα may be interacting with mGluR1a in this area of the brain as well. Indeed, pharmacological inhibition of mGluR1a in the presence of estradiol resulted in the loss of rapid estradiol signaling and no MOR internalization (Dewing et al., 2007). As expected, the loss of MOR activation at the time of estradiol treatment in turn resulted in a loss of sexual receptivity at later time points. This underscores the important role that the membrane ERα has in regulating lordosis behavior.

How ERα, which does not have a hydrophobic region(s) typical of membrane receptors, is inserted into the membrane has slowly been answered. It was first discovered that ERα could be palmitoylated (Pedram et al., 2007). The addition of hydrophobic side groups to the receptor eases its insertion into the membrane. Second, *in vitro,* trafficking of ERα to the membrane was shown to rely on scaffold proteins, the caveolin (CAV) family of proteins. These proteins are important for trafficking proteins to specific regions of the cell membrane involved in signal transduction, the lipid rafts (Massimino et al., 2002). In cultured cells, including hippocampal neurons, CAV1 is required for the trafficking of the ERα-mGluR1 complex to the membrane (Boulware et al., 2007). To determine whether CAV1 was required for ERα trafficking *in vivo* and for sexual receptivity, siRNA against CAV1 was infused into the ARH (Chapter 2). Membrane ERα levels were monitored and treated females were tested for sexual receptivity. Results were consistent with the hypothesis that CAV1 is required for the trafficking of ERα to the membrane. The CAV1 knockdown rats had reduced membrane ERα levels and were less receptive. These results supported the idea the trafficking of ERα to the membrane in the ARH is needed for regulating lordosis behavior.

Spinogenesis and sexual receptivity

One of the early discoveries of estradiol action in the brain was the alteration of neuronal morphology, especially in areas involved in the regulation of sexual behavior. In the deafferented ARH, Matsumoto and Arai showed an increase in dendritic spines in response to estradiol treatment (Matsumoto and Arai, 1981). In the preoptic area, estradiol induced the growth of neurites (Toran-Allerand, 1980). In the VMH, estradiol mediated spinogenesis and dendrite length (Calizo and Flanagan-Cato, 2000).

Dendritic spines are small protrusions that extend from dendrites to receive excitatory signals from axons. The major cytoskeletal component of dendritic spines is actin (Hotulainen and Hoogenraad, 2010). As a dendrite forms, single molecules of actin begin to polymerize,

and this newly formed actin filament deforms the membrane to create a thin protrusion known as a filapodia. These filapodial spines are highly labile and are regarded as immature spines searching for synaptic partners to stabilize them. Once an appropriate axon terminal is found, the head of the spine expands through the branching of actin. The edge of the spine closest to the axon terminal begins to fill with receptors and their associated scaffolding proteins and signaling molecules. This area is known as the postsynaptic density because of the dense population of proteins that are located there. Spines with such large heads and thin necks are designated mushroom-shaped and are considered to be mature. Mushroom-shaped spines are generally more permanent than filapodial and participate in synaptic transmission (Trachtenberg et al., 2002; Yasumatsu et al., 2008).

Early studies considered the regulation of spine density an organizational effect of estradiol. Subsequent studies demonstrated that estradiol even in adulthood affected dendritic structure (Frankfurt et al., 1990). Thus, estradiol regulates not only neural transmission by altering levels of neuropeptides, synthetic enzymes and causing the activation of circuits, but also modulates neuronal morphology through the growth of dendritic spines. Although originally described in the hypothalamus, estradiol-mediated spinogenesis has perhaps been best studied in the hippocampus. These elegant studies demonstrated the highly plastic nature of spines in adulthood. Specifically, the number of spines in the CA1 region of the hippocampus increased on proestrus, when estradiol levels are high (Woolley and McEwen, 1992). This effect can be mimicked in ovariectomized animals treated with estradiol (Woolley and McEwen, 1994).

A similar estradiol-induced increase in spines has also been seen in the ARH where it has been proposed to play a role in regulating the LH surge. In the VMH the estradiol-induced increase in spines has been proposed to regulate female sexual behavior, but has not been formally tested (Frankfurt et al., 1990; Calizo and Flanagan-Cato, 2002). Specifically, spines in the VMH were increased in the ventrolateral portion of the VMH, the subdivision responsible for regulating sexual behavior and the location of a significant population of ERα-positive

neurons (Pfaff and Keiner, 1973; Pfaus et al., 1993). My experiments build on these ideas, but sought to formally examine whether estradiol-mediated spinogenesis in the ARH regulates sexual receptivity. Toward this end, cytochalasin D, a β-actin polymerization inhibitor, was infused into the medial basal hypothalamus to prevent spine growth and female rats were tested for sexual behavior. In addition, these experiments tried to elucidate mechanisms through which estradiol was acting. This question is important because there are two ideas about estradiol action. A direct estradiol action in which estradiol regulates spine formation in cells with ER, or a transsynaptic action, in which estradiol changes synaptic signaling resulting in altered spine density. When estradiol acts directly on cells, the receptor may be nuclear or on the membrane. There is evidence to support both hypotheses. ERα has been found in axons and spines in the hippocampus (Milner et al., 2001); and ERα negative neurons in the VMH and hippocampus have been shown to increase spinogenesis. Such neurons receive inputs from neurons that do contain ERα and spine density is modified in response to afferent signaling (reviewed in (Cooke and Woolley, 2005)). Our results point to a direct effect of estradiol via membrane-initiated signaling that modulates actin-regulating proteins inducing spine formation that is needed for the display of sexual receptivity.

Immediate early genes and sexual receptivity

In an effort to continue to dissect the signaling mediating sexual receptivity, we examined the increase in immediate early genes (IEG). The induction of IEGs has long been used to indicate neuronal activation (reviewed in (Kovacs, 2008)). It is no surprise then that many people have investigated the activation of the circuits regulating sexual receptivity by investigating IEGs (Pfaus et al., 1993; Tetel et al., 1993; Calizo and Flanagan-Cato, 2003). The most commonly used has been Fos, since the activation of this protein in neurons has been correlated with activation (Dragunow and Robertson, 1988; Anokhin et al., 1991). However, several issues have emerged with the use of Fos. First, Fos immunoreactivity is not a marker

for all forms of neuronal activation, including axonal and dendritic activity that does not lead to a change in transcription (Dragunow and Faull, 1989). Also, Fos expression requires strong stimulation and the length of Fos activation varies in different brain regions.

More recently, activity regulated cytoskeleton-associated protein (Arc) has been used as a marker of specific activation. Arc is activated by sensory experiences, and sexual behavior is rich in sensory feedback. Moreover, Arc is a complex regulator of neuronal function which has been implicated in a plethora of neuronal events. In particular, Arc affects dendritic spine growth through an interaction with molecules that regulate the formation of filamentous actin, the major cytoskeletal component of spines (Bramham et al., 2008). In so doing, Arc stabilizes existing spines and may facilitate the growth and maturation of new spines. This allows for greater excitability in Arc expressing neurons. In addition, Arc is able to associate with other molecules, endophilin and dynamin, molecules involved in endocytosis, to affect AMPA receptor endocytosis (Waung et al., 2008). Endocytosis of ionotropic glutamate receptors reduces the signaling capabilities of neurons. Both through the addition of spines and the removal of AMPA receptors, Arc modulates glutamatergic signaling.

Since it is induced by novel sensory experiences, Arc was upregulated after sexual behavior in the VMH (Guzowski et al., 1999; Guzowski et al., 2001; Flanagan-Cato et al., 2006). Animals that were treated with steroids but not subjected to a mating test did not have an increase of VMH Arc. These results indicate that hormone treatment alone was not sufficient to induce Arc, and that the experience of sexual behavior was required. Animals that were behaviorally tested showed a decrease in overall spine density in the VMH (Flanagan-Cato et al., 2006). The authors did not suggest a causal relationship between Arc and the decreased spine density or the modest increase in sexual receptivity of repeatedly tested animals. Although Arc is upregulated by sexual behavior in the VMH, little is known about how Arc might mediate sexual behavior.

The VMH studies indicated that Arc was regulated by behavioral experience, but did not address its role in signaling sensory input to the ARH. In order to determine the sensory role of the ARH, animals were tested once (naïve) or repeatedly (experienced). Spinogenesis was examined in the ARH in experienced animals and compared to naïve females to determine whether spine density would vary between these groups. In addition to lordosis behavior, different sensory tests were used to determine the sensory modality that led to Arc upregulation: social contact with a novel animal or male olfactory stimuli. Other than the mating test, none of the stimuli increased Arc expression. These experiments provided important information about the sensory activation and plasticity of the circuit that regulates female sexual behavior.

## Summary

Together, these experiments elucidated several of the molecular components required for the regulation of sexual receptivity. The study of trafficking of ERα by CAV1 will be the first to elucidate ERα trafficking in the in vivo brain (Chapter 2). It will also reiterate the importance of ERα on the membrane for the regulation of sexual behavior. Estradiol mediated spine density increases have been seen in the ARH previously, but that the inhibition of spinogenesis resulted in behavioral deficits was the first demonstration of the importance of the formation of new spines for female sexual behavior (Chapter 3). The modulation of the IEG Arc and the reproductive circuit by reproductive behavior demonstrated the importance of sensory feedback for the regulation of the estradiol-induction of lordosis behavior (Chapter 4).

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## Chapter 2. CAV1 siRNA Reduces Membrane Estrogen Receptor-α Levels and Attenuates Sexual Receptivity

### Introduction

Cell membranes have been demonstrated to have specialized areas, referred to as caveolae, in which cell signaling machinery is clustered (Li et al., 1995; Li et al., 1996). Neurons do not have caveolae-like invaginations, but their membranes are rich in caveolin proteins that define functional microdomains of the cellular membrane. Such lipid rafts allow for the segregation of signaling pathways (Head et al., 2006). Caveolins, a family of scaffold proteins, participate in targeting receptors and associated proteins to the membrane (Anderson, 1998). Estrogen receptor-α (ERα) has been demonstrated to interact with caveolin proteins, which are involved in trafficking of this receptor to the cell membrane and coupling it with its signaling partners (Razandi et al., 2002; Boulware et al., 2007).

In vitro, ERα has been shown to interact with different mGluRs through specific caveolin proteins (Boulware et al., 2007). Caveolin-1 (CAV1) mediates the ERα interaction with type 1 mGluRs, mGluR1 and 5, while type 2 mGluRs, mGluR2 and 3, are associated with caveolin-3 (reviewed in (Micevych and Mermelstein, 2008)). The specificity of these interactions promotes different types of signaling. ERα transactivation of mGluR1 stimulates PLC dependent pathways leading to activation of the mitogen-activated protein kinase (MAPK) pathway that phosphorylates cAMP response element binding protein (CREB) and releases internal calcium stores (Boulware et al., 2007; Kuo et al., 2009). However, when estradiol activated ERα interacts with type 2 mGluRs, adenylyl cyclase is blocked and flux through the L-type calcium channel is reduced (Boulware et al., 2005; Chaban et al., 2011). In this way both facilitation and inhibition mediated by ERα is explained.

ERα is an important component of the induction of sexual receptivity by estradiol (Ogawa et al., 1999; Micevych et al., 2003). Estradiol has been shown to act through membrane receptors in the arcuate nucleus of the hypothalamus (ARH) to rapidly activate a circuit that extends from the ARH to medial preoptic nucleus (MPN) to ventromedial hypothalamic nucleus (VMH) mediating lordosis behavior (reviewed in (Micevych and Dewing, 2011)). A hallmark of estrogen membrane signaling is ERα transactivation of metabotropic glutamate receptors (mGluRs) in the ARH ((Dewing et al., 2007; Dewing et al., 2008; Christensen et al., 2011); reviewed in (Micevych and Mermelstein, 2008)) leading to the release of β-endorphin (β-END) and the internalization of μ-opioid receptor (MOR) in the MPN (Eckersell et al., 1998).

In vitro, levels of ERα and mGluR1 on the membrane are regulated by estradiol treatment through activation of the ER-mGluR1a complex (Bondar et al., 2009; Dominguez and Micevych, 2010). Estradiol transiently increases both receptors on the membrane at a time point that coincides with increased receptor activation and downstream signaling. To determine if there is a physiological role for caveolin-mediated ERα trafficking to the membrane in the ARH, we used a site-specific knockdown of CAV1, the most likely caveolin protein mediating the association with mGluR1a, an interaction that is important for estradiol stimulation of neuroprogesterone synthesis and lordosis behavior (Dewing et al., 2007; Kuo et al., 2010). siRNA treatment reduced CAV1 expression and prevented the trafficking of ERα to the cell membrane. Estradiol activation/internalization of MOR was abrogated in rats with CAV1 knocked down in the ARH, and the lordosis quotient, a measure of sexual receptivity, was significantly reduced.

## Material and Methods

*Animals*. Male and ovariectomized (ovx; by the supplier) female (200–250 g) Long-Evans rats were purchased from Charles River (Portage, MI). Upon arrival, rats were housed

two per cage in a climate-controlled room, with a 12:12 hr light:dark cycle (lights on at 6:00AM). Food and water were available *ad libitum*. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

*Steroid priming*. Animals were allowed to survive 2-3 weeks after ovx prior to steroid treatment. For all experiments, 17β-estradiol benzoate (EB) dissolved in safflower oil was injected subcutaneously (sc) in a volume of 0.1 ml per rat. Females received 5 µg EB every four days between 8:00AM and 9:00AM for 3 cycles to mimic the natural estrous cycle of female rats (Micevych et al., 1994).

*Guide cannulae implantation surgery*. Bilateral guide cannulae (22 gauge; Plastics One Inc., Roanoke, VA) directed at the ARH (coordinates from bregma: anterior –2.8 mm, lateral 0.8 mm, ventral –7.4 mm from dura; tooth bar: –3.3 mm) were implanted using standard stereotaxic procedures while female rats were anesthetized with isoflurane (2–3% in equal parts oxygen and nitrous oxide). Cannulae were secured to the skull with dental acrylic and stainless steel bone screws. Stylets were placed in the guide cannulae, which protruded less than 0.5 mm beyond the opening of the guide cannulae. Animals were individually housed after surgery, received oral antibiotics (trimethoprim and sulfamethoxazole, 0.4 mg/ml; Hi-Tech Pharmacal, Amityville, NY) in the drinking water and allowed to recover 6-7 days before infusion.

*Microinjection*. For ARH site-specific microinjections 2 µg/µL of CAV1 siRNA or control siRNAs (ON-TARGETplus SMARTpool siRNAs; Dharmacon, Lafayette, CO) were dissolved in aCSF. One µL per side of the siRNA was microinjectioned with an infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.25 µl/min. Microinjection needles (28 gauge) protruded 1 mm or less beyond the opening of the cannula and were allowed to remain in place for 1 min after infusion to allow for diffusion away from the injector. After microinjection, the obturators were reinserted into the guide cannulae and animals were returned to their home cage.

To ensure a significant knockdown of CAV1, animals received four microinfusions, one each day: the first was 30 minutes prior to the second EB injection; and the second was the

next day. The third microinfusion was 3 days after the EB injection, which was the day before the final EB injection. The final microinfusion was 30 minutes prior to the third, and last, EB injection.

*Behavioral testing*. Sexually experienced males were acclimated to the Plexiglas testing arenas for 15 min before testing. Thirty hours after the third EB injection, female sexual receptivity was measured by placing each female rat into an arena with a stud male. Stimulus males were allowed to mount females 10 times and the number of times the female displayed lordosis (lifting of the head, arching of the back, movement of the tail to one side) was recorded. For each female, the sexual receptivity was quantified as a lordosis quotient (LQ), the number of lordosis displays/number of mounts x 100 (Dewing et al., 2007).

*Confirmation of guide cannulae placement*. Animals used for immunohistochemical studies only were anesthetized and transcardially perfused with chilled 0.9% saline, followed with a fixative, 4% paraformaldehyde dissolved in 0.2 M Sorenson's phosphate buffer (pH 7.4). Brains were removed and placed in fixative overnight at 4° C and then replaced with 20% sucrose in phosphate buffer for cryoprotection. Brains were blocked, sectioned (20 µm) on a cryostat (Leica CM 1800, Bannockburn IL), and collected into chambers filled with PBS. Sections were mounted onto SuperFrost/Plus slides (Fisher, Pittsburgh, PA), stained with thionin, dehydrated, and coverslipped with Krystalon (EMD Chemicals, Gibbstown, NJ). Injection sites were mapped and verified with bright-field illumination. In animals used for Western blot analysis, cannula position was confirmed visually during dissection.

Rats with cannulae that were not positioned in the ARH (i.e., located above, lateral to the ARH, or where microinjections had compromised the wall of the third ventricle) were excluded from the study.

*Immunohistochemistry.* Sections from the medial preoptic nucleus (MPN) were collected as above. Sections were incubated with antibody directed against MOR (1:24,000; Neuromics, Edina, MN) with 0.5% Triton-X, 1% BSA, and 1% NGS in PBS for two nights at 4°C. Sections

were washed before being incubated in biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. The sections were washed in TBS with 0.05% Tween-20 (TNT) before being incubated in streptavidin-horseradish peroxidase (TSA kit; Perkin Elmer, Waltham, MA) for 30 min at room temperature. The sections were washed again in TNT. Finally, the sections were incubated in fluorescein (TSA kit; Perkin Elmer) for 5 min. The sections were washed in TNT and Tris buffer before being mounted onto slides. The slides were allowed to dry on a slide warmer and coverslipped with Aqua Poly/Mount (PolySciences Inc, Warrington, PA)

*MOR Internalization.* To quantify the internalization of MOR, pictures of the MPN were taken using a Zeiss Axioskop 2 equipped with epifluorescent illumination and an Axiocam CCD camera at 360x magnification using Zeiss Axiovision (version 4.8). Pictures were converted to grayscale in Adobe (San Jose, CA) Photoshop and adjusted for brightness and contrast to remove the background staining. To obtain an estimate of relative internalization, optical density was then quantified using Image J (version 1.44p) in the pixel inverter function as has been described previously (Dewing et al., 2007). Briefly, a circle of about 60 μm was imposed on the MPN and a measurement was taken. The circle was then moved to an area of the image outside the MPN to determine the background was taken. The background measurement was subtracted from the MPN measurement to obtain the optical density of MOR staining in the MPN alone. Optical density has been correlated with receptor internalization (Mills et al., 2004; Dewing et al., 2007).

