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# Latent Sex Differences in Mechanisms of Estradiol-Induced Excitatory Synaptic Potentiation in the Hippocampus

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**Abstract**

One of the most fascinating observations in the brain is that the neural connections change with experience and this phenomenon is called synaptic plasticity. Patterns of activity or neuromodulators can acutely induce changes in the synaptic strength in the brain. My thesis is focused on understanding the mechanisms of plasticity at the CA3-CA1 synapses in the hippocampus. Specifically, we studied the role of 17β-estradiol (E2) as a neuromodulator. Although, E2 is classically studied as a sex hormone, recent evidence shows that E2 can be locally synthesized in the hippocampus. Moreover, E2 can acutely potentiate excitatory synaptic transmission in the hippocampus. However, the mechanisms that underlie E2-induced potentiation are not well understood. More fundamentally in the hippocampus, the mechanisms of synaptic plasticity have been compared between males and females only in limited studies. In our experiments, we compared the acute E2 effect on excitatory synaptic transmission in both sexes. Through different electrophysiological experiments we found that E2 can acutely potentiate excitatory synaptic transmission very similarly in both sexes. Furthermore, this potentiation is synapse specific and occurs largely by independent pre or postsynaptic mechanisms in both sexes.

While investigating molecular signaling that underlies E2-potentiation in both sexes, we found that although the overall magnitude of E2-potentiation is similar between sexes, the underlying molecular mechanisms differ. First, we observed a sex difference in the requirement of different estrogen receptors (ERs). We found that different ERs not only participate in pre or postsynaptic components of potentiation, but their

requirements are different between sexes. Downstream of ERs, we found that the requirement of kinases like PKA and calcium sources like internal stores and L-type calcium channels also differ between sexes. Conversely, the requirement of some kinases like Src, ROCK, MAPK and CAMKII was similar between sexes. Investigating downstream postsynaptic mechanisms revealed that either an increase in AMPAR number or conductance could underlie E2-potentiation. In females, majority of E2-responsive spines show an increase in conductance, while in males half of the E2-responsive spines show an increase in conductance and the other half show an increase in number. Moreover, we found that in females this increase in conductance occurs due to replacement with calcium permeable AMPARs at the synapses following E2-application. Interestingly, we found that the sex difference in the requirement of PKA is generalizable to long term potentiation, which is one of the most commonly studied synaptic plasticity phenomenon at these synapses.

Overall, our studies have described the role of different molecular signaling components that underlie E2-induced excitatory synaptic potentiation. We found that E2-potentiation can occur via activation of multiple signaling cascades and moreover, some of these signaling pathways are different between sexes. Sex differences in the mechanisms of synaptic plasticity in a non-reproductive brain region like the hippocampus fills a gap in our understanding of synaptic plasticity. Moreover, it aids future research to broaden the therapeutic strategies with the possibility of sex-specific therapeutics to treat neurological and neuropsychiatric disorders.

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# Chapter 1: General Introduction

## 1.1 Synaptic plasticity

The human brain has 86 billion neurons that are believed to have approximately quadrillion synapses between them. Although within a species the overall brain structure is genetically hardwired, but the connections can change with external stimuli. This process of experience-dependent changes in synaptic connectivity is called synaptic plasticity. The synaptic plasticity phenomenon is conserved across species- from invertebrates to humans and it gives the brain an immense capacity to process, learn and remember new information, acquire new skills and develop thoughts throughout life. Synaptic transmission can either be enhanced or depressed by stimuli, and these alterations span wide temporal domains ranging from seconds to long-term modifications. In 1890, William James first adopted the term plasticity in his book “the Principles of Psychology”, which denoted changes in nervous paths associated with the establishment of habits. Ramon y Cajal further conceptualized synaptic plasticity in his neuronal doctrine. Polish neuroscientist Jerzy Konorski first used the term ‘neuroplasticity’ in context of memory formation in 1948. Konorski studied how reflexes are conditioned upon specific stimulus and proposed a theory in which neurons that have been activated by closeness of an active neural circuit, change and incorporate themselves into that circuit (Berlucchi and Buchtel, 2009). Neurophysiologist Donald Hebb in 1949 further developed theoretical models explaining how memories are formed in the brain by a process of synaptic modification that strengthens connections when presynaptic activity correlates with postsynaptic firing (Morris, 1999). This

proposed function for synaptic plasticity, forming a memory trace following the detection of two coincident events, suggests a cellular basis for behavioral phenomena such as Pavlovian classical conditioning (Pavlov, 2010). In the late 1950s, through studies on patient Henry Molaison, the hippocampus was recognized as a brain region important to form new memories (Scoville and Milner, 1957) and gave researchers a brain locus to test theories of synaptic modification. Early experiments demonstrated that synaptic plasticity can be induced by specific patterns of electrical activity. Experiments by Bliss and colleagues in rabbit hippocampus showed that repeated high frequency electrical stimulation resulted in an increased synaptic transmission in the hippocampus, which lasted up to 14 hours after stimulation (Bliss and Lomo, 1973). They called this phenomenon of increase in the synaptic strength “long-term potentiation” (LTP). Interestingly, a few years later researchers found that if the electrical stimulation is given at a low frequency at the same synapses, it depresses the synaptic transmission and is called long-term depression (LTD) (Dunwiddie and Lynch, 1978). Apart from high frequency electric stimulation, synaptic plasticity could also be induced based on the timing of action potential firing at presynaptic neurons compared to postsynaptic neurons. For example, synaptic potentiation occurs when a presynaptic neuron fires before the postsynaptic neuron and synaptic depression when a postsynaptic neuron fires before presynaptic neuron (Markram et al., 1997; Bi and Poo, 1998). Although synaptic plasticity has been observed in almost all parts of the brain, given how well defined the electrical inputs and outputs are, the hippocampus has remained the model brain region to study principles of different synaptic plasticity forms. Since its discovery,

LTP was proposed as a cellular correlate of memory formation in the hippocampus, but experimental evidence of whether LTP underlies memory formation came much later. Indirect occlusion experiments showed that inducing LTP in the hippocampus where synapses can't get further potentiated impaired spatial learning (Moser et al., 1998). *In vivo*, LTP was observed by recording field excitatory potentials in the hippocampus of animals following a single trial inhibitory avoidance task (Whitlock et al., 2006).

Another mechanism to induce synaptic plasticity is neuromodulation. In addition to neurotransmitters like glutamate, GABA and acetylcholine that are required for synaptic transmission, there are chemicals that when released, modulate the strength of the synaptic connections and these are called neuromodulators. There are now more than 20 neuromodulators identified in the hippocampus that alter synaptic transmission when released. Some of these chemicals are synthesized outside the hippocampus, in other brain regions or even peripheral organs, and get released in the hippocampus. These are classified as extrinsic neuromodulators (Marder, 2012). Some examples of extrinsic neuromodulators are dopamine, serotonin, norepinephrine. Other chemicals modulate synapses within a brain region where they are synthesized and are called intrinsic neuromodulators (Marder, 2012). Example of intrinsic neuromodulators in the hippocampus are endocannabinoids, cholecystokinins and neuropeptide Y (Kosaka et al., 1985; Colmers et al., 1987; Marsicano and Lutz, 2006). In some instances, described later, even chemicals like glutamate, acetylcholine can act as in intrinsic neuromodulators. Principles of neuromodulation were first conceptualized in the invertebrate sensory and motor circuits. One of the first studies using extracellular

recordings of the neuro-muscular junction potentials, described the faciliatory actions of serotonin in the muscle of crayfish (Dudel, 1965). In addition to altering baseline synaptic transmission, neuromodulators can also regulate the threshold, magnitude or duration of activity dependent synaptic plasticity.

It is fascinating that diverse mechanisms exist in the brain to induce synaptic plasticity. Are some these mechanisms redundant to influence neural networks or does each mechanism possess a distinct role in the brain? Transient forms of synaptic plasticity have been associated with short-term adaptation to sensory inputs, transient changes in behavioral states, and short-lasting forms of memory. More lasting changes are thought to play important roles in the maturation of neural circuits during development and with long-term forms of memory in the adult nervous system. Another way to understand the distinctions between synaptic plasticity mechanisms would be to understand what downstream mechanisms gets activated and underlie different forms of plasticity. In the next section, using hippocampus as a model brain region, I will describe some commonalities and some distinctions in the underlying mechanisms of different forms of plasticity. Moreover, I will describe the electrophysiological, pharmacological and genetic tools developed by researchers to study these mechanisms.