*Western blots.* To verify caveolin-1 knockdown and loss of membrane ERα in the ARH, animals that had been infused with scrambled or CAV1 siRNA were anesthetized 30 minutes after the last EB injection and decapitated. The brain was rinsed in cold PBS, the ARH dissected on ice and put in homogenization buffer containing protease inhibitors (from the Plasma Membrane Protein Extraction Kit; Abcam, Cambridge, MA). The tissue was homogenized using a dounce homogenizer and membrane proteins were extracted according to

the manufacturer's protocol. After extraction, the whole population of membrane proteins was resuspended in RIPA (RadioImmunoPrecipitation Assay) lysis buffer and protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were then heated to 95°C for 5 minutes in Laemmli sample buffer (Bio-Rad, Hercules, CA). Protein was run on a 10% acrylamide gel and transferred to a PVDF membrane overnight. Membranes were blocked in 5% milk in 0.1% TBS-Tween for 1 hr before being transferred to primary antibody against ERα (MC-20; Santa Cruz Biotechnology), made in 5% milk. The membrane was incubated in primary antibody overnight at 4°C. The next day, membranes were washed and incubated in peroxidase conjugated goat anti-rabbit secondary antibody (1:2000; Vector) for 1 hr. To remove the antibodies, membranes were washed again for at least 1 hr and developed using GE Healthcare ECL for 1 min before being exposed to film. To reprobe the membranes, antibodies were stripped (60mM Tris-HCl, 2.4mM Tris, 2% SDS, 0.7% β-mercaptoethanol) at 55° for 5-7 minutes. Membranes were rinsed in dd$H_2O$ (500 mL), incubated in 5% milk for 1 hr, and placed in primary antibody against CAV1 (1:1000; BD Biosciences, San Jose, CA) with 5% milk overnight. A horse anti-mouse secondary antibody (1:2000; Vector) was used and visualized as above. Finally, membranes were stripped, as before, and incubated in 5% milk with primary antibody directed against flotillin-1 (1:1000; Abcam) and visualized as described. Flotillin was used both as a loading and experimental control for both because it is a membrane protein found in lipid rafts and is not affected by EB administration (Boulware et al., 2007).

*Statistics.* All data are expressed as the mean ± SEM. All data were analyzed by two-tailed t-tests. Differences were considered significant at $P < 0.05$. Statistical analysis was conducted using GraphPad Prism 5 (version 5.02, GraphPad Software, Inc., La Jolla, CA) software. The number of animals used in each experiment is specified in *Results*.

## Results

*Knockdown of Caveolin-1 and Membrane ERα.* Ovx female rats were injected every four days with 5 μg EB and were infused four times with siRNA directed against CAV1 or scrambled siRNA. The protocol was designed to knockdown both endogenous and estradiol-induced levels of CAV1 mRNA and protein. In the ARH, CAV1 siRNA reduced membrane caveolin protein by 64% compared with scrambled siRNA controls (Fig 1B; 100 ± 19.2% vs 36.1 ± 13.0%; P= 0.0237, t=2.878, df= 7; n= 4-5).

Since CAV1 was suspected to be the scaffold protein responsible for trafficking ERα to the cell membrane, we also examined the levels of ERα in the membrane fraction. Indeed, the loss of CAV1 affected the population of membrane ERα proteins, but did not affect intracellular levels (Fig 1A; intracellular ERα: 100 ± 25.2% vs 107.5 ± 4.3%). As previously reported, the cell membrane fraction contained two ERα immunoreactive proteins, the full length 66 kDa ERα and the 52 kDa ERαΔ4 alternatively splices protein (Gorosito et al., 2008; Bondar et al., 2009; Dominguez and Micevych, 2010). Interestingly, unlike in primary cultures of hypothalamic neurons or astrocytes, in this ex vivo experiment, full length ERα was more abundant on the membrane than the ERαΔ4, as demonstrated by the longer exposure times needed to see the 52 kDa splice variant. Although both were decreased, only the reduction in the full length ERα was significant (Fig 1C; mERα: 100 ± 5.9% vs. 53.9 ± 14.1%; P= 0.0209, t= 2.793, df= 9; n= 5-6; ERαΔ4: 100 ± 9.2% vs. 77.1 ± 15.4%)

*Knockdown of Caveolin-1 Affects MOR Internalization and Sexual Receptivity.* A hallmark of estradiol activation of the circuit that regulates sexual receptivity is the activation/internalization of MORs in the MPN (Eckersell et al., 1998; Mills et al., 2004). MOR internalization is a consequence of estradiol membrane signaling (Dewing et al., 2007; Dewing et al., 2008) that activates a microcircuit in the ARH that ultimately stimulates the release of β-endorphin (β-END) into the MPN. β-END causes rapid activation and internalization of MOR in the MPN (Sinchak and Micevych, 2001). To determine if blocking ERα trafficking prevents circuit activation MOR

internalization, animals killed 30 minutes after the final EB injection were immunostained for MOR. Estradiol-induced MOR internalization was observed in scrambled siRNA treated rats, but in CAV1 siRNA treated rats, MOR internalization was reduced (Fig 2; P= 0.0116, t= 3.257, df= 8; n= 4-6).

Transient MOR signaling is required for the full display of female sexual receptivity 30 to 48 hours after EB treatment (Torii et al., 1999; Sinchak and Micevych, 2001). When CAV1 siRNA treated rats were tested for lordosis behavior 30 hours after the final EB injection, sexual receptivity was attenuated (Fig 3; LQ: 93.3 ± 4.4 vs. 30.0 ± 12.3; P= 0.0004, t= 5.745, df= 8; n= 4-6).

## Discussion

The major finding of the present study was that disruption of a proposed mechanism for ERα trafficking resulted in a loss of this receptor on the cell membrane. All of the effects of estradiol membrane signaling were abrogated. The CAV1 knockdown resulted in the loss of MOR activation and attenuation of female sexual receptivity. To our knowledge this was the first study to demonstrate the physiological relevance of CAV1 in maintaining a population of ERα on the cell membrane.

Caveolins may play several roles in neurons including: trafficking receptors, mediating receptor-receptor interactions, and segregating signaling microdomains (reviewed in (Stern and Mermelstein, 2010)). It appears that with regards to ERα, caveolins may be performing all three functions. As shown in this study, caveolins are responsible for the transport of ERα to the membrane, since knockdown of CAV1 resulted in a decrease in the population of membrane ERα. This is not due to a loss of ERα but a deficit of trafficking since intracellular ERα levels were not impacted by CAV1 siRNA treatment. Caveolin proteins mediate ERα association with specific mGluRs on the membrane (Boulware et al., 2007). This association allows the ERα transactivation of mGluR initiating G protein signaling; in this way a receptor that is not a G

protein-coupled receptor induces cell signaling cascades. Finally, caveolins specify which mGluR is associated with ERα leading to facilitative or inhibitory signaling ((Boulware et al., 2007); reviewed in (Micevych and Mermelstein, 2008)). Whether estradiol facilitates or inhibits downstream signaling is dependent on which specific mGluR is associated with ERα. Estradiol binding to ERα transactivates mGluR1a to initiate G protein dependent signaling. In hypothalamic cells this activation of mGluR1a leads to the activation of the MAPK pathway or the release of $IP_3$ receptor sensitive calcium stores. The association with mGluRs is mediated by specific caveolin proteins (Boulware et al., 2007).

In the present experiments, using a circuit in which cell signaling was dependent on ERα-mGluR1a, we confirmed that CAV1 was responsible for establishing the ERα-mGluR1a signaling unit. (Dewing et al., 2007; Dewing et al., 2008; Christensen et al., 2011). Antagonism of mGluR1a blocked estradiol membrane-initiated downstream cell signaling, morphological plasticity and activation of the ARH – MPN circuit. (Boulware et al., 2005; Dewing et al., 2007; Dewing et al., 2008; Kuo et al., 2009; Christensen et al., 2011).

Although the loss of membrane ERα was only ~50%, it significantly impacted the ARH-MPN circuit as demonstrated by both the attenuation of MOR internalization and sexual receptivity. The present results confirm that membrane-initiated estradiol signaling is an important component of estradiol facilitation of lordosis behavior. The loss of the ERα-mGluR1 interaction through the inhibition of mGluR1 resulted in a reduction of estradiol-mediated signaling and ultimately the attenuation of female sexual behavior (Dewing et al., 2007). Here, we found that a reduction of the membrane ERα population results in the same deficits.

Although our experiments were focused on the ARH to MPN circuit and sexual receptivity, estradiol in the ARH affects many other systems. Estradiol-mediated spinogenesis in the ARH is at least partially regulated by the ERα-mGluR1 interaction (Christensen et al., 2011). Another estradiol-sensitive circuit in the ARH regulates energy balance (Gao et al.,

2007). How reducing membrane ERα signaling will affect these and other circuits will require additional experiments.

Surface biotinylation experiments done to identify the location of ERα within the membrane revealed that this receptor has an extracellular portion protruding from the cell membrane (Gorosito et al., 2008; Bondar et al., 2009; Dominguez and Micevych, 2010). In addition, to the full length, 66 kDa ERα another, more abundant immunoreactive protein was identified. This ~52 kDa immunoreactive ERα was observed in primary cultures of neurons and astrocytes. Using primary cultures of embryonic and immortalized hypothalamic neurons (N-38), which express the 52 kDa ERα, we identified an mRNA missing exon 4 of *ESRI* (Dominguez et al., Submitted). This ERαΔ4 mRNA previously had been identified in brain as an abundant alternatively spliced variant (Skipper et al., 1993). In the present ex vivo study, ERαΔ4 was not as abundant as ERα. This difference does not appear to be a developmental phenomenon since greater membrane levels of ERαΔ4 were found in embryonic neurons and adult astrocytes. However, the greater ERα relative abundance may reflect a difference between cells in vitro compared with ex vivo (Bondar et al., 2009; Dominguez and Micevych, 2010). Previous co-immunoprecipitation demonstrated an mGluR1a interaction with the full length, 66 kDa ERα and not the ERαΔ4. While we still do not know its function, ERαΔ4 trafficking and internalization parallels ERα: estradiol transiently increases cell membrane levels and its internalization. Further experiments will be needed to address these questions.

The current study highlighted another distinction between the ERα and ERαΔ4: CAV1 knockdown significantly reduced cell membrane ERα, but not ERαΔ4 levels. There may be several potential explanations. First, CAV1 siRNA was a partial knockdown, reducing CAV1 protein only by half and this may not have been sufficient to affect ERαΔ4 trafficking. Second, CAV1 may not be the scaffold protein responsible for trafficking ERαΔ4. Third, exon 4 codes for the nuclear translocation signal, perhaps ERαΔ4 is not as dependent on CAV1 for trafficking as the ERα. Future experiments will address these possibilities.

In summary, specific CAV1 knockdown in the ARH reduced levels of ERα due to a loss of CAV1-mediated trafficking to the cell membrane. The loss of membrane ERα in vivo resulted in deficits of circuit activation, demonstrated by an attenuation of MOR internalization and loss of lordosis behavior. The present results paralleled attenuating estradiol activation by blocking of mGluR1a in the ARH, suggesting an important role for CAV1 in maintaining the ERα-mGluR1a complex. CAV1 knockdown did not alter intracellular levels of ERα further strengthening the case for membrane-initiated estradiol signaling in mediating sexual receptivity, a vital physiological function.

Figure Legends

**Fig 1. siRNA reduced CAV1 and ERα on the membrane. (A)** Western blots illustrating the loss of CAV1 and ERα in ARH membrane fractions after CAV1 siRNA Flotillin, an integral membrane protein, was not affected by CAV1 knockdown and was used to normalize the results. The ERα splice variant, ERαΔ4, which is only found on the membrane, and intracellular ERα levels were unaffected by CAV1 knockdown. Intracellular ERα was normalized to GAPDH. **(B)** CAV1 siRNA reduced CAV1 by ~64% and **(C)** ERα by ~50% compared with scrambled controls. * = $P < 0.05$ vs. scrambled siRNA (n = 4-6)

**Fig 2. Knockdown of CAV1 attenuated MOR internalization.** CAV1 siRNA microinfused into the ARH reduced MOR activation/internalization in the MPN, a measure of lordosis circuit activation which requires estradiol membrane signaling. * = $P < 0.05$ vs. scrambled siRNA (n = 4-6)

**Fig 3. Sexual receptivity is diminished when CAV1 is knocked down**. Lordosis quotients (LQ) of female rats tested with stimulus males 30 hours after their final EB injection. Control females, treated with scrambled siRNA, were fully sexually receptive. Sexual receptivity of those treated with CAV1 siRNA was significantly diminished. * = $P < 0.05$ vs. scrambled siRNA (n = 4-6)

Figure 1

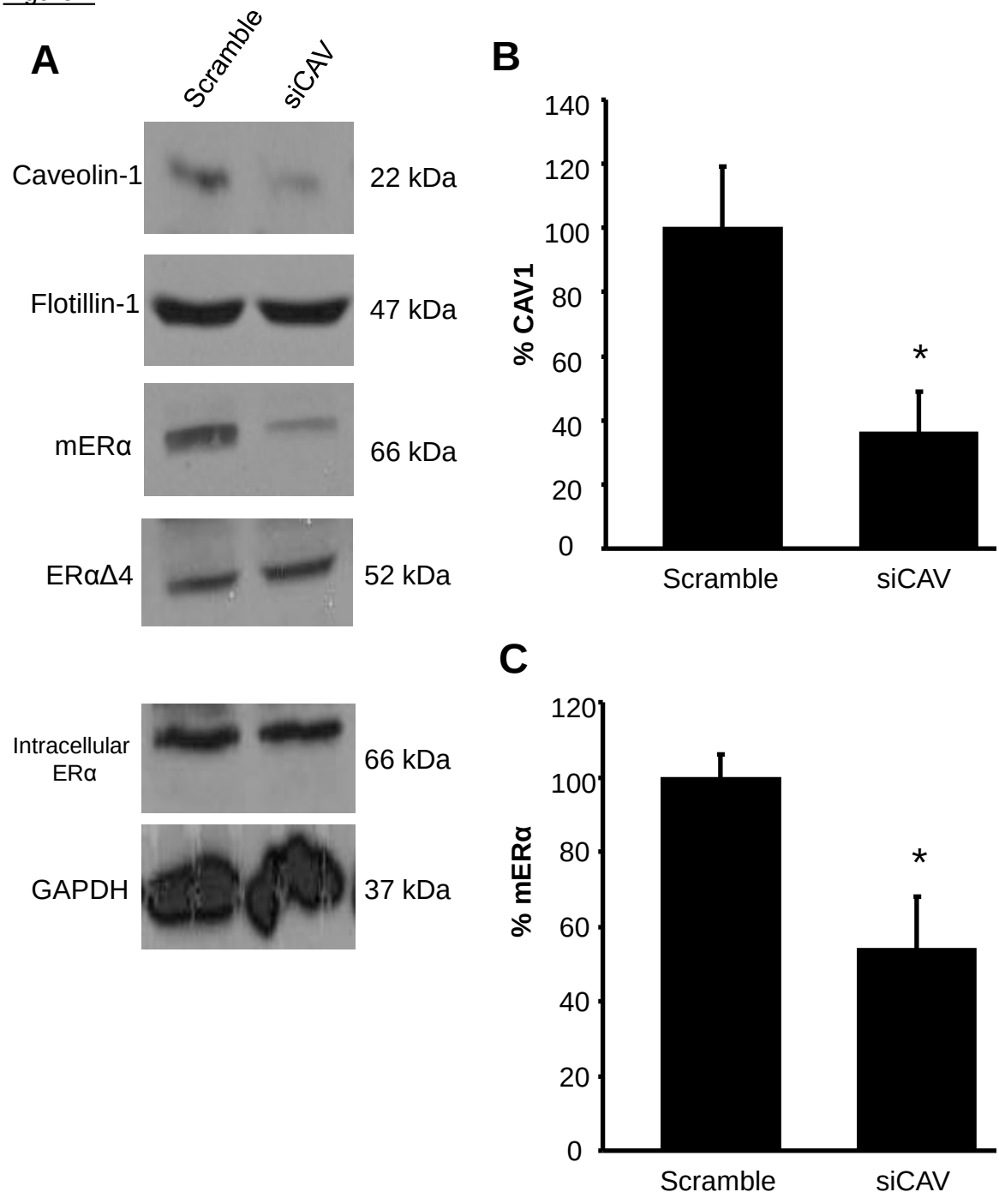
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Figure 2

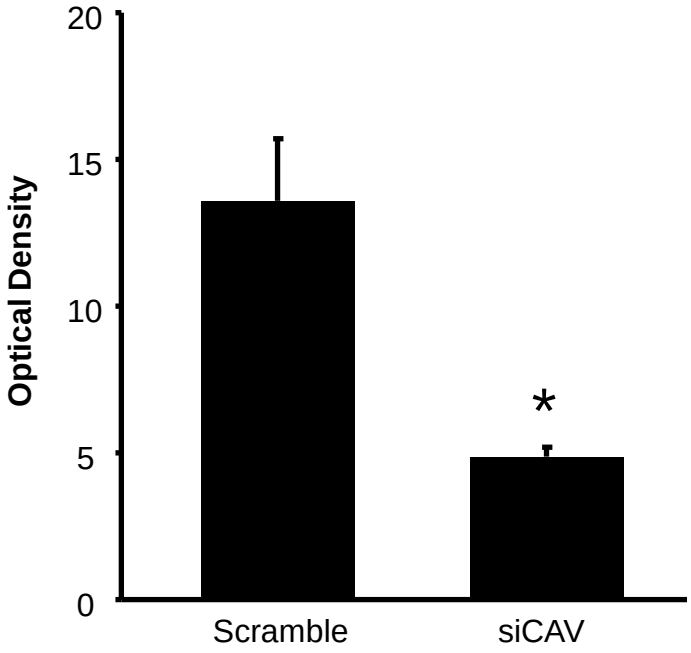
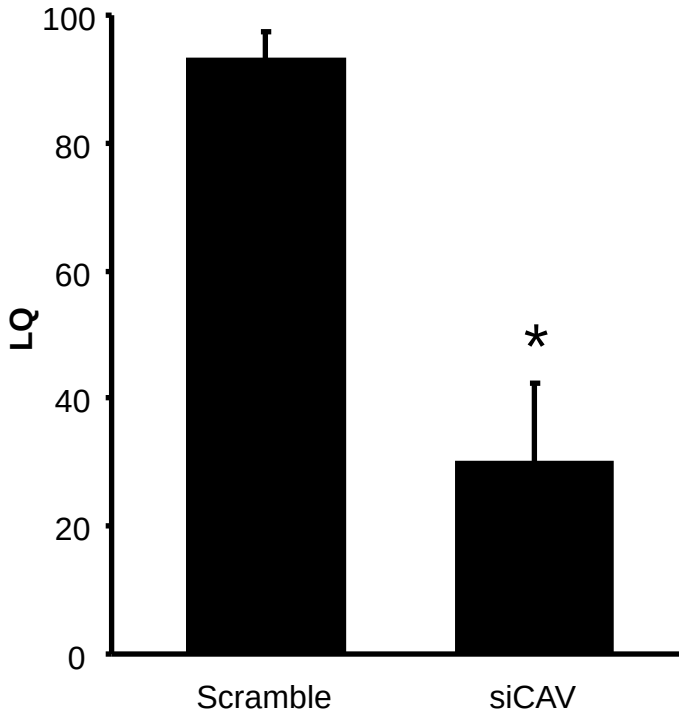


Figure 3



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Behavioral/Systems/Cognitive

# Membrane-Initiated Estradiol Signaling Induces Spinogenesis Required for Female Sexual Receptivity

**Amy Christensen, Phoebe Dewing, and Paul Micevych**

Department of Neurobiology and Laboratory of Neuroendocrinology of the Brain Research Institute, David Geffen School of Medicine at UCLA, Los Angeles, California 90095

**Estrogens have profound actions on the structure of the nervous system during development and in adulthood. One of the signature actions of estradiol is to alter the morphology of neural processes. In the hippocampus, estradiol modulates spines and cellular excitability that affect cognitive behaviors. In the hypothalamus, estradiol increases spine density in mediobasal hypothalamic nuclei that regulate reproduction. The hypothalamic arcuate nucleus (ARH), an important site for modulation of female sexual receptivity, has a sexual dimorphism in dendritic spine density that favors females. In the present study, we used both β-actin immunostaining and Golgi staining to visualize estradiol-induced changes in spine density in Long–Evans rats. Golgi impregnation was used to visualize spine shape, and then β-actin immunoreactivity was used as a semiquantitative measure of spine plasticity since actin forms the core of dendritic spines. At 4 h after estradiol treatment, both β-actin immunofluorescence and filopodial spines were increased (from 70.57 ± 1.09% to 78.01 ± 1.05%, $p < 0.05$). Disruption of estradiol-induced β-actin polymerization with cytochalasin D attenuated lordosis behavior, indicating the importance of estradiol-mediated spinogenesis for female sexual receptivity (81.43 ± 7.05 to 35.00 ± 11.76, $p < 0.05$). Deactivation of cofilin, an actin depolymerizing factor is required for spinogenesis. Membrane-initiated estradiol signaling involving the metabotropic glutamate receptor 1a was responsible for the phosphorylation and thereby deactivation of cofilin. These data demonstrate that estradiol-induced spinogenesis in the ARH is an important cellular mechanism for the regulation of female sexual behavior.**

## Introduction

Estradiol regulates CNS functions ranging from reproduction to energy balance to cognition. Underlying these actions, estradiol has the ability to regulate plasticity by affecting dendritic structure, particularly spinogenesis. Although the actions of estradiol on dendritic spines were initially described in the hypothalamus, they have been extensively characterized in the hippocampus (Matsumoto and Arai, 1981; Frankfurt et al., 1990; Gould et al., 1990; Woolley and McEwen, 1992; Calizo and Flanagan-Cato, 2000). In regions associated with the regulation of reproduction in females, such as the ventromedial nucleus (VMH) and the arcuate nucleus (ARH) of the hypothalamus, estradiol increases spine density, suggesting a relationship between these events that has never been tested (Matsumoto and Arai, 1981; Garcia-Segura et al., 1986; Calizo and Flanagan-Cato, 2000). In the ARH, the increase of spines was assumed to mediate estrogen positive feedback, which regulates luteinizing hormone release and ovulation (Matsumoto and Arai, 1981; Parducz et al., 2002, 2006; Csakvari et al., 2007, 2008; Naftolin et al., 2007). Additionally, the ARH is also important for estradiol induction of female sexual receptivity (Mills et al., 2004; Dewing et al., 2007, 2008), actions mediated by membrane-initiated estradiol signaling (MIES), in which estrogen receptor-α (ERα) transactivates a group 1 metabotropic glutamate receptor (mGluR1a) (Dewing et al., 2007, 2008). These events activate an opioid circuit mediated by β-endorphin (β-END) that inhibits μ-opioid receptor (MOR)-expressing neurons in the medial preoptic nucleus (MPN), which project to the VMH. Within minutes of estradiol treatment, this circuit produces a transient inhibition needed to elicit the full display of lordosis behavior 48 h later (Sinchak and Micevych, 2001; Mills et al., 2004).

The present studies test the hypothesis that estradiol induction of spinogenesis in the medial basal hypothalamus (MBH) is necessary for lordosis behavior. Actin remodeling underlies the establishment and maturation of dendritic spines, and β-actin-immunoreactive levels are a measure of dendritic spine density (Matus et al., 1982; Kaech et al., 1997). To begin analyzing the mechanism of estradiol action, phosphorylated cofilin was measured (Schubert et al., 2006; Carlisle et al., 2008). Cofilin, an F-actin severing protein, is inactivated by phosphorylation. Estradiol increased both phosphorylated cofilin and spine density. To test whether p-cofilin was regulated by MIES, mGluR1a was blocked, preventing spinogenesis. Visualization of spines with Golgi impregnation revealed that the majority of spines formed within the first 4 h after estradiol treatment had an immature, filopodial morphology. After the initial increase, the total number of spines did not increase for 48 h, but after 20 h, there was an increase in the number of functional, mushroom-shaped spines (Kasai et al., 2003). Finally, a behavioral study was used to deter-

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Correspondence should be addressed to Paul Micevych, Department of Neurobiology, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095-1763. E-mail: pmicevych@mednet.ucla.edu.

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mine whether blocking spinogenesis in the MBH prevented lordosis behavior. Cytochalasin D (CD) blocked estradiol-induced actin polymerization, which significantly attenuated lordosis behavior. Together these results provide strong evidence that MIES regulates spinogenesis, which is critical for the induction of sexual receptivity in the female rat.

## Materials and Methods

*Animals.* Male and ovariectomized (ovx; by the supplier) female (200–250 g) Long–Evans rats were purchased (Charles River). Upon arrival, rats were housed in a climate-controlled room, two per cage in a 12 h light:12 h dark cycle room (lights on at 6:00 A.M.) and provided food and water *ad libitum*. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

*Steroid priming.* Animals were allowed to survive 2–3 weeks after ovx before steroid treatment. For all experiments, 17β-estradiol benzoate (EB) dissolved in safflower oil was injected subcutaneously in a total volume of 0.1 ml per rat. For determination of spine density and morphology, ovx rats injected with either 50 μg of EB or oil vehicle were perfused at the appropriate survival time (4, 20, 30, or 48 h after injection). For cofilin immunostaining, animals were perfused 1 h after a single injection of 5 μg of EB. Females that were treated with cytochalasin D or behaviorally tested received 5 μg of EB every 4 d between 9:00 and 10:00 A.M. for three cycles to mimic the natural estrous cycle of female rats (Micevych et al., 1994). A large 50 μg bolus of EB was used initially to ascertain the effects a single dose of estrogen had in the ARH. Later experiments use a smaller dose to be more physiologically relevant.

*Guide cannula implantation surgery.* Bilateral guide cannulae (22 gauge; Plastics One) directed at the ARH (coordinates from bregma: anterior −2.0 mm, lateral 0.8 mm, ventral −6.9 mm from dura; tooth bar: −3.3 mm) or single guide cannulae directed at the lateral ventricle (coordinates from bregma: anterior −1.0 mm, lateral ±1.4 mm, ventral −3.5 mm from dura; tooth bar: −3.3 mm) were implanted using standard stereotaxic procedures while female rats were anesthetized with isoflurane (2–3% in equal parts oxygen and nitrous oxide). Cannulae were secured to the skull with dental acrylic and stainless steel bone screws. Stylets were placed in the guide cannulae, which protruded <0.5 mm beyond the opening of the guide cannulae. Animals were individually housed after surgery, received oral antibiotics (trimethoprim and sulfamethoxazole, 0.4 mg/ml; Hi-Tech Pharmacal) in the drinking water, and allowed to recover 7 d before behavioral testing or infusion.

*Microinjection.* For ARH site-specific microinjections 50 nmol of the β-actin polymerization inhibitor, CD (Enzo Life Sciences) was dissolved in DMSO. For infusions into the lateral ventricle, 400 nmol of CD was used. mGluR1a antagonist LY367,385 (400 nmol) was dissolved in 10 mM NaOH and aCSF (1:1, pH 7.8) and was infused into the lateral ventricle. Microinjections/infusions were performed with an infusion pump (Harvard Apparatus) at a rate of 0.5–1.0 μl/min. Microinjection needles (28 gauge) protruded 2 mm beyond the opening of the cannula and were allowed to remain in place for 1 min after infusion to allow for diffusion of drug treatment or control vehicle from the injector. After microinjection, the obturators were reinserted into the guide cannulae and animals returned to their home cage until testing.

*Behavioral testing.* Animals received two cycles of EB (5 μg) injections. Thirty minutes before the third EB injection, β-actin polymerization in the ARH was blocked by site-specific injections of CD. Control animals were infused with aCSF rather than CD. Lordosis behavior was tested 30 h after the last injection EB. Sexual receptivity was measured by placing each female rat in a Plexiglas testing arena with a stud male. Sexually experienced males were acclimatized to the arenas for at least 15 min before testing. Males were allowed to mount females 10 times and the number of times the female displayed lordosis (lifting of the head, arching of the back, movement of the tail to one side) was recorded. For each female, the sexual receptivity was quantified as a lordosis quotient (LQ), the number of lordosis displays/number of mounts × 100.

*Confirmation of guide cannulae placement.* Animals were anesthetized after the series of behavioral experiments and transcardially perfused with chilled 0.9% saline, followed with a fixative, 4% paraformaldehyde dissolved in 0.2 M Sorenson's phosphate buffer, pH 7.4. Brains were removed and placed in fixative overnight at 4°C and then replaced with 20% sucrose in phosphate buffer for cryoprotection. Brains were blocked, sectioned (20 μm) on a cryostat (Leica CM 1800), and collected into chambers filled with PBS. Sections were mounted onto SuperFrost/Plus slides (Fisher), stained with thionine, dehydrated, and coverslipped with Krystalon (EMD Chemicals). Injection sites were mapped and verified with bright-field illumination. Rats with cannulae that were not positioned in the ARH (i.e., located above, lateral to the ARH, or where microinjections had compromised the wall of the third ventricle) were excluded from the study.

*Golgi staining.* Animals were perfused, as described above, 4, 20, 30, and 48 h after a 50 μg EB injection and immediately processed for Golgi staining using the FD Rapid Golgi Stain Kit (FD Neurotechnologies) according to the manufacturer's protocol. After staining, brains were sectioned at 120 μm and direct mounted on gelatin-coated SuperFrost/Plus slides. The sections were then stained, dehydrated, and coverslipped using Permount (Fisher).

*Golgi image analysis.* In both analyses, only neurons whose cell bodies could be visualized within the ARH and whose dendrites were unobstructed along their entire visible length were chosen for analysis. Spines were morphologically characterized under 630× magnification (Axioskop 2; Zeiss) and classified as filopodial, stubby, mushroom, or cup shaped (Hering and Sheng, 2001; Bourne and Harris, 2008). Filopodial spines were defined as thin protrusions with a uniform diameter (~1–4 μm long). Stubby spines were thicker and shorter (~1–4 μm long with a head of ~0.5–1 μm diameter). Spines with a thin neck that ended in a bulbous head were classified as mushroom shaped (~1–2 μm long and 1–2 μm wide), and those with a U-shaped head were classified as cup shaped. Percentages were calculated by totaling the number of each shape and dividing by the total number of spines counted and multiplying by 100.

To determine spine density, Golgi-stained sections were analyzed using a Zeiss Axioskop 2 with at a magnification of 397× equipped with Neurolucida program (MBF Bioscience). The cell body and full length of all dendrites in a section were traced. Spines were placed where they were visualized along the dendrite. Density was normalized as the number of spines per 10 μm of dendrite length.

*Immunohistochemistry.* For β-actin staining, animals were perfused 4 h after estradiol injection and immediately processed for immunohistochemistry. Rabbit primary antibody directed against β-actin (1:1000; Abcam) was used. Sections processed for fluorescence were incubated in blocking buffer (tyramide signal amplification kit; NEN Life Science Products) and then in biotin-conjugated goat anti-rabbit IgG (Vector Laboratories; 1:200) for 1 h. Tissue was then washed in Tris-buffered saline and incubated in streptavidin-horseradish peroxidase (NEN Life Science Products; 1:100) for 30 min, washed, and incubated for 5 min in fluorescein-conjugated tyramide (1:50; tyramide signal amplification kit; NEN Life Science Products). Sections were washed again in 0.1 M Tris buffer and mounted on SuperFrost/Plus slides. Mounted sections were air dried and coverslipped using Aqua Polymount mounting medium (Polysciences).

For p-cofilin staining, animals were perfused 1 h after injection and processed for immunohistochemistry. Sections were incubated overnight with an antibody directed against p-cofilin (1:250, Cell Signaling Technology). Immunoreactivity was visualized with diaminobenzidine (DAB) histochemistry kit (VECTASTAIN Elite ABC kit; Vector Laboratories). Sections from control and estradiol-treated rats were incubated in parallel to reduce variability of staining. Mounted sections were air dried and dehydrated before being coverslipped with Krystalon.

*Image analysis.* All fluorescent sections were examined using a Zeiss Axioskop 2 equipped with epifluorescent illumination, Axiocam digital camera, and AxioVision digital image analysis system (Carl Zeiss North America). Fluorescein isothiocyanate was imaged with a 488 nm excitation and 550 nm emission filter. Images were adjusted for brightness and contrast using the Zeiss LSM-PC and PhotoShop (version 6.0; Adobe) programs. DAB sections were viewed with bright-field illumination. Pic-

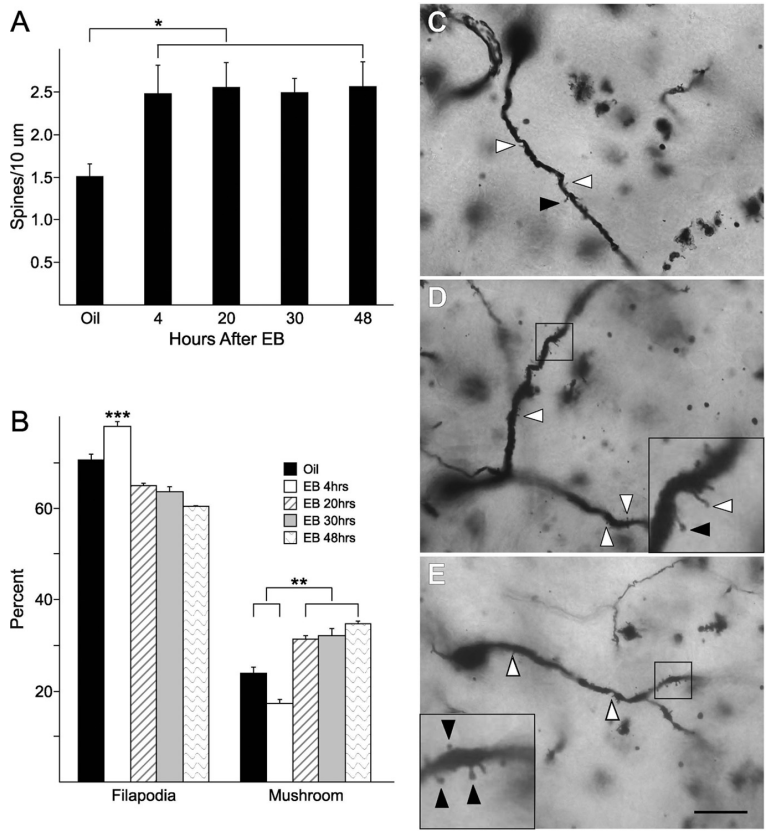


**Figure 1.** Estradiol increases spine density and maturity. ***A***, Ovx animals treated for 4 h with EB show an increased spine density compared to oil-treated controls. At later time points the density remains elevated ($n = 4–6$). ***B***, Four hours of EB treatment significantly increases the number of spines with a filopodial appearance in the ARH compared to oil-treated animals, as well as all other EB time points. Spine morphology matured at later time points after estradiol treatment (i.e., 20, 30, and 48 h). Significantly more mushroom spines were present at time points later than 4 h or in the oil-treated controls ($n = 4–6$). ***C–E***, An oil-treated animal (***C***), an animal treated with EB for 4 h (***D***), and an animal treated with EB for 48 h (***E***). White-filled arrowheads indicate filopodial spines, and black arrowheads indicate mushroom-shaped spines. Insets show close ups of filopodial (in ***D***) and mushroom (in ***E***) shaped spines. Since cup-shaped and stubby spines accounted for <5% of the population of spines in the ARH, they were not included in the figure. Scale bar measures 20 $\mu$m. Error bars represent the SEM. *$p < 0.05$. **$p < 0.001$. ***$p < 0.0001$.

tures were taken using the Axiocam digital camera in grayscale using the same exposure time for all sections without further adjustment.

To determine a relative measurement of immunoreactivity, fluorescent images (×360) of the regions under study were converted to grayscale and adjusted for brightness and contrast in Adobe Photoshop. ImageJ (version 1.32j; NIH) was set to the Pixel Inverter function and calibrated. For calibration, a negative region was measured on each section. For $\beta$-actin immunostaining, an area outside the ARH and for p-cofilin the internal capsule was used as the negative control because this region did not respond to estradiol and was found in all sections in which the ARH was present. The p-cofilin optical density (OD) in the internal capsule was found to be similar in all treatment groups (A. Christensen, unpublished observation). For $\beta$-actin and p-cofilin immunostaining in the ARH, the OD within a 75 $\mu$m circle was measured. The OD measurement from the negative control areas was subtracted to determine the OD of the staining in the ARH.

*Western blot.* N-38 cells were cultured in high-glucose DMEM (Invitrogen) containing 10% FBS, 0.15% $NaHCO_3$, and 1% penicillin/streptomycin. Cells were harvested and lysed by pulling through a syringe in RIPA lysis buffer system, which included protease inhibitors (Santa Cruz Biotechnology) supplemented with a protein phosphatase inhibitor set (Millipore). The cells were then centrifuged and the pellet was discarded. Twenty micrograms of protein was loaded onto a 15% polyacrylamide gel and run for 1 h at 120 V. The protein was transferred overnight onto a PVDF membrane. The membrane was blocked for 1 h with 5% milk, then incubated overnight in primary antibody against p-cofilin (1:500) in 5% milk. The membrane was washed in 0.1% TBS-Tween and incubated with peroxidase anti-rabbit secondary for 1 h. The membrane was washed for at least 1 h in TBS-Tween before being rinsed in TBS. It was covered in GE Healthcare ECL for 1 min before being exposed to film for 30 s.