## 1.2 Principles of synaptic plasticity

### 1.2.1 *Basics of excitatory synaptic transmission at CA3-CA1 synapses*

In order to understand the mechanisms of synaptic plasticity, it is important first to recognize some basic properties of synaptic transmission at CA3-CA1 synapses. CA1 pyramidal neurons receives ~30,000 excitatory connections from CA3 neurons at the dendrites (Megias et al., 2001). At the presynaptic axonal terminals of these synapses, action potential generation leads to calcium influx. Proteins associated with glutamate synaptic vesicle sense this calcium influx and this results in exocytosis of the vesicles. The vesicular release probability at these synapses is low (about 0.1-0.2, (Megias et al., 2001)). The presynaptic synaptic vesicular cycle is a highly regulated process and is discussed in greater detail later. Once glutamate is released, it binds to postsynaptic glutamate receptors. Ultra-structurally, a postsynaptic site is defined by the presence of a postsynaptic density (PSD) and the synaptic receptors are clustered at the PSD region. The three most common glutamate receptors are named after the specific pharmacological agents that activate them- α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPARs), N-methyl-d-aspartate (NMDARs) and kainate receptors (Traynelis et al., 2010). Different subunits of AMPAR, NMDAR and kainate receptors have been identified. There are at least five forms of NMDA receptor subunits (NMDA-R1, and NMDA-R2A through NMDA-R2D); different synapses have distinct combinations of these subunits, producing a variety of NMDA receptor-mediated postsynaptic responses (Traynelis et al., 2010). Similarly, AMPARs are composed of four types of subunits, designated GluA1, GluA2, GluA3, and GluA4. Out of these, three AMPARs predominantly participate in baseline synaptic transmission (Traynelis et al., 2010). AMPARs are voltage insensitive, ligand gated ion channels that exist as hetero-

tetramers and are mostly composed of two different subunits. Once bound to glutamate, they undergo a conformational change and most subunits allow influx of Na+ and K+ ions, which result in the depolarization of the postsynaptic cell (Traynelis et al., 2010). In adult hippocampal synapses, NMDARs are calcium permeable and most AMPARs are calcium impermeable. However, there are some calcium permeable subunits of AMPARs which will be discussed later. Glutamate released by vesicles reaches a maximum concentration of 1mM (Clements et al., 1992) and within a millisecond is released from the synaptic cleft actively by the nearby glutamate transporter system in the glial cells.

It is a fascinating observation that a few seconds change in the electrical activity is transduced into distinct biochemical signaling that can vary from few minutes to days. It is now well established that the activation of distinct protein signaling cascades can either increase or decrease the synaptic strength of neurons. Additionally, the type of signaling that gets activated also determines the magnitude and duration of synaptic plasticity. Theoretically, synaptic plasticity occurs via direct regulation of pre- and/or post synaptic transmission machinery. For example, synaptic plasticity could arise due to changes (a) in the number of neurotransmitter receptors in the postsynaptic membrane, (b) in the quantity of neurotransmitters released from the presynaptic neuron, or (c) in the probability of vesicular release that contain neurotransmitters. The pre and/or postsynaptic locus of plasticity provide the first distinction between the different forms of plasticity. For example, at CA3-CA1 synapses, short term plasticity like paired-pulse facilitation occurs due to an increase in the presynaptic glutamate

release probability, whereas LTP at CA3-CA1 synapses occurs due to postsynaptic increase in the glutamate sensitivity.

### 1.2.2 *Electrophysiology tools to study synaptic physiology*

The methods to study synaptic plasticity have evolved over the last century. However, direct measurements of electrical activity are still the most reliable measurements of changes in synaptic strength. The early LTP experiments were done *in vivo* using field excitatory post-synaptic potential (fEPSPs) recordings in rabbit hippocampus, but to tease apart the cellular and molecular mechanisms in greater detail a more controlled system was required. Almost 15 years later, LTP experiments began to be done in acute transverse brain slices with intact hippocampal circuit. Some of the advantages of slices are- access to specific cells, intracellular recording, pharmacological access, control of the internal and external concentration of ions, thereby controlling the driving force of ions. To study LTP at CA3-CA1 synapses, fEPSPs can be recorded by placing the stimulating electrode and the recording electrode close to each other in the stratum radiatum region. These fEPSPs are average responses from many synapses. Increase in the fEPSP slope or amplitude will suggest an increase in synaptic strength. Another method to study LTP on a fewer groups of synapses within a cell is by performing whole cell electrical recording, where the recording electrode is to gain full access inside the cell. With the access to the whole cell, the cell membrane can be voltage clamped at different potentials. In the whole cell voltage clamp recording, excitatory postsynaptic currents (EPSCs) are