*Statistical analysis.* All data are expressed as the mean ± SEM. Mean differences between groups in behavior and p-cofilin data were determined using two-way ANOVAs followed by Bonferroni *post hoc* analysis in which the main effect or interaction was significant at $p < 0.05$. Golgi and $\beta$-actin data were analyzed by two-tailed *t* tests and one-way ANOVAs followed by Newman–Keuls *post hoc* analysis in which the main effect or interaction was significant at $p < 0.05$. Differences were considered significant at $p < 0.05$. Statistical analysis was conducted using GraphPad Prism 5 (version 5.02, GraphPad Software) software. The number of animals used in each experiment is specified in Results.

## Results

### Estradiol induces spinogenesis in the ARH

Golgi staining indicated that EB treatment significantly increased the density of spines on neurons in the ARH at all time points examined when compared to control levels (Fig. 1; two-tailed *t* test, oil vs 4 h EB, $p = 0.05$; $t = 2.284$; df = 8; oil vs 20 h, $p = 0.02$; $t = 3.120$; df = 6; oil vs 30 h, $p = 0.003$; $t = 4.335$; df = 7; oil vs 48 h EB, $p = 0.02$; $t = 3.210$; df = 6; $n = 4–6$ animals per group). After the initial increase at 4 h, the density of dendritic spines did not change at any of the time points examined. On the other hand, the morphology of the spines did change. Spine morphology has been correlated with maturation (Matsuzaki et al., 2001; Kasai et al., 2003; Smith et al., 2003). Filopodial spines are considered more immature than stubby and mushroom-shaped spines and not as likely to be contacted by a presynaptic element (Dailey and Smith, 1996; Ziv and Smith, 1996). At 4 h, a significant increase in the percentage of filopodial spines was associated with a significant decrease in the percentage of mushroom spines compared with controls and with later time points (Fig. 1; one-way ANOVA, $p < 0.0001$; $F = 53.23$; df = 4; SNK comparison test, 20 h EB vs 4 h EB, $p < 0.0001$; $q = 13.64$; 30 h EB vs 4 h EB, $p < 0.0001$; $q = 15.95$; 48 h EB vs 4 h EB, $p < 0.0001$; $q = 18.28$). At 20+ h after estradiol treatment, the number of mature mushroom-shaped spines significantly increased (one-way ANOVA, $p < 0.0001$; $F = 40.85$; df = 4; SNK comparison test, oil vs 4 h EB, $p < 0.0001$; $q = 5.992$; 20 h EB vs 4 h EB, $p < 0.0001$; $q = 12.31$; 30 h EB vs 4 h EB, $p < 0.0001$; $q = 13.88$; 48 h EB vs 4 h EB, $p < 0.0001$; $q = 15.52$). The number of stubby and cup-shaped

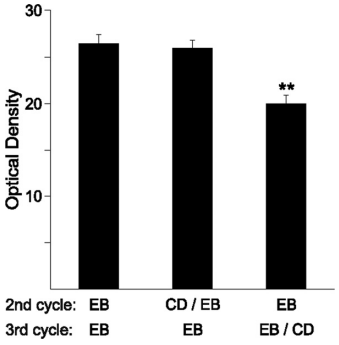


**Figure 2.** CD inhibits β-actin polymerization. Each female was given three cycles of 5 μg of EB and perfused 4 h after their final dose. Before either the second or third cycle, some animals received a 400 nmol infusion of the β-actin polymerization inhibitor CD into the lateral ventricle. Animals given CD on the second cycle were able to recover the ability to make new spines by the third cycle and showed an increase in spinogenesis equal to those that had received three doses of EB only. In contrast, CD given on the third cycle, before the final dose of EB had significantly reduced levels of β-actin optical density indicating decreased spinogenesis ($n$ = 5–7). Error bars represent the SEM. $^{**}p < 0.001$.

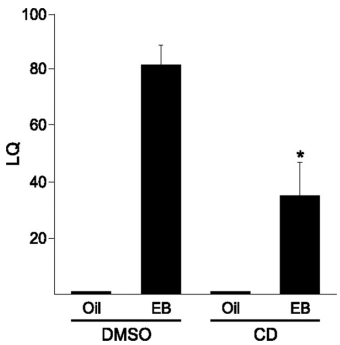


**Figure 3.** Spinogenesis is important for female sexual behavior. Ovx animals were given 5 μg of EB every 4 d for three cycles. Thirty minutes before the final estradiol treatment, females had 50 nmol of CD microinfused into the ARH. Thirty hours later, they were tested for sexual receptivity. Those that were given CD and EB had a significantly reduced LQ compared to DMSO + EB controls ($n$ = 4–7). Error bars represent the SEM. $^{*}p < 0.01$.

spines observed was very low and no difference in the numbers of stubby or cup-shaped spines was measured throughout the 48 h time course of the experiment.

### CD inhibition of β-actin polymerization decreased spinogenesis

In the gonadally intact female rat, estradiol peaks every 4 d as part of the estrous cycle. Our paradigm of treating animals every 4 d with EB mimics this pattern. To determine whether the EB is inducing new ARH spines during every cycle, CD, a blocker of β-actin polymerization, was infused into the lateral ventricle to avoid damaging the arcuate nucleus. The bulk of neuronal β-actin is located in dendritic spines, where it is the major cytoskeletal component supporting dendritic spines (Hotulainen and Hoogenraad, 2010). Thus, immunohistochemical staining for β-actin was used as a semiquantitative indicator of spine density (Matus et al., 1982; Kaech et al., 1997). In our cyclic paradigm, CD was infused 1 h before the second EB injection or 1 h before the third EB injection. Inhibiting β-actin polymerization with CD during the second cycle did not decrease β-actin density when the rats were treated for a third cycle compared to control animals (Fig. 2). However, infusing CD before the third EB injection decreased the amount of β-actin density, suggesting that during the estrous cycle, EB can induce new spines every 4 d (Fig. 2; one-way ANOVA, $p < 0.0001$; $F = 15.55$; SNK comparison test, EB vs third cycle CD, $p < 0.0001$; $q = 6.97$; EB vs second cycle CD, $p > 0.05$; $q = 0.494$; second cycle CD vs third cycle CD, $p < 0.0001$; $q = 6.79$, $n$ = 5–6 animals per group). Moreover, the data support the idea that CD inhibited spine growth acutely without toxic effects to the neurons, and that dendrites retain the ability to continue to make spines with future treatments of EB.

### Spinogenesis in the MBH is required for female sexual receptivity

To determine the behavioral consequence of estradiol-induced spinogenesis in the MBH, we tested the lordosis reflex in ovx rats treated with estradiol for 3 cycles. Rats treated this way are maximally receptive (Dewing et al., 2007). Thirty minutes before the third injection, CD or DMSO vehicle was microinfused directly into the ARH. Animals were tested 30 h later. Animals primed with EB and treated with the DMSO vehicle were maximally receptive. Those that received CD microinfusions, however, had significantly attenuated LQs (Fig. 3; two-way ANOVA, drug, $p = 0.0052$; df = 1; $F = 9.843$; steroid, $p < 0.001$; df = 1; $F = 61.90$; drug × steroid, $p = 0.0052$; df = 1; $F = 9.843$). CD infusions without EB treatment had no affect on the receptivity of the animals. These data suggest that estradiol-induced spinogenesis is a necessary component of the neural mechanism controlling female sexual receptivity.

### MIES regulates cofilin phosphorylation

Dendritic spine development and morphology is dependent on regulating the actin cytoskeleton (Matus, 2000) (for review, see Hotulainen and Hoogenraad, 2010). An important actin regulating protein is cofilin. Cofilin disassembles filamentous actin and is inhibited by phosphorylation. In order for new spines to form, cofilin must be deactivated through phosphorylation (Bamburg, 1999; Meng et al., 2002). When ovx females were injected with estradiol, p-cofilin immunoreactivity increased in the ARH compared with oil-injected controls (Fig. 4; two-tailed $t$ test, $p = 0.01$; $t = 3.113$; df = 9; $n$ = 5–6 animals per group). In addition to an increase in OD, there was also an increase in the number of large cells in estradiol-treated animals. These cells were located generally in the ventral and lateral portion of the ARH. The neurons were consistent in both size and location with β-endorphin-positive cells.

To determine whether MIES regulated spinogenesis was part of the mechanism of estradiol-induced lordosis, the interaction of MIES with deactivation of cofilin was examined. Previous work has demonstrated that blockade of mGluR1a with LY367,385 prevented MIES in the ARH and blocked the estradiol-induced lordosis behavior (Dewing et al., 2007). Here, LY367,385 infused 1 h before injection with EB significantly reduced estradiol-induced p-cofilin levels in the ARH (Fig. 4; two-way ANOVA, drug, $p = 0.136$; df = 1; $F = 2.41$; steroid, $p = 0.0403$; df = 1; $F = 4.81$; drug × steroid, $p = 0.0075$; df = 1; $F = 8.84$; $n$ = 5–7 animals per group), suggesting that MIES regulated spinogenesis through a modulation of cofilin activity.

## Discussion

The major finding of the present study was that MIES-mediated spinogenesis in the ARH was necessary for the induction of sexual receptivity. Using a well established cyclical administration of estradiol, once every 4 d to mimic changing estradiol levels dur-

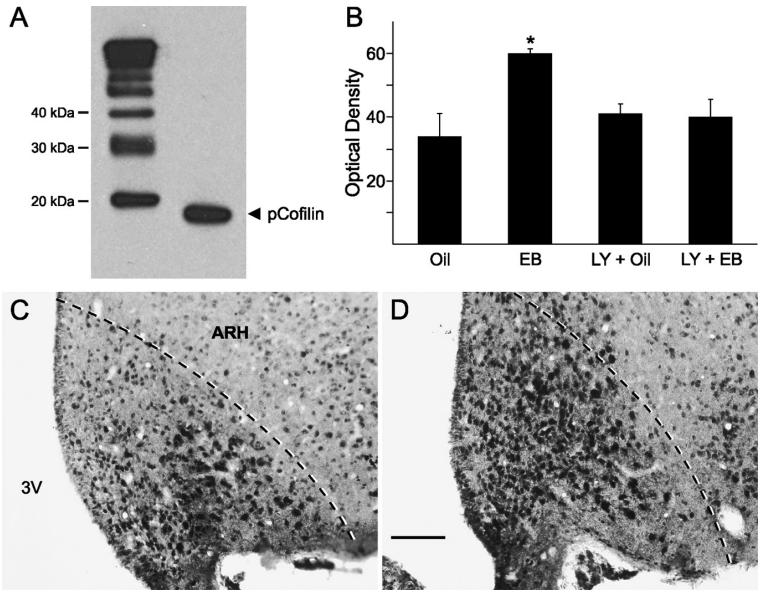


**Figure 4.** Cofilin is deactivated by estrogen receptor interaction with mGluR1a. ***A***, A Western blot showing a single band for p-cofilin migrating at an apparent molecular weight of ~19 kDa. ***B***, Ovx animals were injected with 5 $\mu$g of EB or oil and perfused 1 h later. Animals that received the mGluR1 antagonist, LY367,385, were infused 1 h before injection. ***C***, ***D***, There is significantly less p-cofilin in the oil- (***C***) than in the EB- (***D***) treated animals. This increase was blocked by inhibition of mGluR1, suggesting that the interaction between ER$\alpha$ and mGluR1 is important for the initiation of spinogenesis ($n = 5$–7). The ARH is outlined by the dotted line. Scale bar measures 100 $\mu$m. Error bars represent the SEM. *$p < 0.01$. 3V, Third ventricle.

ing the estrous cycle, we observed that estradiol induced new spines every cycle, which is necessary for estradiol induction of lordosis behavior. The initial action of estradiol was to increase the percentage of spines with filopodial morphology. With increasing time after estradiol priming, the percentage of filopodial spines decreased and mushroom shaped spines increased. This shift in morphology suggested that a population of immature filopodial spines matured to mushroom shapes. This is the natural development of spines, which begin as a filopodial extension of a dendrite. If a synaptic partner is found, receptors will be recruited into the spine membrane as well as scaffold proteins that anchor these receptors at the postsynaptic specialization (Matsuzaki et al., 2001; Holtmaat et al., 2006; Knott et al., 2006; Yoshihara et al., 2009). As all of these new proteins are trafficked into the spine, the spine morphology changes—the tip becomes wider with a growing postsynaptic density. Thus, stubby and mushroom spines are considered to be functional, i.e., forming a synapse that processes incoming synaptic signals. Indeed, the present results are consistent with a model in which estradiol treatment induces spinogenesis in a relatively rapid manner, but the appearance of mature spines requires more time and coincides with the period of female sexual receptivity, which can be induced some 20 h after estradiol priming and is readily apparent 30–48 h after estradiol.

There is increasing evidence that estradiol activates cell signaling through transactivation of mGluRs. In the ARH, ER$\alpha$ at the plasma membrane interacts directly with mGluR1a to activate a G-protein signaling cascade that leads to the phosphorylation and activation of the novel protein kinase, PKC$\theta$ (Dewing et al., 2007, 2008). This interaction between ER$\alpha$ and mGluR1a has been shown to be important for the control of both female sexual receptivity and the release of neuroprogesterone from astrocytes in the hypothalamus (for review, see Micevych and Mermelstein, 2008; Micevych et al., 2009). In fact, recent work has shown that ERs may be able to interact with several different kinds of mGluRs in brain-region-specific patterns to activate many G-protein-coupled signaling cascades (Meitzen and Mermelstein, 2011).

Several factors point to MIES as the mechanism through which estradiol induced spinogenesis. These results are congruent with our previous work that demonstrated that MIES is an important component of ARH regulation of lordosis behavior (Dewing et al., 2007, 2008). First, estradiol was able to rapidly increase spinogenesis within the ARH. Only 4 h were needed to observe a significant increase in spine density. It is possible that an increase could have been seen even earlier, but we wanted to focus on time points when lordosis is expected (30–48 h) and chose only one time point during the refractory period, before sexual receptivity is expressed (i.e., 4 h). This idea of rapid estradiol action involved in spinogenesis is supported by the observation that estradiol induced p-cofilin within 1 h. Since the formation of spines is dependent on polymerization of G-actin, the primary structural component of dendritic spines, the deactivation of an actin depolymerizing factor is critical for spinogenesis. Second, the phosphorylation of cofilin is attenuated by mGluR1a antagonism. Thus, the mechanism through which estradiol is acting involves the mER$\alpha$-mGluR1a complex and cell signaling that potentially activates LIM kinase-1, which in turn phosphorylates cofilin, deactivating it and allowing the establishment of new spines (Bamburg, 1999; Meng et al., 2002). Such regulation of the actin cytoskeleton provides a mechanism through which estradiol can induce the formation of filopodial dendritic spines, as observed from our studies in the ARH. Moreover, p-cofilin has been associated with the stabilization of long-term potentiation in the hippocampus synapses through the expansion of synaptic contacts (Fedulov et al., 2007). Therefore, the deactivation of cofilin provides a mechanism to explain the generation and maturation of dendritic spines associated with lordosis behavior, because it may act during both the early increase in spine density and the later maturation of spines. We did not look at later time points to see whether it was still phosphorylated.

Estradiol-stimulated spinogenesis has been well documented in multiple brain areas (Matsumoto and Arai, 1981; Frankfurt et al., 1990; Gould et al., 1990; Woolley and McEwen, 1992; Calizo and Flanagan-Cato, 2000). The increase in spine density in the hippocampus has been shown to affect hippocampus-dependent working memory (Daniel and Dohanich, 2001; Sandstrom and Williams, 2001, 2004). In the ventral striatum, estradiol decreased the spine density in the core of the nucleus accumbens (Matsumoto and Arai, 1981; Frankfurt et al., 1990; Gould et al., 1990; Woolley and McEwen, 1992; Calizo and Flanagan-Cato, 2000). In addition, estradiol was seen to shift the population of spines from a more mature to less mature morphology, suggesting a decrease in synaptic excitability. This outcome is diametri-

cally opposed to our results but suggests that MIES in the striatum is inhibitory. In the ARH, membrane ER interacts with mGluR1a and activates stimulatory cell signaling. In the striatum, on the other hand, MIES involves the mGluR3, which is negatively coupled to adenylyl cyclase and inhibits L-type voltage-gated calcium channels (Mermelstein et al., 1996; Grove-Strawser et al., 2010). Thus, depending on the mGluR transactivated by membrane ER, MIES can either stimulate or inhibit spinogenesis.

Estradiol-regulated spinogenesis in the ARH is a well established effect, originally observed by Matsumoto and Arai (1979). However, this regulation of spine density had not been correlated with behavior. Estradiol induction of ARH spines has been proposed as a mechanism for regulating the estrogen-induced LH surge (Ojeda and Urbanski, 1994), but this has never been formally tested. Our data establish the relevance of spines to behavior by demonstrating that estradiol induction of spines is necessary for sexual receptivity as part of the ARH-MPN-VMH circuit (Mills et al., 2004; Sinchak et al., 2010). The specificity of the ARH effect was demonstrated by our site-specific infusion of CD. Estradiol activated spinogenesis throughout the brain together with all of the lordosis-regulating circuitry, including in the ARH and VMH. But inhibiting spinogenesis specifically within the ARH greatly attenuated the lordosis behavior, suggesting that morphological plasticity in the ARH is necessary for female sexual receptivity.

Our model of estradiol activation of the lordosis-regulating circuit is based on the interaction of NPY and β-endorphin through NPY-Y1 receptors in the ARH (Mills et al., 2004). At the present time, it is difficult to predict whether estradiol-induced spinogenesis occurs in NPY, β-endorphin or as yet an unidentified population of neurons. While the p-cofilin increase in large cells suggests that β-endorphin neurons are increasing spines, it does not rule out an increase in NPY or other cell types. One scenario is that new spines occur predominantly on β-endorphin-containing neurons in the ARH, which would have an enhanced response to NPY. If spines were prevented from forming and subsequently maturing, β-endorphin neurons would be more refractory to the NPY signal. Alternatively, estradiol may stimulate spinogenesis in NPY neurons, which would be more receptive to excitatory stimulation and activate β-endorphin neurons in turn. The ARH also mediates energy balance through NPY and β-endorphin. Estradiol is anorexic and has been reported to reduce NPY mRNA *in vivo* and inhibit NPY release from immortalized hypothalamic neurons (Silva et al., 2010; Dhillon and Belsham, 2011). In terms of feeding regulation, NPY and β-endorphin neurons appear to have a parallel organization with both sets of neurons affecting melanin-concentrating hormone neurons in the lateral hypothalamus (Schwartz and Gelling, 2002). For sexual receptivity, the NPY and β-endorphin neurons appear to be arranged in series. One likely possibility is that there are two populations of NPY and β-endorphin, one mediating energy balance and the other sexual receptivity. Further studies are needed to resolve this question.