measured from CA1 neurons before, during and after LTP stimulation. Like fEPSPs, increase in EPSC amplitude reflects an increase in synaptic strength. One advantage with whole cell voltage clamp recording is that we can study the role of different voltage sensitive ion channels and in isolation and changes in their properties during LTP induction. Another advantage of whole cell recording is that the drugs to block specific proteins can be applied intracellularly, specifically into the cell from which the EPSCs are being recorded. In the whole cell configuration, action potential independent miniature EPSCs (mEPSCs) can also be recorded in the presence of TTX (tetradotoxin), a sodium channel blocker. Each mEPSC occurs due to a single spontaneous (action potential-independent) glutamate vesicular release. Following LTP, changes in the amplitude of mEPSCs suggest a postsynaptic change in glutamate sensitivity and changes in frequency of mEPSCs suggest a presynaptic change in the glutamate release probability. A disadvantage of using whole cell recording is dialysis. When micropipette containing internal solution is impaled into the cell, it dialyses or dilutes the cellular contents and can affect synaptic plasticity. For example, in whole cell LTP experiments, it was shown that LTP induction was impaired, if the induction protocol was given 15 mins after whole cell access was achieved (Malinow and Tsien, 1990). Methods to study the molecular signaling and tools to measure changes in the presynaptic component of plasticity are described later.

### 1.2.3 *Activity dependent synaptic plasticity requires calcium*

One of the common features of different forms of synaptic plasticity is activity-dependent calcium influx. Calcium acts as a transducer or a second messenger, that converts electrical activity to specific biochemical signaling. Experiments with calcium chelators demonstrated that hippocampal LTP is calcium dependent. In acute hippocampal slices, intracellular injection with ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) in the postsynaptic cell, blocked LTP at CA3-CA1 synapses (Lynch et al., 1983). We now know that many different sources of calcium are required for activity dependent synaptic plasticity.

### 1.2.3 (a) *NMDAR as source of calcium in activity dependent synaptic plasticity*

Since the pharmacological characterization of different glutamate receptors (Mayer and Westbrook, 1984), it was recognized that calcium influx in many forms of LTP occurs via NMDARs at CA3-CA1 synapses for inducing potentiation (Luscher and Malenka, 2012). Studies in hippocampal slices show that NMDARs antagonist, 2R)-amino-5-phosphonovaleric acid (APV), blocks LTP induction at CA3-CA1 synapses (Collingridge et al., 1983). NMDARs are voltage dependent glutamate receptors and require cell depolarization to remove the magnesium block and subsequently increase the postsynaptic calcium concentration. To activate NMDARs during LTP induction, the postsynaptic depolarization is usually established either by high frequency stimulation of presynaptic axons or by experimentally depolarizing the postsynaptic cell and giving low frequency stimulations to the presynaptic axons. Because its contribution to

postsynaptic responses requires both presynaptic release of glutamate and postsynaptic depolarization, the NMDAR is often referred to as a ‘coincidence detector’. Surprisingly, at the same synapses, calcium influx through the NMDARs are also required for LTD (Luscher and Malenka, 2012). Theories first modelled by John Lisman and later experimentally proven, showed that it is not just the calcium influx but the peak amplitude, duration and location of calcium currents that determines whether the synapses will be potentiated or depressed (Luscher and Malenka, 2012). Indeed, it is now fairly well accepted that modest activation of NMDARs leading to modest increases in postsynaptic calcium are optimal for triggering LTD, whereas much stronger activation of NMDARs leading to much larger increases in postsynaptic calcium are required to trigger LTP (Malenka, 1994).

1.2.3 (b) *Other sources of calcium in activity dependent synaptic plasticity*

There are some forms of LTP that are NMDAR-independent. They are still calcium dependent and calcium in these forms of LTP comes from other sources like L-type voltage sensitive calcium channels. They get activated at depolarized potentials and are shown to be expressed at both pre and postsynaptic terminals in the hippocampus. Initial observations made by Roger Nicoll’s lab showed that calcium influx specifically by L-type calcium channels can also potentiate excitatory synaptic transmission (Kullmann et al., 1992). In the following studies, recording fEPSPs in rat hippocampal slices and using specific antagonists, they showed that distinct theta burst stimulation paradigms (TBS) recruit either NMDARs or L-type calcium channels (Morgan and Teyler, 2001).