In summary, these experiments have shown that estradiol-mediated plasticity is important for regulating female sexual receptivity. This structural plasticity occurs every 4 d coincident with the rise of estradiol during the estrous cycle, and relies on MIES.

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## Chapter 4. Lordosis behavior is suppressed in sexually experienced females and is rescued by inhibition of activity regulated cytoskeletal-associated protein (Arc)

### Introduction

The CNS regulation of female sexual behavior requires integration of steroid hormone information from the gonads and somatosensory stimuli in a circuit within the limbic system and hypothalamus (Kow et al., 1979; Micevych and Sinchak, 2011). Tactile information from the flanks and the perineum reaches the ventromedial nucleus of the hypothalamus (VMH; (Flanagan-Cato et al., 2001)) and increases the excitation of neurons in this final common output from the limbic-hypothalamic module (Chan et al., 1984; Pfaff et al., 2008), as demonstrated by upregulation of immediate early genes (IEG; (Tetel et al., 1993; Polston and Erskine, 1995; Calizo and Flanagan-Cato, 2003; Flanagan-Cato et al., 2006)).

Throughout the brain, the induction of the IEG, Arc, has been correlated with salient sensory stimuli (e.g., (Matsuoka et al., 2002)). In the female, mating results in elevated Arc expression in the VMH (Flanagan-Cato et al., 2006), but little is known about whether such sensory stimuli influence the limbic-hypothalamic circuit upstream of the VMH. Estradiol in the arcuate nucleus of the hypothalamus initiates the induction of lordosis behavior through the release of β-endorphin (β-END) into the medial preoptic nucleus (MPN), activating local μ-opioid receptor-expressing neurons (MOR), which in turn project to the VMH (Mills et al., 2004; Sinchak et al., 2010; Micevych and Dewing, 2011).

Does Arc activation in the arcuate nucleus modulate sexually receptive behavior? In hippocampus, Arc is involved in long term depression (LTD) through its association with and removal of AMPA receptors from the membrane, and with LTP by regulating the actin core of dendritic spines, assisting their growth and maturation. In the arcuate nucleus, we hypothesized that either estradiol stimulation that increased the mGluR1a activation and spinogenesis (Mills

et al., 2004; Christensen et al., 2011) would activate Arc, as demonstrated in vitro (Chamniansawat and Chongthammakun, 2009), or sensory feedback from a mating (Flanagan-Cato et al., 2006).

In ovariectomized rats, estradiol treatment alone did not increase Arc expression in the arcuate nucleus or VMH. A single mating bout increased the population of Arc expressing cells in both nuclei. Female rats treated with estradiol every four days and tested after each treatment showed an increase in Arc expressing cells on only the first behavioral test. Surprisingly, sexual experience reduced lordosis behavior compared with rats treated with an equal dose of estradiol, but only tested for lordosis behavior once. To determine if Arc upregulation after a single mating bout interfered with the induction of sexual receptivity in subsequent matings, Arc antisense oligodeoxynucleotides (asODNs) were infused into the arcuate nucleus. In Arc knockdown rats, the estradiol-induction of sexual receptivity was not compromised by repeated mating experiences.

## Materials and Methods

*Animals***.** Male and ovariectomized (ovx; by the supplier) female (200–250 g) Long-Evans rats were purchased (Charles River, Portage, MI). Upon arrival, rats were housed in a climate-controlled room, two per cage in a 12-hr light:12-hr dark cycle room (lights on at 0600) and provided food and water *ad libitum*. All experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

*Steroid priming***.** Animals were allowed to survive 2-3 weeks after ovx prior to steroid treatment. For all experiments, 17β-estradiol benzoate (EB) and progesterone (P) were dissolved in safflower oil and injected subcutaneously (sc) in a volume of 0.1 ml per rat. Animals that were tested for lordosis behavior received 5 µg EB every four days between 0900 and 1000 to mimic the natural estrous cycle of female rats (Micevych et al., 1994).

Progesterone (500 µg; s.c.) was injected 24-26 hrs after EB injections to mimic the surge of progesterone in a naturally cycling female, as previously described (Sinchak and Micevych, 2001).

*Guide cannulae implantation surgery***.** Bilateral guide cannulae (22 gauge; Plastics One Inc., Roanoke, VA) directed at the arcuate nucleus (coordinates from bregma: anterior –2.8 mm, lateral 0.8 mm, ventral –7.4 mm from dura; tooth bar: –3.3 mm) were implanted using standard stereotaxic procedures while female rats were anesthetized with isoflurane (2–3% in equal parts oxygen and nitrous oxide). Cannulae were secured to the skull with dental acrylic and stainless steel bone screws. Obturators (Plastics One) were placed in the cannulae, which protruded less than 0.5 mm beyond the opening of the guide cannulae. Animals were individually housed after surgery, received oral antibiotics (trimethoprim and sulfamethoxazole, 0.4 mg/ml; Hi-Tech Pharmacal, Amityville, NY) in the drinking water and allowed to recover 6-7 days before infusion. Rats with cannulae not positioned in the arcuate nucleus (i.e., located above, lateral to the arcuate nucleus, or where microinjections had compromised the wall of the third ventricle) were excluded from the study.

*Arc antisense.* Arc oligodeoxynucleotides (ODNs) were designed to knockdown Arc mRNA and protein. The antisense ODNs were a mix of two sequences: 5'-ATGGTCCAGCTCCATCTG-3' and 5'-GGAGGCCGCCGGTCGTCAT-3'. The scramble ODNs were random sequences with no known mRNA targets: 5'-GGTGCATTCCCGAGTCCA-3' and 5'-GCGGACGGTCGCGCTACGT-3' (Life Technologies, Grand Island, NY). All ODNs had ends modified with phosphorothioate to protect against degradation. ODNs were dissolved in artificial cerebral spinal fluid to a final concentration of 1 µg/µL.

Animals were infused with arcuate nucleus site-specific microinjections of Arc ODN or scramble ODN with an infusion pump (Harvard Apparatus, Holliston, MA) at a rate of 0.25 µl/min 90 minutes prior to the start of each behavior test. Microinjection needles (28 gauge) protruded

not more than 1 mm beyond the opening of the guide cannula. The injectors were allowed to remain in place for 1 min after infusion to allow for diffusion away from the injector tip. After microinjection, the obturators were reinserted into the guide cannulae and animals were returned to their home cage to wait for behavior testing.

***Behavioral testing***. Testing began 30 hr after EB injection. Stimulus males were acclimated to the testing arenas for at least 15 min. Sexual receptivity was measured by placing each female rat in a Plexiglas testing arena with a sexually experienced male. Males were allowed to mount females 10 times and the number of times the female displayed lordosis (lifting of the head, arching of the back, movement of the tail to one side) was recorded. For each female, the sexual receptivity was quantified as a lordosis quotient (LQ), the number of lordosis displays/number of mounts x 100.

*Social testing.* Females tested for the effects of female socialization on Arc activation were put into a new clean cage that was neither animal's home cage, without access to food or water. The animals were allowed to explore one another for 30 minutes. One animal from the cage was perfused as described below. The other remained in the cage and was perfused 30 minutes later.

*Male odor testing.* Soiled male bedding from a single male was placed into an empty cage, so there would be no marking patterns. A female was allowed to explore this cage without access to food or water for 30 minutes before being perfused as described below.

*Immunohistochemistry.* Females in which Arc levels were to be monitored after behavior testing were perfused immediately after the conclusion of the test. Animals were anesthetized and transcardially perfused with chilled 0.9% saline, followed with a fixative, 4% paraformaldehyde dissolved in 0.2 M Sorenson's phosphate buffer (pH 7.4). Brains were removed and placed into fixative, stored overnight at 4° C and then replaced with 20% sucrose in phosphate buffer for cryoprotection. Brains were blocked, sectioned (20 µm) on a cryostat

(Leica CM 1800, Bannockburn IL), and collected into chambers filled with PBS. Sections from the arcuate nucleus were incubated overnight with an antibody directed against Arc (1:250, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoreactivity was visualized with diaminobenzidine (DAB) histochemistry kit (VECTASTAIN Elite ABC kit; Vector Laboratories). Sections were mounted onto SuperFrost/Plus slides (Fisher, Pittsburgh, PA), air dried and dehydrated before being coverslipped with Krystalon (EMD Chemicals, Gibbstown, NJ).

*Image analysis.* Sections were examined using a Zeiss Axioskop 2, Axiocam digital camera, and AxioVision digital image analysis system (Carl Zeiss North America, Thornwood, NY). Images were adjusted for brightness and contrast using the Zeiss LSM-PC and PhotoShop (version 10.0; Adobe, San Jose, CA) programs. DAB sections were viewed with bright-field illumination. Every fourth section from the arcuate nucleus was analyzed for Arc immunoreactivity. Cells were considered Arc-positive if their cell body showed positive staining. The arcuate nucleus and VMH from only one side from each section was counted in an alternating pattern, including both the left and the right sides of the brain in the final count. Sections in which the arcuate nucleus or VMH from both sides was damaged during immunohistochemistry were not used for analysis.

*Western blotting.* Animals were anesthetized, decapitated and their brains removed and rinsed with cold PBS. The arcuate nucleus was dissected out on ice and put in RIPA (RadioImmunoPrecipitation Assay) lysis buffer with protease inhibitors (Santa Cruz Biotechnology). The tissue was sonicated before being centrifuged. The supernatant was collected and 10-50 µg of protein was heated for 5 minutes at 95°C in Laemmli sample buffer (Bio-Rad, Hercules, CA) and run on a 10% acrylamide gel. The protein was transferred overnight to a Hybond-P PVDF membrane (GE Healthcare). The membrane was blocked in 5% milk in 0.1% TBS-Tween for 1 hr before being transferred to primary antibody against Arc (1:250; Santa Cruz Biotechnology), made in 1% BSA, spinophilin (1:1000; Millipore, Billerica,

MA) or synapsin (1:15,000; Santa Cruz Biotechnology), made in 5% milk. The membrane was incubated in primary antibody overnight at 4°C. The next day the membrane was washed and incubated in peroxidase conjugated secondary antibody (1:2000; Vector) for 1 hr. The membrane was washed again for at least 1 hr and developed using GE Healthcare ECL. The membrane was exposed to film for 5 seconds to 2 minutes. The membrane was then stripped of antibody using stripping buffer (60mM Tris-HCl, 2.4mM Tris, 2% SDS, 0.7% β-mercaptoethanol) at 55° for 5-7 minutes, rinsed in 500mL of dd$H_2O$ and blocked in 5% milk for 1 hr. The membrane was then incubated in primary antibody against GAPDH (1:2000; Millipore) in 5% milk overnight. The membrane was visualized as above with exposure to film for 5-10 seconds.

To examine membrane levels of ERα, experienced and naïve animals were anesthetized and decapitated. The brain was rinsed in cold PBS, and the arcuate nucleus dissected on ice and put in homogenization buffer containing protease inhibitors (from the Plasma Membrane Protein Extraction Kit; Abcam, Cambridge, MA). The tissue was homogenized using a dounce homogenizer and membrane proteins were extracted according to the manufacturer's protocol. After extraction, the whole population of membrane proteins was resuspended in RIPA lysis buffer and protease inhibitors (Santa Cruz Biotechnology). Proteins were run as stated above. Membranes were blocked in 5% milk in 0.1% TBS-Tween for 1 hr before being transferred to primary antibody against ERα (MC-20; Santa Cruz Biotechnology), made in 5% milk. After detection, the membrane was stripped and incubated in primary antibody flotillin-1 (1:1000; Abcam) and visualized as described. Flotillin was used both as a loading and experimental control because it is a membrane protein and is not affected by EB administration (Boulware et al., 2007).

*Statistical analysis.* All data are expressed as the mean ± SEM. All data were analyzed by two-tailed t-tests or one way-ANOVA, as specified in *Results*. Differences were considered

significant at $P < 0.05$. Statistical analysis was conducted using GraphPad Prism 5 (version 5.02, GraphPad Software, Inc., La Jolla, CA) software. The number of animals used in each experiment is specified in *Results*.

## Results

*Sexually experienced females showed less receptivity than naïve females.* Females were injected with 5 µg EB every four days. Animals designated "naïve" were injected for the requisite number of cycles and tested once for lordosis behavior 30 hours after the final injection. Naïve animals showed a characteristic ramping of their lordosis quotients: increasing the number of EB injections increased their sexual receptivity. Maximum receptivity was reached within 3 or 4 cycles (Fig 1). Females designated "experienced" were tested 30 hours after each injection. These repeatedly mated females did not reach maximal receptivity and instead plateaued at a moderate lordosis quotient (~50) despite having the same amount of estradiol as their naïve counterparts (Fig 1; t-test cycle 4: $P = 0.04$, $t = 2.269$ $df = 19$; cycle 5: $P = 0.03$, $t = 2.446$, $df = 11$; $n = 4$-32). All further experiments were designed to determine the differences between these groups and to examine the mechanism underlying the loss of receptivity in experienced females.

*Sexual behavior upregulated Arc in the arcuate nucleus.* Since Arc is upregulated by salient sensory experience (Guzowski et al., 1999; Guzowski et al., 2001), this IEG may mediate the difference between the level of sexual receptivity of experienced and naïve rats. Previous work had also shown that sexual behavior and not hormone treatment upregulated Arc expression in the VMH (Flanagan-Cato et al., 2006). More recently, estradiol was reported to induce Arc in vitro (Chamniansawat and Chongthammakun, 2009). To examine the steroid and behavioral regulation of Arc in the arcuate nucleus, animals were injected with oil, EB, or EB followed by

progesterone and sacrificed one hour later. None of these treatments alone significantly upregulated Arc in the arcuate nucleus or the VMH (Fig 2; t-test arcuate nucleus: $P = 0.92$; VMH: $P = 0.88$; $n = 3$-8). However, when animals were injected with 4 cycles of estradiol and tested for sexual behavior 30 hours after the final injection (naïve), there was a significant upregulation of Arc in the arcuate nucleus (Fig 3; one-way ANOVA: $P = 0.002$; Newman-Keuls Multiple Comparison Test: naïve vs. experienced: $P < 0.05$; naïve vs. naïve + P: $P < 0.05$; naïve vs. experienced + P: $P < 0.05$; naïve vs. Exp + P + EB: $P < 0.05$; $n = 4$-5) and VMH (Fig 3; one-way ANOVA: $P = 0.0007$; Newman-Keuls Multiple Comparison Test: naïve vs. experienced: $P < 0.005$; naïve vs. naïve + P: $P < 0.005$; naïve vs. experienced + P: $P < 0.005$; naïve vs. Exp + P + EB: $P < 0.005$; $n = 4$-5), when measured both immunohistochemically and by Western blot (Fig 3; one-way ANOVA: $P = 0.02$; Newman-Keuls Multiple Comparison Test: untested vs. naïve: $P < 0.05$; experienced vs. naïve: $P < 0.05$; $n = 3$-4). This upregulation was not seen in females tested after every EB injection (experienced).

Interestingly, a single injection of oil, followed by a behavior test was able to elicit the same amount of Arc upregulation as naïve animals in both the arcuate nucleus and VMH (Fig 3; arcuate nucleus: Newman-Keuls Multiple Comparison Test: oil vs. experienced: $P < 0.05$; oil vs. naïve + P: $P < 0.05$; oil vs. experienced + P: $P < 0.05$; oil vs. Exp + P + EB: $P < 0.05$; VMH: Newman-Keuls Multiple Comparison Test: oil vs. experienced: $P < 0.05$; oil vs naïve + P: $P < 0.05$; oil vs. experienced + P: $P < 0.05$; oil vs. Exp + P + EB: $P < 0.05$; $n = 4$-6). Since these oil-treated animals had a lordosis quotient of 0, this indicated that the level of sexual receptivity was not associated with increased Arc expression, but the male induced mating behavior was related to the upregulation of Arc protein.

A naïve animal, primed with EB and given progesterone 4 hrs prior to the behavior test did not have an increase in the number of Arc-positive neurons even though the mating experience was novel (Fig 3). Progesterone has been suggested to “reset” neuronal circuits

controlling sexual behavior after estradiol treatment (Allrich, 1994; Sinchak and Micevych, 2001; Griffin and Flanagan-Cato, 2008). We expected progesterone to allow Arc upregulation in experienced animals. However, when experienced females were treated with progesterone 4 hrs before their final behavior test, LQ was increased, but Arc protein was not induced (Fig 3). To determine if progesterone action required a longer time course (i.e., progesterone would allow Arc induction on subsequent tests), experienced animals were treated with progesterone on the third cycle, and tested for lordosis behavior. On the fourth cycle, the same rats were primed with EB-only and again given a lordosis behavior test (Exp + P + EB). As before, we did not detect a statistically significant upregulation of Arc protein (Fig 3; $P > 0.05$). All the rats treated with progesterone had LQs of nearly 100, even when progesterone was given on the previous cycle. Together, these experiments indicate that in gonadally intact, normally cycling female rats, progesterone negatively regulates Arc induction and abrogates its effect on sexual receptivity.

*Other factors involved in Arc upregulation.* To test whether aspects other than the mating component regulate Arc, females were given several novelty tests. In the first, females were housed for 30 minutes with unknown females to test whether socializing with a new animal upregulated Arc. Compared to oil-treated untested controls, there was no increase in the numbers of the Arc-positive neurons in the arcuate nucleus, but there was some increase in the VMH (Fig 2B). To test whether the scent of the male was sufficient to activate Arc, females were placed in cages with used male bedding for 30 minutes. Again, no increase in Arc-positive neurons was observed in the arcuate nucleus (Fig 2B; one-way ANOVA: $P = 0.0005$; Newman-Keuls Multiple Comparison Test: naïve vs. novel female: $P < 0.0005$; naïve vs. male bedding: $P < 0.0005$; $n = 4$-5). Interestingly, in the VMH, male-soiled bedding induced a large increase in Arc-positive neurons to levels similar to those seen in tested naïve animals (Fig 2B; one-way

ANOVA: P = 0.07; n = 4-5). As illustrated in the figures, this is the only time the pattern of Arc activation was different between the arcuate nucleus and the VMH (compare Figs 3 and 2B).

Arc has been shown to affect spinogenesis and spine density (Peebles et al., 2010; Moonat et al., 2011). Spinophilin is a postsynaptic protein, highly concentrated in spines, and is a marker for spine density (Amateau and McCarthy, 2004; Orlowski et al., 2012). Synapsin-1, a marker for presynaptic terminals, was used to estimate functional spines. An increase in spinophilin should have a corresponding increase in synapsin-1 if mature, functional spines are being formed (Borges et al., 2010). Both spinophilin and synapsin were upregulated in experienced compared to untested EB-treated females (Fig 4; spinophilin: one-way ANOVA: P = 0.04; Newman-Keuls Multiple Comparison Test: experienced vs. untested; P < 0.05; experienced vs. naïve: P < 0.05; synapsin: one-way ANOVA: P = 0.02; Newman-Keuls Multiple Comparison Test: experienced vs. untested; P < 0.05; experienced vs. naïve: P < 0.05; n = 4). The increase in both these markers indicates an experience-induced increase in dendritic spines.

In LTD, Arc internalizes AMPA receptors (Waung et al., 2008). Sexual receptivity is partially dependent on estradiol membrane signaling in the arcuate nucleus (Dewing et al., 2007; Christensen and Micevych, In Press). A loss of membrane estrogen receptor-α (mERα) in experienced females would reduce the strength of membrane signaling pointing to an underlying deficit that may explain the low levels of sexual receptivity in these females. To determine levels of mERα after behavioral testing, the arcuate nucleus from naïve and experienced females was collected after the fourth behavior test (or the behavior test after the fourth cycle for naïve animals). Experienced animals had ~25% less mERα than naïve (Fig 5; t-test: P = 0.04, t = 2.534, df = 6; n = 4) suggesting that experience negatively regulated mERα, which resulted in a deficit of membrane-initiated estradiol signaling leading to lower lordosis behavior scores.

*Knockdown of Arc restored sexual receptivity in experienced animals.* To formally test whether Arc was involved in the loss of sexual receptivity in experienced females, animals were infused 90 minutes before each behavior test with either asODNs directed against Arc mRNA or scrambled controls. Arc protein levels were reduced by about 50% in Arc asODN treated animals compared to scrambled controls (Fig 6; t-test: $P = 0.04$, $t = 2.823$, $df = 5$; $n = 3$-4). By reducing Arc protein, and not allowing an increase due to experience in the arcuate nucleus, sexual receptivity was rescued. When Arc levels were reduced, experienced females displayed lordosis behavior that resembled naïve females and was significantly different from scramble treated controls (Fig 6; t-test cycle 2: $P = 0.04$, $t = 2.317$, $df = 12$; cycle 3: $P = 0.005$, $t = 3.545$, $df = 11$; cycle 4: $P = 0.0004$, $t = 5.026$; $df = 11$; $n = 6$-7). Although not quantified, animals treated with Arc asODNs tended to show proceptive behaviors (e.g., ear wiggling, hopping and darting), which were never observed in scrambled controls. In addition, in some Arc knockdown rats, proceptive behaviors were seen as early as cycle 2, a point at which naïve EB-only treated animals do not have these behaviors.

Finally, the knockdown experiments indicated that Arc was not regulating spines needed for lordosis behavior. Arcuate nucleus spine density, as measured by spinophilin and synapsin, in Arc asODN treated rats was not statistically different than those females treated with scrambled controls (data not shown; t-test spinophilin $P = 0.57$; synapsin $P = 0.85$; $n = 4$-5).

## Discussion

The major finding of these experiments was that sexual behavior is regulated by experience. More experienced females have significantly reduced receptivity compared to females that are treated with an equal amount of EB but are tested only once. Several factors

were observed to be changed by sexual experience: Arc was upregulated, dendritic spines were increased, but membrane levels of ERα were decreased.

Although we initially predicted that pairing multiple mating tests with EB treatment would result in "learning" that would be evident as females became sexually receptive with fewer estradiol treatments, we found the opposite. Experienced females were less receptive than their naïve (tested once) counterparts. In order to explain this observation, we examined an IEG, Arc, which is upregulated by salient sensory experience in other parts of the brain and by estradiol in vitro (Guzowski et al., 2001; Chamniansawat and Chongthammakun, 2009). Arc was increased in both the VMH and the arcuate nucleus after the initial behavior test, regardless of the level of sexual receptivity or the amount of estradiol. In fact, estradiol was not needed to stimulate Arc in the arcuate nucleus. Oil-treated females mated with stimulus males had an upregulation of Arc that was equivalent to estradiol-treated naïve females.

Estradiol in vivo did not activate Arc as previously reported in vitro (Chamniansawat and Chongthammakun, 2009). This is interesting since estradiol membrane signaling in the arcuate nucleus is dependent on mGluR1a (Dewing et al., 2007). Arc, on the other hand, has been shown to be activated by another member of the same family, mGluR5, which is sparsely distributed in the arcuate nucleus (van den Pol et al., 1995; Kumar et al., 2012). If Arc is being regulated by glutamatergic signaling, there is a high degree of specificity between estradiol, which does not activate, and salient sensory input, which does activate Arc.

Glutamate that induces Arc has been implicated in hippocampal LTP and LTD (Bramham et al., 2008). In the arcuate nucleus, large doses of DHPG, the mGluR1a agonist, could substitute for estradiol in activating the lordosis circuit, measured by MOR internalization in the MPN (Dewing et al., 2007; Dewing et al., 2008). Activation of MOR in the MPN inhibits lordosis acutely, but is necessary for ultimately inducing maximal sexual receptivity. In estradiol treated ovx or normally cycling females, MOR is activated the day before lordosis behavior and

this inhibition is attenuated with time or is removed by progesterone. It is possible that sexual behavior reactivates this circuit, releasing glutamate, which stimulates arcuate nucleus circuits leading to MOR activation and producing an inhibition of lordosis behavior. Although such a mechanism may explain an acute attenuation of lordosis, it does not explain the long lasting Arc effect. Mating also reduced the levels of membrane ERα. Further experiments will be needed to ascertain the mechanism, but one possibility is that Arc increases mERα endocytosis, removing it from the membrane and reducing membrane-initiated signaling. Such a LTD-related mechanism is proposed in the hippocampus for AMPA receptors (Waung et al., 2008).

However Arc is regulated in the arcuate nucleus, it remains activated for a prolonged period. After the initial, novel mating experience that activates Arc, in subsequent mating tests, levels retreated to those in untested females. Sexual receptivity, however, remained attenuated for the duration of the experiment. When the upregulation of Arc was prevented before mating, receptivity was rescued, suggesting that Arc upregulation prevented the full display of sexual behavior in experienced females. The Arc knockdown notwithstanding, only progesterone treatment blocked Arc activation and its downstream consequences, which allowed for full sexual receptivity in experienced females.

Similarly, it is difficult to hypothesize that Arc acts to decrease sexual receptivity of experienced rats by acting on spines. During LTP induction protocols in the hippocampus, Arc has been shown to associate with the spine actin core. Arc mediates actin growth and branching, assisting in the growth and maturation of spines (Messaoudi et al., 2007). Indeed, in the hippocampus, increasing Arc increases dendritic spines, and overexpression of Arc increases spine density in vitro (Peebles et al., 2010; Moonat et al., 2011). In the arcuate nucleus, sexual experience increased dendritic spines measured by a parallel increase in spinophilin and synapsin levels, reflecting a greater number of functional synapses (Borges et al., 2010). However, in females with more spinophilin and synapsin, sexual receptivity was

never maximal. In our previous studies, spinogenesis was induced by estradiol and this increase in the arcuate nucleus was necessary for sexual receptivity (Christensen et al., 2011). An interesting observation from those studies was that estradiol-induced spinogenesis plateaus. This may point to an optimal spine density: too few or too many spines and lordosis behavior is attenuated. However, spinophilin and synapsin levels are equivalent in Arc asODN experienced females and control treated females, indicating that Arc regulation of spines is probably not responsible for the difference in sexual receptivity between naïve and experienced females.

As with Arc, spine levels are restored by progesterone. Progesterone has been reported to reduce estradiol-induced spines in the hippocampus and the VMH (Woolley and McEwen, 1992; Murphy and Segal, 2000; Griffin and Flanagan-Cato, 2008). Such a reduction in spines is proposed as one mechanism through which progesterone could reset the lordosis regulating circuits, allowing them to once again respond fully to an estradiol induction with maximal receptivity. In the present study, progesterone administered 4 hrs before the behavior test inhibited the synthesis of Arc, even in naïve females. Even more striking, the synthesis of Arc seemed to coincide with times when the female did not have the endocrine profile that is associated with ovulation and thus, the interaction with a male can be considered an “empty mating”. In this model, Arc may be a molecular gate moderating lordosis behavior: Arc is upregulated when females are not ovulating and continues to decrease sexual receptivity during estradiol-only conditions, since these mating bouts cannot result in fertilization. They are reproductively worthless. High levels of peripheral progesterone, signaling ovulation, inhibit Arc expression allowing maximal sexual receptivity. In this way, Arc mediates hypothalamic “learning,” which prevents sexual receptivity in the absence of ovulation. The behavioral effect of Arc is not apparent until future cycles. Progesterone, when released, blocks Arc. This is modeled in experienced estradiol treated rats that had attenuated lordosis behavior. These animals treated with estradiol and progesterone became maximally receptive and had low levels

of Arc. Interestingly, they remained maximally sexually receptive on the following cycle, even though they were treated with an estradiol-only dose. If, however, progesterone was not present during a cycle and mating bout, Arc was induced and inhibited future sexual encounters until ovulation or progesterone was present. Such a circumstance may be present before puberty, when the female is just beginning to develop an estrus cycle and it is somewhat erratic. Arc is thereby gating the behavior, allowing the female to be sexually receptive when mating is likely to be beneficial, but inhibiting receptivity when the timing is not optimal.

Our results confirmed earlier studies that showed hormone treatment alone was not able to activate Arc in the VMH and extended this observation to the arcuate nucleus (Flanagan-Cato et al., 2006). However, where we observed an Arc increase only in naïve females treated with estradiol, Flanagan-Cato and colleagues (2006), reported an increase in both naïve and experienced animals. Since, the experimental design of those studies differed from ours, it is difficult to rationalize the differences. Their rats were treated with 10 μg EB (for two days) and progesterone before the mating test. In our paradigm, 5 μg EB was given every four days, designed to mimic the estrus cycle (Micevych et al., 1994). Additionally, we tested behavior 30 hrs after estradiol priming and Flanagan-Cato et al. tested after 48 hrs. Which of these conditions contributed to the disparate results is presently unknown, but may help to elucidate the role of Arc in the physiology of sexual receptivity,

In summary, mating behavior drastically changed the hypothalamic nuclei that regulate it. In the arcuate nucleus, the IEG Arc, which is known to play a role in synaptic plasticity, was upregulated after the first bout of sexual behavior. Although it was not upregulated again, experienced animals show a severe reduction in sexual receptivity. Downregulation of Arc with antisense repaired the deficit. While the evidence is overwhelming that Arc is involved in the inhibition of receptivity, understanding the mechanism underlying this regulation will require further investigation.

Figure Legends

**Fig 1. Experience reduced sexual receptivity.** Naïve females were injected with 5 µg EB every four days for the stated number of cycles, but tested for sexual behavior only once, 30 hrs after the final injection. They showed the characteristic ramping of receptivity that is expected by this treatment. Females reached maximal receptivity by about 3 cycles. Experienced females were tested 30 hrs after each injection of EB. They never reached maximal receptivity and instead plateaued at an LQ of about 50. LQ = lordosis quotient, * = $P < 0.05$, n = 4-32

**Fig 2. Steroid hormone treatment and novel experiences did not affect Arc levels in arcuate nucleus. (A)** Female rats treated with oil, EB or EB followed by progesterone for one hour showed no change in levels of Arc protein in either the arcuate nucleus or the VMH. ARH = arcuate nucleus of the hypothalamus, VMH = ventromedial hypothalamus, n = 3-8. **(B)** Differential effects of novelty tests on Arc+ neurons in the arcuate nucleus and VMH. Females were housed for 30 minutes with females they had never interacted with before. Arc was upregulated in the VMH, but not in the arcuate nucleus. When exposed to soiled male bedding no change in Arc staining was observed, but Arc in the VMH was upregulated. The data from these tests were compared to animals that had been tested for sexual receptivity only once (Naïve; from Fig 3). The upregulation of Arc in the VMH by a novel female or the scent of male bedding was not significantly different from that elicited by a sexual behavior test. ARH = arcuate nucleus of the hypothalamus, VMH = ventromedial hypothalamus, * = $P < 0.05$, n = 4-5

**Fig 3. Naïve females upregulate Arc protein in the arcuate nucleus and VMH.** Naïve females were injected four times with 5 µg of EB and tested 30 hrs after the final injection. Arc protein was significantly upregulated by the novel sexual behavior test as seen by both

immunohistochemistry **(D)** and western blot **(E)**. Arc staining was localized to the cell body and could be seen out into the dendrites in naïve animals **(A, B)**. After the fourth behavior test, there was no Arc upregulation in experienced animals **(D, E)**. A representative section of the immunostaining of Arc can be seen in **(C)**. Treatment of naïve animals with progesterone 4 hrs before testing for sexual receptivity abolished the increase in Arc immunoreactivity (Naïve + P). Likewise, progesterone was unable to rescue Arc upregulation when given 4 hrs before the behavior test in experienced animals (Exp + P). If progesterone was given on the third cycle and animals were tested again four days later after another dose of EB (Exp + P + EB), Arc was also not upregulated. Estradiol treatment was not required for the upregulation of Arc, as oil-treated animals were just as capable of increasing Arc protein levels. ARH = arcuate nucleus of the hypothalamus, VMH = ventromedial hypothalamus, scale bar in B measures 20 μm, scale bar in C measures 100 μm, * = $P < 0.05$, n = 4-6 in (D), n = 3-4 in (E)

**Fig 4. Dendritic spines are increased in experienced animals. (A)** Spinophilin, a postsynaptic marker of spines, is increased in experienced animals. **(B)** A marker of presynaptic terminals, synapsin-1, is also increased in experienced females. GAPDH was used as the loading control. * = $P < 0.05$, n = 4

**Fig 5. Membrane estrogen receptor levels are reduced by sexual experience.** In a membrane preparation of the arcuate nucleus, animals tested 30 hrs after each behavior test (Experienced) showed a significant reduction in the amount of ERα on the membrane (mERα) compared to animals injected the same number of times but tested only once (Naïve). Measurements were made in comparison to the integral membrane protein flotillin-1, which is not affected by estradiol treatment. * = $P < 0.05$, n = 4

**Fig 6. Arc knock down restores sexual receptivity. (A)** Arc protein was reduced in animals treated with asODNs against Arc mRNA compared to scrambled controls. GAPDH was the loading control. **(B)** When animals were infused into the ARH with asODNs against Arc 90 minutes before each behavior test, their lordosis quotient was significantly increased compared to scrambled controls. The scores of antisense treated animals are similar to naïve. * = $P < 0.05$, n = 3-4 in (A), n = 6-7 in (B)

Figure 1

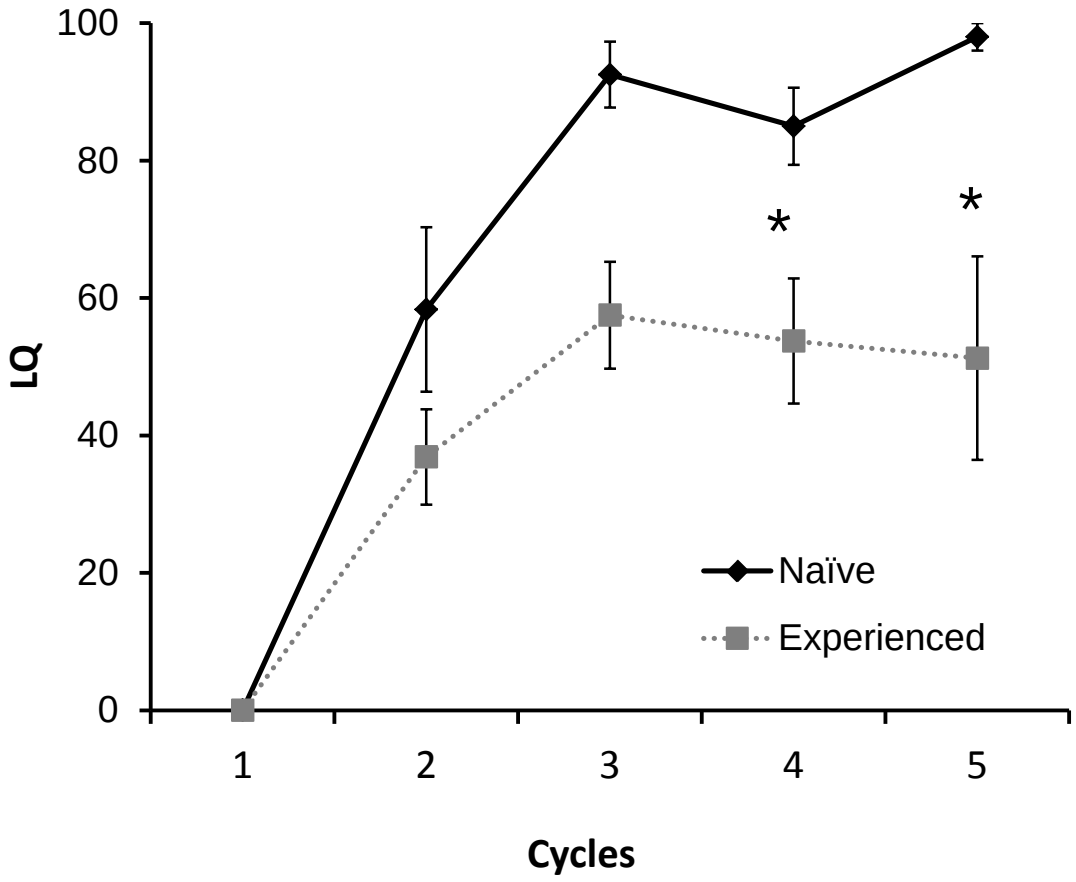
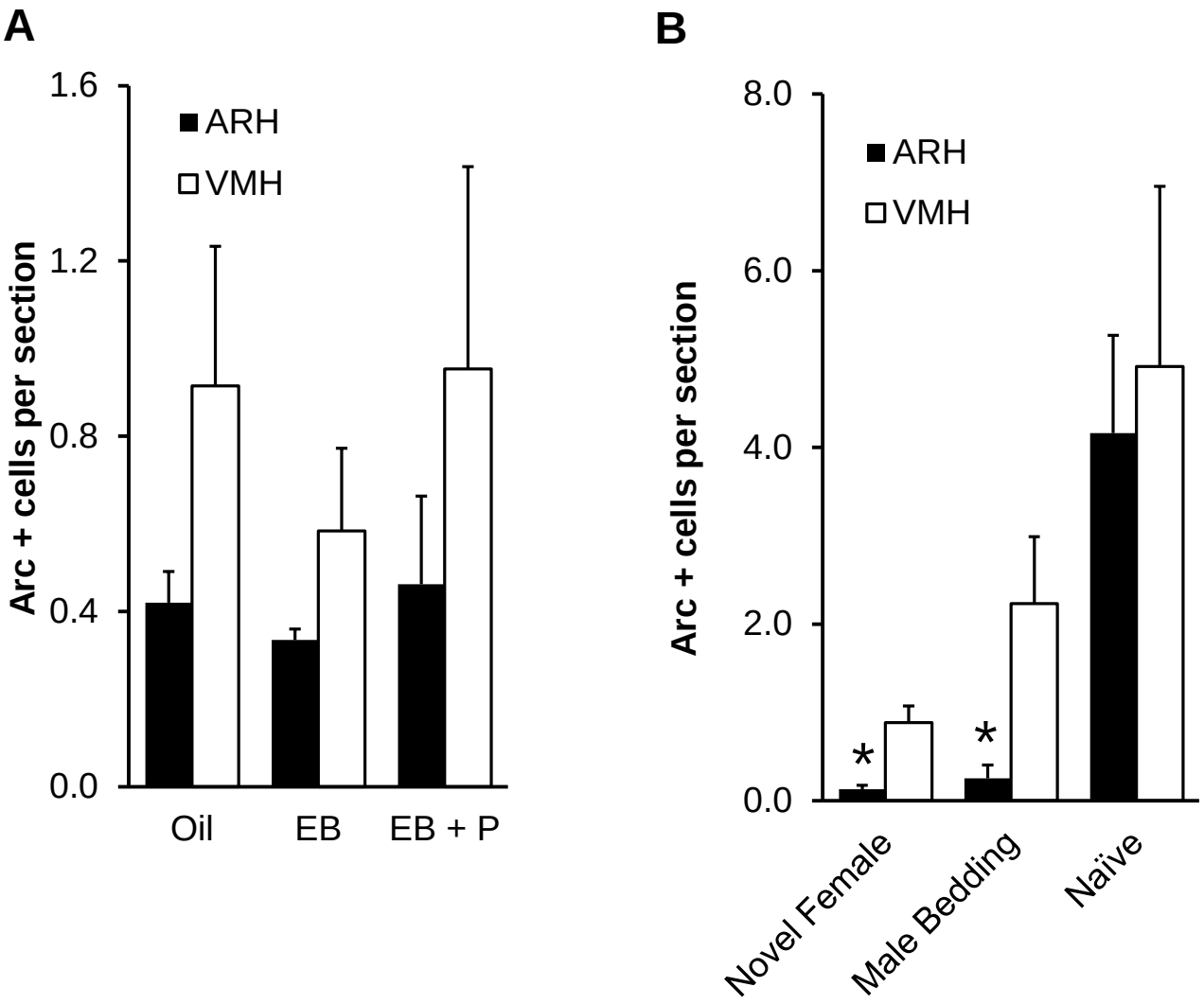