Another calcium source that exist at the synapses of hippocampal neurons is the endoplasmic reticulum. Initially described as the “spine apparatus” in the dendritic spines observed by the electron microscopy studies, was later found to be an extension of a smooth endoplasmic reticulum (Spacek and Harris, 1997). Endoplasmic reticulum or internal calcium stores are also present in the presynaptic axonal terminals. Inositol Phosphate-3 receptor (IP3R) and Ryanodine Receptor (RyR) are the two receptors that are expressed on the internal calcium stores (Segal and Korkotian, 2014). Initial studies showed that CA1 pyramidal neuron spines have only RyR and only dendrites have both IP3R and RyR, thus hinting at the possibility that they differentially regulate the calcium release from the internal stores (Sharp et al., 1993). These receptors can be targeted using specific pharmacological drugs and have been studied in different forms of plasticity at CA3-CA1 synapses. Unlike the role of NMDARs and L-type calcium channels, the role of internal calcium stores is debatable. Early studies using SERCA pump blocker- thapsigargin, which depletes calcium stores, blocks the induction of LTP at these synapses (Harvey and Collingridge, 1992). Later studies show that internal calcium stores are required in constraining the magnitude of LTP. In separate studies, specific mouse mutants for IP3R and RyR, show an increase in the magnitude of LTP as compared to wild types (Futatsugi et al., 1999; Fujii et al., 2000). Even though their role in LTP is speculative, these calcium sources could be required for other forms of synaptic plasticity.

Although the magnitude of potentiation is similar across LTP forms that requires different calcium sources, differences have been reported in the synaptic locus of LTP,

that is, whether there is an increase in presynaptic glutamate release or a postsynaptic increase in glutamate sensitivity. NMDAR dependent LTP at CA3-CA1 synapses is shown to be postsynaptic in that it increases excitatory synaptic transmission mainly by increasing glutamate sensitivity. On the other hand, L-type calcium channels induced LTP is shown to be presynaptic (Stricker et al., 1999; Grover et al., 2009). Thus, to understand the mechanisms of synaptic plasticity, both pre and postsynaptic mechanisms are discussed below.

### 1.2.4 *Neuromodulator requires specific receptors*

In comparison to the activity-dependent synaptic plasticity, the mechanisms of neuromodulation are more diverse. Some factors that bring diversity are as follows. There can be intrinsic neuromodulation that depends on the ongoing synaptic activity whereas extrinsic neuromodulation whose release is independent of the ongoing activity and is influenced by external stimuli. There can be volume transmission, where the neuromodulator affects synapses of many cell types in a brain region versus synaptic transmission where it affects only one or few synapses within a cell. There can transient effect of a neuromodulator versus long lasting effect. Given how diverse the effects of neuromodulators can be, we can imagine the diversity of mechanisms that underlies neuromodulation. However, whether the modulation is driven by extrinsic or intrinsic sources, similar to activity dependent plasticity, the pre or post synaptic loci of action is an essential factor and can include modification of (1) the properties of presynaptic neurotransmitter release, (2) the modification of postsynaptic responsiveness/receptor

signaling, and/or (3) the modulation of the postsynaptic intrinsic electrical and biochemical properties or gene regulation.

One of the common features of most neuromodulators is that they act on neurons via specific receptors. These receptors are not necessarily at the synapses and can be extra-synaptic, but still most of these receptors are membrane associated. Based on their structure and whether they can pass currents, these receptors can be broadly categorized into two main types- ionotropic or metabotropic. Ionotropic receptors are ligand gated ion channels like NMDARs, and when bound to a neuromodulator allow flow of ions through the same protein complex that binds the ligand. For example, 5HT-3 receptors are ionotropic serotonergic receptors that are primarily expressed in the interneurons in hippocampus. Raphe nuclei synthesizes serotonin in the brain, and it gets released in the hippocampus. 5HT-3 upon activation at the presynaptic terminals increase inhibitory synaptic transmission on CA1 pyramidal neurons (Passani et al., 1994). More common neuromodulator receptors are metabotropic receptors that belong to a seven trans-membrane receptor family called the G-protein coupled receptor (GPCRs) family. Upon binding of neuromodulators, they undergo conformational change and activate myriad of downstream molecular signaling via secondary messengers. Although calcium is the most common secondary messenger, many other secondary messengers have now been discovered like cyclic adenosine mono phosphate (cAMP), DAG (diacylglycerol) and IP3, that activate specific downstream signaling in the brain. Metabotropic glutamate receptors (mGluRs) are examples of metabotropic receptors that get activated when bound to glutamate. mGluR1 and

mGluR5 subtypes are primarily expressed in different parts of CA1 neurons. Activation of mGlur1 triggers increase in intracellular calcium concentrations, depolarization of pyramidal neurons in the CA1 region and an elevation in the frequency of spontaneous inhibitory post-synaptic potentials (Mannaioni et al., 2001).