Figure 2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Novel Female
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Figure 3

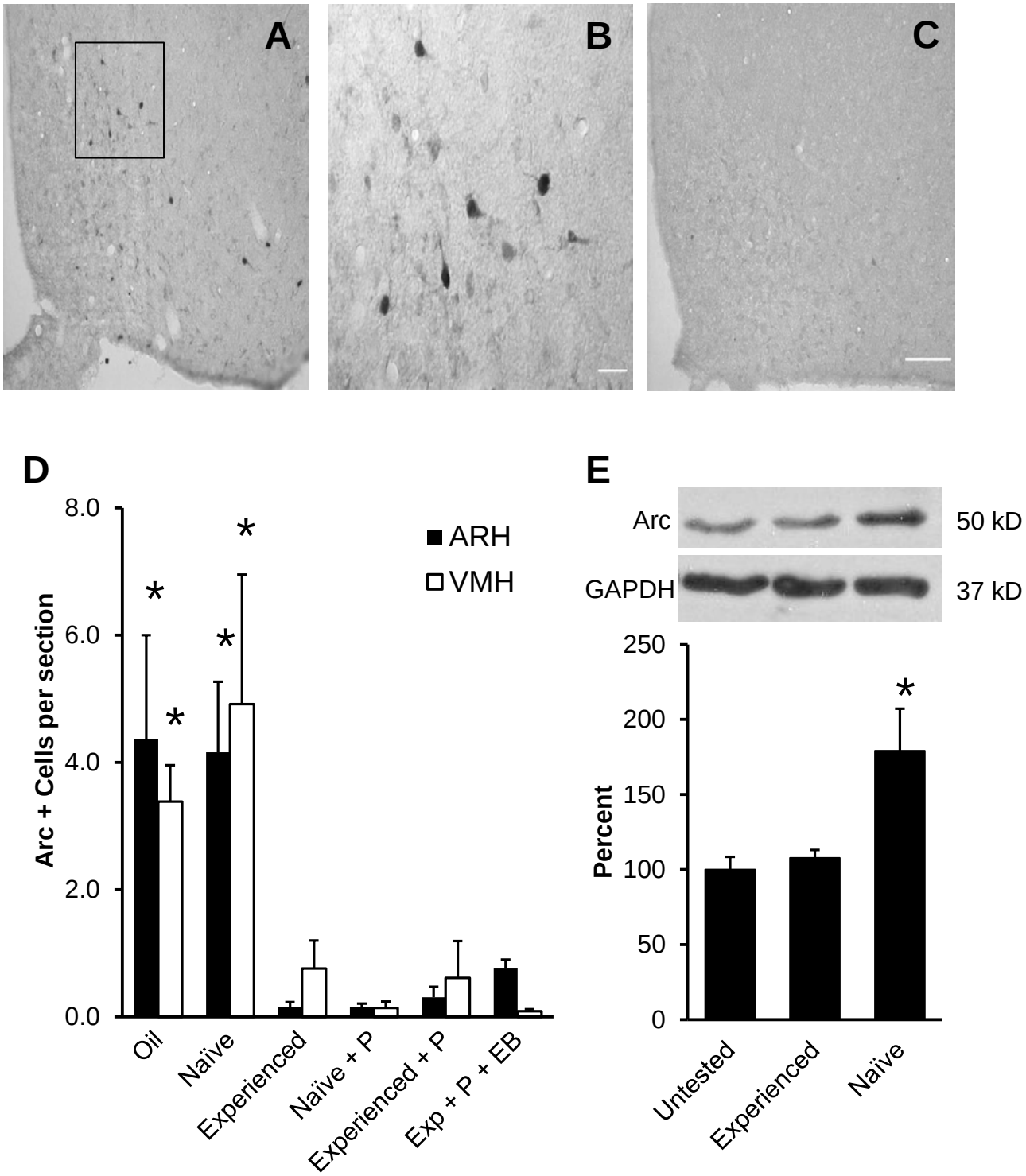
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Figure 4

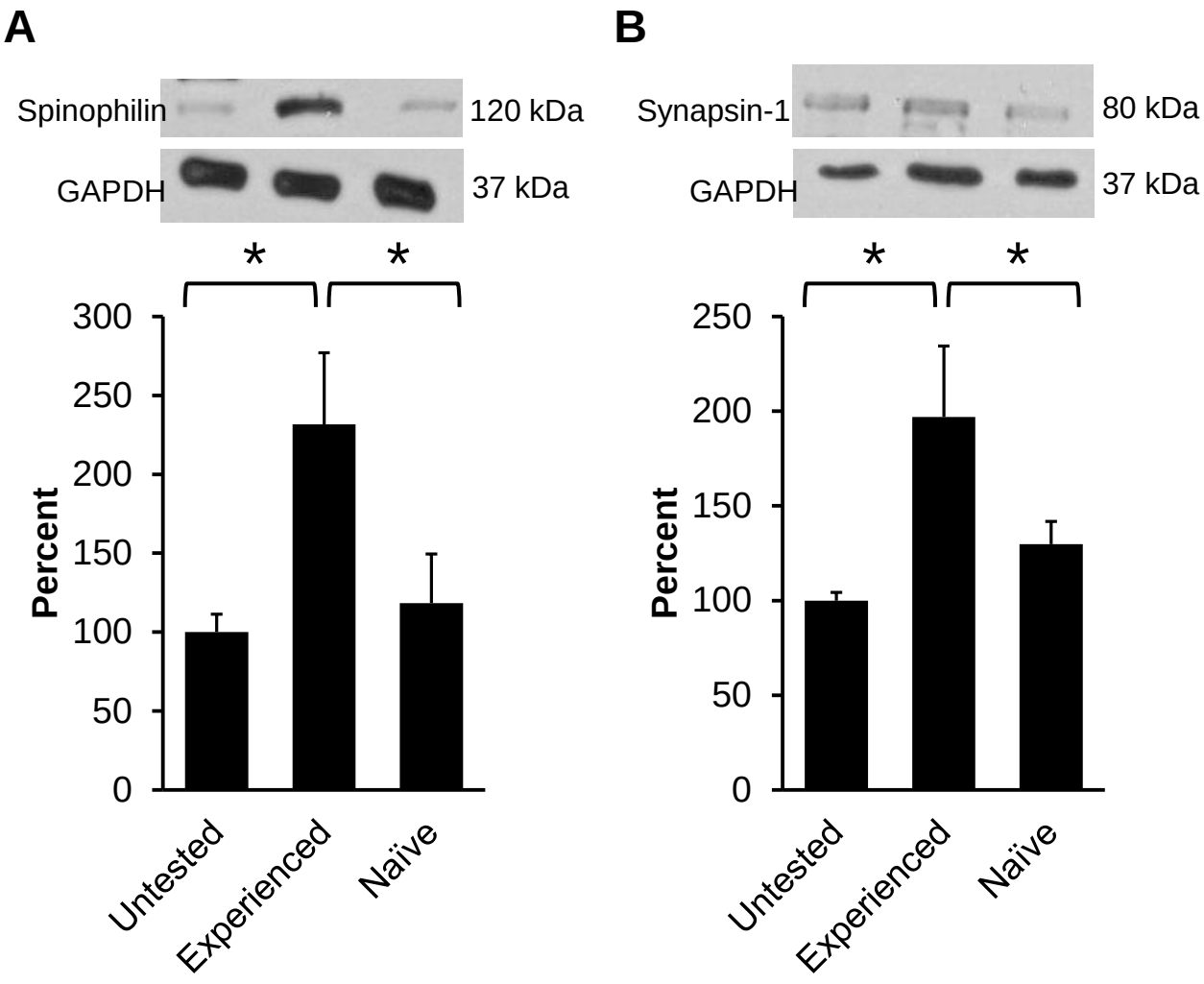
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Figure 5

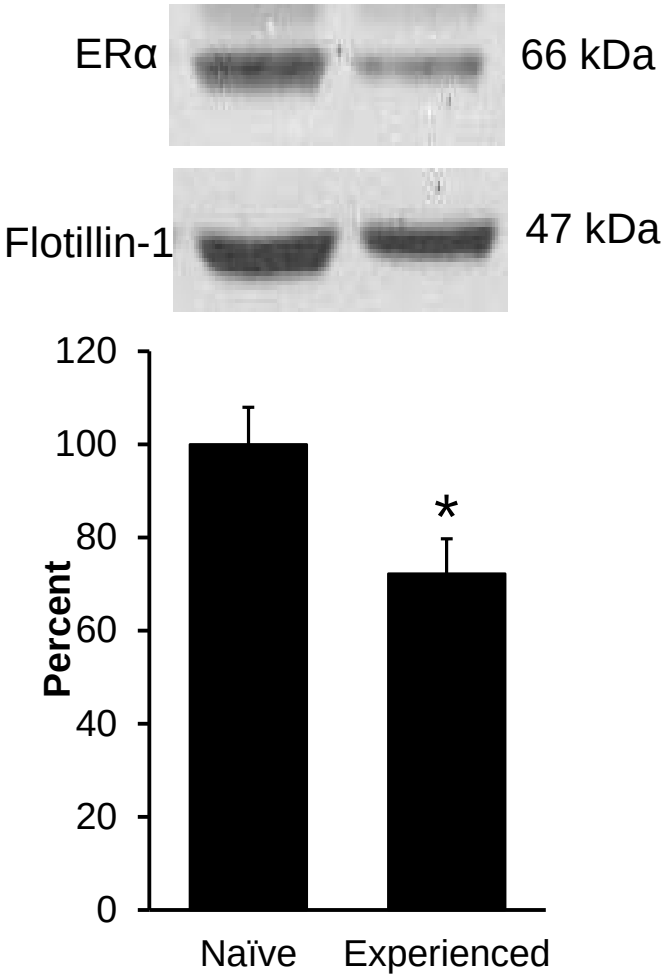
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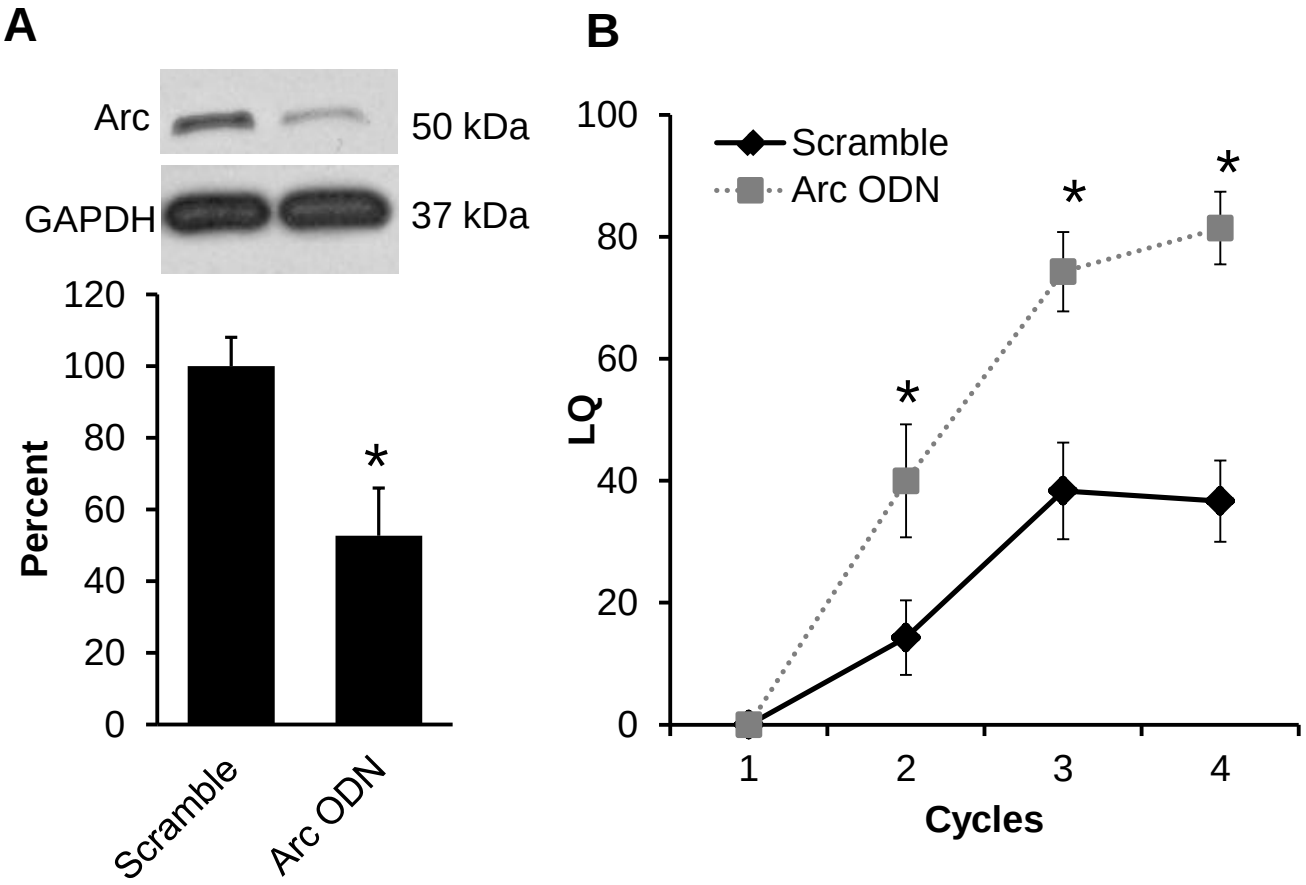
Figure 6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Cycles

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## Chapter 5. Discussion

My overarching interest has been to understand estradiol signaling regulating sexual receptivity in the female rat. Before I started on my dissertation project, I had participated in several studies that defined the rapid actions of estradiol. Specifically, we demonstrated that that full expression of sexual receptivity in female rats requires an interaction between the membrane ERα (mERα) and metabotropic glutamate receptor type 1a (mGluR1a) in the arcuate nucleus of the hypothalamus (ARH; (Dewing et al., 2007; Dewing et al., 2008)). The dissertation experiments are built on these original experiments and were designed to examine how this signaling is integrated into molecular events in the ARH that are required for the regulation of sexual receptivity. In the first experiment, presented in Chapter 2, I examined the mechanism of mERα trafficking to the cell membrane. The hypothesis was that caveolin-1 (CAV1), a protein that is important for mERα-mGluR1a interaction, mediated this membrane insertion. The results were consistent with the hypothesis: knockdown of CAV1 reduced mERα and lordosis behavior. This reiterates the importance of mERα in the ARH for sexual receptivity and demonstrates a molecular mechanism for receptor trafficking *in vivo*. In the second set of experiments in Chapter 3, I sought to test whether the same membrane-initiated estradiol signaling mediated spinogenesis in the ARH, and whether this morphological plasticity was required for sexual receptivity. Estradiol-induced spinogenesis was dependent on mGluR1a signaling that regulated the actin cytoskeleton. Blocking spinogenesis prevented estradiol-induced lordosis behavior, demonstrating for the first time the importance of newly formed spines in the regulation of sexual receptivity by the hypothalamus. Finally in Chapter 4, I examined the effect of sensory feedback on regulation of ARH function and sexual behavior. The experience of mating induced the immediate early gene activity regulated cytoskeleton-associated protein (Arc/Arg3.1). This protein has been associated with several forms of

synaptic plasticity. Paradoxically, Arc was induced only once, and it reduced mERα levels and attenuated lordosis behavior on all subsequent tests. Blocking Arc expression prevented the experience-induced attenuation of sexual receptivity in estradiol only treated female rats. Together, these experiments have elucidated several molecular events necessary for the estradiol-induction of lordosis behavior and demonstrated the importance of experiential feedback in regulating the hypothalamic response to estradiol membrane signaling.