Most of the neuromodulators are released from the presynaptic terminal and affect the physiology of either its own axonal terminal (autocrine) or the postsynaptic spines, dendrites or cell (paracrine). However, there are a few neuromodulators that affect the presynaptic physiology in a retrograde fashion. An example of these is endocannabinoids, which are synthesized and released by the postsynaptic cell and binds to CB1 receptors on the presynaptic terminal and suppresses glutamate release probability.

In addition to influencing the synaptic transmission, neuromodulators can also change the intrinsic properties of neurons. Studies have shown that activating muscarinic acetyl choline receptors (mAChRs) activates calcium release from internal stores which then activates calcium-dependent potassium channels. The potassium channel activation then results in the hyperpolarization of the cell membrane (Gulledge and Kawaguchi, 2007). Other neuromodulators also affect intrinsic properties like after-hyperpolarization currents, calcium-dependent potassium conductance, spike-frequency adaptation, etc.

As briefly mentioned above, apart from having an independent effect on the neurotransmission, neuromodulators can also modulate activity dependent synaptic plasticity. One well studied example of this is the effect of brain derived neurotropic

factor (BDNF) on LTP at CA3-CA1 synapses. Through many *in vitro* and *in vivo* studies using pharmacological and genetic engineering tools, it is now established that BDNF contributes to LTP induction and is thought to be required for maintenance of LTP at CA3-CA1 synapses (Lu et al., 2008). Thus, both activity dependent synaptic plasticity and neuromodulation could work hand in hand to affect neuronal physiology.

### 1.3. Molecular signaling that underlies synaptic plasticity

So far, I have described how electrical activity or neuromodulators affect neuronal physiology and require NMDAR-dependent calcium influx or specific receptor-dependent recruitment of secondary messengers respectively. What happens downstream are numerous biochemical signaling cascades both at pre and/or postsynaptic terminals that when activated influence specific aspects of synaptic physiology. Common themes in the molecular signaling emerge depending on the type and duration of synaptic plasticity. For instance, similar kinase and phosphatase signaling gets activated during mechanisms of excitatory synaptic potentiation. Similarly, for synaptic mechanisms that require long-lasting changes, similar molecular signaling that increases specific gene expression will be recruited. As it is difficult to cover all the protein signaling cascades that underlie synaptic plasticity mechanisms, in this section molecular signaling pathways that are required for LTP at CA1 excitatory synapses in the hippocampus are discussed and compared it with other forms of plasticity wherever required. Most of the evidence of molecular signaling comes from fEPSP or EPSC LTP experiments *in vitro* acute slices or neuronal hippocampal cultures

in the presence of drugs that either block or activate the protein of interest. Another approach to study proteins of interest that is described is to manipulate genes that make these proteins in the whole animal or specific cells in the animal using genetic manipulations.

### 1.3.1 *Molecular mechanisms underlying LTP*

#### 1.3.1 (a) *Postsynaptic mechanisms to induce LTP*

Based on the duration of potentiation, LTP can be divided into early and late component. Early component is the protein synthesis independent phase while late LTP is the protein synthesis dependent phase (Frey et al., 1993; Huang and Kandel, 1994). Calcium-calmodulin dependent protein kinase II (CaMKII) and protein kinase C (PKC) are two calcium dependent kinases that were identified to be required in early LTP. These kinases are required to induce LTP, but their ongoing activity is not required for the maintenance of LTP (CaMKII/ PKC). Protein kinase A (PKA) was shown to be required for the protein synthesis dependent or Late LTP and not required for the early LTP (Huang and Kandel, 1994). Although some studies showed that PKA could modulate other kinases like CAMKII even during early LTP (Blitzer et al., 1998). PKA is activated by the cAMP signaling that is canonically downstream of GPCR signaling, and can also be activated by calcium via calcium sensitive adenylyl cyclase (Wong et al., 1999). PKA in L-LTP then in crosstalk with other kinases phosphorylates a transcription factor called cAMP response element binding protein (CREB) to activate expression of different genes to maintain potentiation (Roberson et al., 1999). The activity of these

kinases is short-lived as they are only active in the presence of the active signal (calcium or other secondary messengers). Interestingly, CaMKII has an autophosphorylation property and can remain active even in the absence of an active signal (Lisman et al., 2012). Due to this unique property of CAMKII, it was initially thought to be required for the maintenance of early LTP at CA3-CA1 synapses, however no experimental data was able to prove this. In addition to these kinases getting deactivated in the absence of an active signal, other proteins called phosphatases, that are also activated by calcium, can actively de-phosphorylate these kinases. Phosphatases like Protein Phosphatase 1 (PP1) and Calcium/calmodulin-dependent phosphatase 2B (calcineurin) have been shown to be involved in LTP (Wang and Kelly, 1997; Allen et al., 2000). Several other kinases like Mitogen activated protein kinases (MAPKs), Tyrosine receptor activated kinase (Src), Rho associated kinases (ROCK) and Phospho-inositide-3 kinase (PI3K) were each shown to be required for induction of early LTP (English and Sweatt, 1997; Lu et al., 1998; Opazo et al., 2003). Each of these kinases has been shown to play different function to initiate, express or maintain potentiation. Similarly, apart from CREB, several other transcription factors like CAAT-Enhancer Binding Proteins (C-EBP), Early growth response protein (Egr), Activator Protein-1 (AP-1) and c-rel have been identified to be important in L-LTP (Alberini, 2009). Two of the immediate early genes that get activated following binding of some of these transcription factors are now established markers of L-LTP; these are activity regulated cytoskeletal (Arc) protein (Waltereit et al., 2001) and c-fos protein (Dragunow et al., 1989).

To make biochemical signaling even more interesting new optical methods have now provided direct evidence that LTP is synapse specific. A dendritic spine can now be identified and stimulated using two-photon glutamate uncaging. Such uncaging gives rise to a glutamate pulse with submicron dimensions that directly targets the identified spine and induces LTP at the synapse on that spine. Nearby synapses that are only microns away are not potentiated (Matsuzaki et al., 2004). Given the complex biochemical cascade that is involved in LTP and the small distances that separate different synapses, it is remarkable that synapse specific LTP can be achieved. Furthermore, it is believed that there is compartmentalization of molecular signaling within each dendritic spine (given the morphology of spines, it makes it biophysically possible to contain kinases and phosphatases) that gives it the capacity to potentiate independent of the effects on neighboring spines (Colgan and Yasuda, 2014).

Are the downstream effects of the kinases redundant or do they all regulate different parts of synaptic plasticity? Many studies after the early observation of the requirement of these kinases in LTP went into understanding their specific function in LTP. Studies have shown that upon LTP stimulus, CaMKII gets activated by calcium entry and is translocated from the cytoplasm to the synapses (Lee et al., 2009). Again, this translocation is synapse specific. Electron microscopy immunolabelling and co-immunoprecipitation studies have shown that once activated in the synapses CaMKII binds to the post synaptic density (PSD) and NR2B subunit of NMDARs (Leonard et al., 1999). This CaMKII-NR2B complex contributes to the induction of LTP (Zhou et al., 2007). The exact role of CaMKII-NR2B complex is not yet known but CAMKII at the

PSD is proposed to play many different roles during LTP induction that are described below. Calcium influx also activates many other kinase-signaling pathways. Ras-ERK-Raf signaling for example gets activated and is required to increase in AMPARs exocytosis onto the membranes (Qin et al., 2005). It is now well established that there are structural changes that underlies LTP at CA3-CA1 synapses. In hippocampal cultures, initial studies showed that following HFS there is an increase in the dendritic spine size (Matsuzaki et al., 2004). Src/ROCK signaling is shown to play role in the facilitating actin polymerization and thereby increasing the spine size during LTP (Saneyoshi et al., 2010; Nakahata and Yasuda, 2018). One of the working models it that the increase in the spine size would allow more AMPAR to be incorporated at the synapses thereby increasing the response to glutamate release. Studies using super-ecliptic pHluorin tagged with AMPAR subunits show an increase in the surface expression of AMPARs following high frequency stimulation (Kopec et al., 2006). Although these new AMPARs are expressed at the membrane, they are still extra-synaptic and would not participate in the synaptic transmission until they get translocated to the synapse. Two mechanisms have been described experimentally via which more AMPARs could be inserted at the synapses. First mechanism is the CaMKII phosphorylation of auxiliary AMPAR subunits like stargazin to translocate extra synaptic AMPAR to the PSD (Opazo et al., 2010). Another property of AMPAR is that they are mobile and even during basal synaptic transmission they laterally move in and out of the synapses (Tardin et al., 2003). Thus, the second mechanism is to immobilize these AMPARs at the synapse and this is also thought to be mediated by CAMKII (Opazo et

al., 2010). Apart from kinases indirectly regulating different aspects of postsynaptic physiology, some of them directly regulate the single channel properties of the glutamate receptors. PKC has been shown to phosphorylate S1303 and S1323 of NR2B subunit of NMDARs to reduce magnesium block and increase NMDAR mediated currents (Ben-Ari et al., 1992; Liao et al., 2001). CaMKII has been shown to phosphorylate S831 of subunit of AMPAR GluA1 subunit and increase the single channel conductance (Kristensen et al., 2011). PKA has been shown to increase the mean open probability of AMPAR by phosphorylating S831 subunit (Banke et al., 2000).