The ARH-MPN-VMH circuit that regulates sexual receptivity

The ARH projects to the medial preoptic nucleus (MPN) which projects to the ventromedial hypothalamus (VMH) to regulate sexual behavior (Mills et al., 2004; Sinchak et al., 2010). According to our model of estradiol activation of circuits regulating sexually receptive behavior, estradiol in the ARH binds to mERα causing it to transactivate mGluR1a (Dewing et al., 2007). This initiates G protein activation and the phosphorylation and activation of PKCθ (Dewing et al., 2008) and the neuropeptide Y (NPY) activation of NPY-Y1 receptors located on neurons expressing β-END (Mills et al., 2004). These neurons project to the dorsomedial MPN. Activation of μ-opioid receptors (MORs) by β-END inhibits lordosis (Sinchak and Micevych, 2001). The transient MOR inhibition induced by estradiol is required for full lordosis behavior at time points when the female is receptive, 30-56 hours later.

In the ARH, estradiol signaling requires mGluR1a, and about 25% of all ERα immunoreactive cells also contain mGluR1a (Dewing et al., 2007). In addition, the full length 66 kDa ERα and mGluR1a co-immunoprecipitate, suggesting that they are capable of direct interaction (Dewing et al., 2007). When mGluR1a signaling is inhibited in the ARH with the antagonist LY 367,385, MOR activation is reduced. Because the activation of MOR is required

for sexual behavior, the loss of this signaling also results in a reduction of sexual receptivity in animals in which mGluR1a is inhibited.

Membrane estrogen receptor alpha and its trafficking

There are two classical estrogen receptors, ERα and ERβ. Both were originally believed to act only in the nucleus, where they bind DNA and regulate transcription. However, over the past couple decades, experiments have shown that estradiol is able to activate signaling cascades that require its receptor to be localized to either the cytoplasm or the cell membrane. Estrogen receptors were shown to be on the membrane because they can bind to and be activated by membrane impermeant estradiols (Armen and Gay, 2000; Beyer and Karolczak, 2000; Dewing et al., 2007; Gambino et al., 2012). In addition, both ERα and ERβ have been found on the membrane through the use of membrane isolation experiments and more recently by surface biotinylation (Chambliss et al., 2000; Powell et al., 2001; Chaban et al., 2004; Gorosito et al., 2008; Bondar et al., 2009; Dominguez and Micevych, 2010). Because ERα is not a typical seven transmembrane pass receptor, many have questioned the mechanism for its insertion into the membrane as well as its trafficking mechanisms.

Like other steroid receptors that are found in the membrane, including the androgen receptor and progesterone receptor, ERα must be palmitoylated to be moved to the cell surface (Pedram et al., 2007). Palmitoylation increases the hydrophobicity of proteins so that they can be inserted into the lipid bilayer. For ERα, the addition of palmitic acid occurs on cysteine 447 (Pedram et al., 2007). When this amino acid is mutated, ERα is no longer found in the membrane and is exclusively localized to the cytoplasm and nucleus. Likewise, palmitoylated ERα is never found in the nuclear fraction, but is always localized to the membrane. Palmitoylation also mediates the interaction of ER with scaffold proteins of the caveolin family.

In neurons, caveolins have three functions: trafficking receptors, modulating interactions between receptors, and maintaining signaling microdomains (reviewed in (Stern and Mermelstein, 2010)). Several members of the caveolin family have been shown to be responsible for the trafficking of ERα to the membrane both in vitro and in vivo (Boulware et al., 2007). According to the model proposed by Mermelstein and colleagues ((Boulware et al., 2007); reviewed in (Micevych and Mermelstein, 2008)), the dependence of estradiol membrane signaling on mGluRs requires caveolins to mediate the association: CAV1 allows ERα to interact with type 1 mGluRs, mGluR1 and 5, leading to facilitative signaling; CAV3 associates ERα with type 2 mGluRs, mGluR2 and 3, leading to inhibitory signaling.

I hypothesized that the interaction of ERα with CAV1 regulates the interaction with mGluR1a, which we had demonstrated was critical for estradiol signaling mediating lordosis behavior (reviewed in (Micevych and Christensen, In Press)). Using siRNA to specifically knock CAV1 down in the ARH resulted in a 50% deficit in mERα (Figure 1 in Chapter 2) (Christensen and Micevych, In Press). This was interpreted as disrupted trafficking of ERα because intracellular levels were unaffected by the knockdown. Loss of mERα, as expected, led to a decrease in MOR activation and internalization in the MPN and ultimately to attenuated lordosis behavior.

These CAV1 siRNA experiments revealed interesting features of mERα. Studies, including some from this laboratory, demonstrated that ERα has an extracellular portion by labeling the protein using surface biotinylation experiments (Gorosito et al., 2008; Bondar et al., 2009; Dominguez and Micevych, 2010). All of these experiments used primary cultures of hypothalamic neurons and astrocytes. They all identified both the full-length 66 kDa ERα and a more abundant ERα-immunoreactive protein with an apparent molecular weight of approximately 52 kDa. The smaller ERα was missing from ERKO mouse membranes indicating that it was coded by the *ESR1* gene (Bondar et al., 2009). Two ERα mRNA splice variants have

been reported in the brain that code for proteins of the right molecular weight (Skipper et al., 1993; Ishunina et al., 2005). One is missing exon 4 and the other exon 7 and 8. PCR studies of primary hypothalamic neurons and immortalized ARH neurons revealed that the 52 kDa ERα was missing exon 4 and was designated as ERαΔ4 (Dominguez et al., Submitted). The relative abundance of the ERαΔ4 on the membrane was consistent with fact that exon 4 codes for the nuclear translocation domain. However, the function of ERαΔ4 remains nebulous. It does not co-immunoprecipitate with mGluR1a, and therefore, it is probably not involved in initiating G protein signaling. In vivo, the full length ERα, rather than the ERαΔ4, is the predominant form, and CAV1 knock down did not significantly affect membrane ERαΔ4 levels (Figure 1 in Chapter 2) (Christensen and Micevych, In Press). The reasons for this are not clear. One explanation may be that the relative overabundance of the ERαΔ4 is an artifact of culturing cells. Another maybe that ERαΔ4 is developmentally regulated since the in vivo studies used adult rats and the primary neuronal and immortalized neurons were embryonic. The knock down results also suggest that another caveolin protein may interact with ERαΔ4, so knocking CAV1 down would not disrupt its trafficking. Further experiments will be needed to examine the various possibilities.

Estradiol-mediated spinogenesis is required for lordosis behavior

Estradiol-mediated spinogenesis has been seen throughout the brain in areas such as the hippocampus, cerebellum, ARH and VMH (Matsumoto and Arai, 1981; Frankfurt et al., 1990; Gould et al., 1990; Woolley and McEwen, 1992; Calizo and Flanagan-Cato, 2000). In fact, hippocampus-dependent working memory tasks may rely on these spine density changes (Daniel and Dohanich, 2001; Sandstrom and Williams, 2001, 2004). Because this increase in spine density is observed in areas that are relevant to sexual behavior, the ARH and VMH in

particular, it has been proposed that the spines might be used to regulate receptivity. Some have proposed that the increase in spines seen in the ARH mediates the estradiol induced luteinizing hormone surge (Ojeda and Urbanski, 1994). Others have proposed that the spine increase in the VMH is required for sexual behavior (Calizo and Flanagan-Cato, 2000). Neither theory had been formally tested prior to my experiments.

While estradiol was shown to increase spine density decades ago by Matsumoto and Arai (Matsumoto and Arai, 1979), more recent work has established a time course for changes in spine shape, as well as a mechanism for estradiol spine induction. Estradiol has been shown to affect spine density through two methods. First, estradiol may work through direct activation of receptors residing within the neuron that is changing morphology. The direct actions of estradiol may be through a receptor on the membrane or in the nucleus. The second method of estradiol action is transsynaptic, in which neurons receive input from and change spine density in response to other neurons that contain estrogen receptors. Experimental evidence supports both hypotheses. The axons and spines of neurons in the hippocampus have been shown to contain ERα (Milner et al., 2001). Likewise, ERα-negative neurons in the VMH and hippocampus, which receive input from neurons that contain ERα, have been shown to increase spine density in response to estradiol (reviewed in (Cooke and Woolley, 2005)).

A spine develops from a thin dendritic outgrowth that resembles a filapodia. Once a proper synaptic contact is made, all the necessary molecules for a functional synapse are recruited to the spine tip and into the postsynaptic specialization (Matsuzaki et al., 2001; Holtmaat et al., 2006; Knott et al., 2006; Yoshihara et al., 2009). The addition of all these new proteins, including receptors, scaffold proteins and signaling molecules, causes an enlargement of the spine head. Thus, stubby and mushroom shaped spines are considered functional, while filapodial spines are thought to be more labile. Unless stabilized by an appropriate partner, filapodial spines are pruned. In my experiments, estradiol increased spine density in the ARH

within 4 hours. Although we waited 48 hrs, there was no further increase in spines at later time points (Figure 1 in Chapter 3) (Christensen et al., 2011). However, there was a change in spine morphology. Initially, at 4 hours, immature, filapodial spines increased, while mushroom-shaped spines increased at later time points, 20-48 hours after estradiol (Figure 1 in Chapter 3) (Christensen et al., 2011). It is during these later times that sexual behavior can be induced in the female, so functional spines were present when they would be most appropriate for regulating reproductive behavior. When spinogenesis was blocked in the ARH with the β-actin polymerization inhibitor cytochalasin D (CD), females were less receptive (Figure 3 in Chapter 3) (Christensen et al., 2011). This suggests that estradiol-mediated spine density increases in the ARH are required for sexual behavior.

Several factors suggest that spinogenesis in the ARH is mediated by mERα. First, changes in spine density occur within 4 hours of estradiol treatment. It is possible that nuclear receptors could be activated to increase transcription and translation during this time period, but is more likely that membrane receptors, which activate more rapid signaling mechanisms, initiate spinogenesis. Second, the actin depolymerizing factor, cofilin, was phosphorylated within one hour of estradiol treatment (Figure 4 in Chapter 3) (Christensen et al., 2011). The initiation of spine formation requires the formation of filaments of actin, through the polymerization of G-actin. Cofilin breaks down actin filaments. Because cofilin is deactivated by phosphorylation, it must be phosphorylated for spines to form (Meng et al., 2002). In addition, the phosphorylation of cofilin has been associated with the expansion of synaptic contacts which is required for long-term potentiation (LTP; (Fedulov et al., 2007)). Estradiol regulation of the actin cytoskeleton provides a mechanism through which estradiol can induce spinogenesis. Third, the phosphorylation of cofilin was inhibited when mGluR1a was antagonized with LY367,385 (Figure 4 in Chapter 3) (Christensen et al., 2011). Therefore, the mechanism of mERα signaling in the ARH to MPN circuit is the same as that which induces spinogenesis in the ARH.

Arc regulation of sexual behavior in experienced females

In a continuing effort to understand the mechanisms underlying estradiol mediated sexual receptivity and to understand how spines may be regulated, we examined the activation of Arc. Arc is associated with the regulation of filamentous actin (Messaoudi et al., 2007) and is upregulated by novel sensory experiences (Guzowski et al., 1999; Guzowski et al., 2001). Sexual behavior is a rich sensory experience. Both male and female rats engage in anogenital investigation. The male uses this olfactory information to gauge the readiness of the female for mating. It is no surprise, therefore, that Arc was induced in the olfactory bulb of male rats after sexual behavior (Matsuoka et al., 2002). For females, the induction of lordosis behavior, named for the stereotypical arching of the back, requires the male to make physical contact with the perineum, flanks and tail base (Pfaff et al., 1977). Indeed, sexual behavior has been shown to induce Arc in the VMH of female rats (Flanagan-Cato et al., 2006). More recent work has shown that in the VMH, it may not be the tactile stimuli from the male that induced Arc upregulation, but rather the male odor that was processed by the VMH (Figure 2 in Chapter 4) (Christensen et al., In Prep). Significantly, an upstream regulator of the VMH, the ARH where estradiol information initially stimulates the circuit, seemed to receive information about the other parts of the sexual behavior and upregulated Arc.

The upregulation of Arc in the ARH regulated sexual behavior in experienced female rats. Ovariectomized rats were injected with 5 μg estradiol benzoate (EB) every four days to mimic the natural estrus cycle of a rat (Micevych et al., 1994). The animals were tested for sexual behavior 30 hours after each injection. These animals showed significantly less sexual receptivity than animals that were injected the same number of times with EB, but tested only once (naïve). Naïve animals were maximally receptive after 3 cycles of EB treatment, but the

experienced females never attained maximal receptivity. Their behavior plateaued at a half-maximal level (Figure 1 in Chapter 4) (Christensen et al., In Prep). As expected, Arc was upregulated following the first behavioral test, regardless of the estradiol treatment. However, it was completely unexpected that subsequent lordosis behavior testing did not induce Arc while lordosis quotient remained half-maximal (Figure 3 in Chapter 4) (Christensen et al., In Prep). If Arc was interfering with the full expression of lordosis behavior, its effects were long lasting. To test this, experienced females were treated before each behavior test with antisense oligodeoxynucleotides (asODNs) directed against Arc mRNA. Sexual receptivity was significantly elevated, resembling lordosis quotients of naïve rather than experienced females (Figure 6 in Chapter 4) (Christensen et al., In Prep). This suggests that Arc negatively regulates sexual receptivity in experienced females.

Sexual experience changed several other features. First, mating experience increased dendritic spine density. This increase was beyond the levels seen with EB alone, as well as that observed in the once tested naïve animals. An increase in spines is critical for sexual receptivity (Christensen et al., 2011), but it is possible that there is an upper limit to the spine density. When this limit is exceeded, sexual receptivity is negatively impacted. Unfortunately, this was not consistent with our Arc model. Arc asODN and scramble ODN (control) treated animals had the same spine density. Second, experience decreased membrane ERα levels, which are required to for the induction of sexual receptivity (Dewing et al., 2007; Christensen and Micevych, In Press). At this point, it is not clear how Arc is interfering with sexual receptivity. One idea is that Arc, which has been shown to regulate endocytosis of AMPA receptors (Waung et al., 2008), also may be increasing the rate of mERα internalization. Further experiments will be needed to explore this idea.

EB treatment alone did not induce Arc in the VMH and ARH (Figure 2 in Chapter 4) (Flanagan-Cato et al., 2006; Christensen et al., In Prep). Although in vitro estradiol was reported

to upregulate Arc (Chamniansawat and Chongthammakun, 2009). In vitro, Arc levels have been reported to be increased by the activation of mGluR5 (Kumar et al., 2012). This suggests a remarkable degree of specificity since estradiol activates mGluR1a in the ARH, and mGluR1a is in the same family of mGluR receptors as mGluR5. Although ERα can also associate with mGluR5, very little mGluR5 immunoreactive staining has been demonstrated in the ARH (van den Pol et al., 1995). Another possibility is that salient sensory information that can induce Arc is carried by glutamate that activates NMDA receptors. In fact the first induction of Arc was attributed to activation of NMDA receptors (Lyford et al., 1995).

Is this Arc-mediated inhibition of sexual receptivity present in the gonadally intact mating female rat? In the normally cycling female rat, estradiol levels rise and reach a peak on proestrus, causing the release of progesterone. This progesterone induces a surge of luteinizing hormone (LH) that results in ovulation. The female is maximally receptive after the progesterone surge, thus optimally timing mating with ovulation. Since my goal was to understand the estradiol signaling, the estradiol and progesterone induced lordosis behavior was not examined very extensively. I tried progesterone treatment 26 hours after estradiol, which abrogated the increase in Arc in naïve and experienced animals (Figure 3 in Chapter 4) (Christensen et al., In Prep). In keeping with previous results, these females were fully sexual receptive. The inhibition of Arc upregulation in naïve animals is interesting because it suggests an important role for progesterone in the regulation of future behavior. In estradiol-only behavior tests, lordosis is eventually inhibited and the experienced animals are half maximally receptive, but if progesterone is added, maximal receptivity can be achieved on a subsequent behavior test, even if the lordosis is induced by only estradiol. These actions may represent the way progesterone “resets” the circuit and allows it be ready for another cycle of estradiol.

My working hypothesis is that Arc, in the intact animal, acts as a molecular gate for lordosis behavior. Arc activation was inhibited in all females treated with progesterone: naïve,

experienced, and experienced treated with progesterone then tested with EB (Figure 3 in Chapter 4) (Christensen et al., In Prep). Surprisingly, Arc activation seems to coincide with "empty matings," occurring when the females are not ovulating. A female that undergoes an empty mating, designated by the lack of circulating progesterone, has Arc upregulated, which attenuates future sexual behavior. When estradiol and progesterone are present, Arc is downregulated and the hypothalamus is responsive - producing full sexual receptivity.

Together, the regulation of the ARH by Arc shows that the hypothalamus is significantly changed by mating. Arc was upregulated by the first behavior test but deficits in sexual receptivity continued for the duration of the experiment. The behavior is restored by blocking Arc expression. Where and on what Arc acts upon is still unclear, and further investigation is required to understand the mechanism of Arc action.

Summary

The regulation of sexual receptivity is complex and requires precise timing and the coordination of multiple molecular changes within the hypothalamus. In addition, the behavior itself seems to modify both the molecular aspects and the synaptic connections of the circuit. Membrane ERα transactivation of mGluR1a in the ARH is required to initiate a signaling cascade that includes PKCθ (Dewing et al., 2007; Dewing et al., 2008). The trafficking of ERα to the membrane requires the scaffold protein, CAV1 (Christensen and Micevych, In Press). The activation of mERα not only activates the ARH to MPN circuit, leading to the activation and internalization of MOR, but also stimulates the phosphorylation of cofilin, an action which is required for the growth and maturation of dendritic spines (Christensen et al., 2011). Estradiol initiates the increase in spinogenesis in the ARH that is required for sexual behavior (Christensen et al., 2011). During sexual behavior, the circuit is modified in part by the

activation of the immediate early gene Arc. Arc is upregulated after the first mating, and if progesterone does not stimulate ovulation, Arc will decrease sexual receptivity (Christensen et al., In Prep). Although I focused on the ARH regulation of sexual receptivity, estradiol regulates a number of physiological functions in this part of the hypothalamus: energy balance, negative feedback onto gonadotropin releasing hormone neurons (GnRH), and temperature regulation (Kellert et al., 2009; Roepke et al., 2011; Mittelman-Smith et al., 2012). How changes in spine density and Arc regulate those actions is at present unknown but is important to understand. Membrane estradiol actions have been implicated in all of these events although the responsible ER remains to be eludicates and may not be ERα. The present studies demonstrate that together the mERα, estradiol-mediated spinogenesis, and Arc upregulation represent a part of the required changes needed for the coordinated control of sexual behavior in females.

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