Although a lot of mechanisms have been described to induce and express LTP, how early LTP is maintained is still debatable. CaMKII is one of the favorite candidates for LTP maintenance because of the unique property of CAMKII to remain active even in the absence of calcium. CaMKII has been studied for the last 30 years and its role in maintenance is still controversial. Initial pharmacology studies used drugs like KN93 that only blocked the activation of CAMKII (bound to the calcium/calmodulin binding site). These drugs had no effect on the LTP induced potentiated synapses. In recent years drugs like tatCN21 have been designed to block the catalytic subunit and the autophosphorylation of CAMKII. Using these drugs, people found that at high concentrations, the potentiation following LTP stimulus was partially reduced, thus suggesting that autophosphorylation of CAMKII could be important in maintenance of LTP (Vest et al., 2010). Second kinase that is argued to play role in LTP maintenance is Protein kinase Mζ (PKMζ). PKMζ is an isoform of PKC and studies show that when the catalytic site of this kinase is blocked, the potentiated synapses come back to their

baseline strength (Sacktor and Hell, 2017). Third protein thought to play a role in LTP expression and maintenance is calcium permeable AMPAR (cpAMPARs). Under basal conditions, it is known that AMPAR mostly contain GluA2 subunits which makes them calcium impermeable. Following LTP however, there is reorganization of AMPAR subunits at the synapse and some of these receptors lack GluA2 subunits which thus become calcium permeable. Moreover, PKMζ is shown to play role in trafficking these cpAMPARs onto the synapse. It is hypothesized that cpAMPAR could be a longer lasting source of calcium and could continually activate downstream kinases, whose ongoing activities may be required for the maintenance of LTP. However, a study has shown that cpAMPAR are only transiently inserted following LTP induction and are responsible for expression but not maintenance of LTP (Plant et al., 2006).

1.3.1 (b) *Presynaptic mechanisms to induce LTP*

Many of the kinases described above have been shown to also play a role in modulating the presynaptic physiology. Directly investigating the presynaptic physiology using conventional electrophysiological tools has been very challenging. Thus, before talking about the mechanisms that underlie presynaptic potentiation, I first describe some of the ways to study/estimate the changes in the presynaptic physiology. It was first discovered that neurotransmitters are released in quanta through observation of spontaneously occurring miniature potentials and the observation that evoked postsynaptic responses correspond to integral multiples of the quantal unit (Fatt and Katz, 1952; Kuno, 1964). Since its introduction, quantal analysis and its derivatives,

such as coefficient of variation (CV) and minimal stimulation, have been used widely to investigate whether a change in neurotransmitter release accompanies long-term changes in synaptic strength. Another way to understand presynaptic physiology is to record mEPSCs from the cell as described earlier. However, since the discovery of the silent synapses, which lack AMPARs (Isaac et al., 1995), it is debatable whether the increase in the changes in miniature frequency is purely due to changes in presynaptic release probability. In evoked experiments, paired pulse ratio (PPR) is another tool to estimate changes in presynaptic physiology, where two electrical stimuli are delivered to a presynaptic axon in rapid succession (few milliseconds apart), the second postsynaptic response often differs characteristically from the first. The ratio of the amplitude of the second EPSC response to that of the first is called the paired-pulse ratio (PPR). Changes in the paired pulse ratio is inversely correlated with the changes in the presynaptic release probability. Other more recent tools directly measure the calcium influx at the presynaptic terminals using chemical dyes like Fura, Fluo, etc. or genetic calcium indicators such as GCamp6. Using these tools in combination with others, scientists have studied the presynaptic functions of proteins.

Presynaptic neurotransmitter release occurs in a tightly regulated fusion of the synaptic vesicular membrane to the plasma membrane and this process is called synaptic vesicle cycle. From the readily releasable pool of vesicles, synaptic vesicles first dock at the active zone, and then an ATP-dependent priming reaction makes them competent for exocytosis when an action potential triggers calcium influx into the presynaptic terminal via voltage-gated calcium channels. Theoretically, two principle

points of regulating release could underlie presynaptic plasticity: (a) the amplitude and duration of calcium influx produced by an action potential, i.e., the conversion of an action potential to calcium; and (b) the release probability per given calcium concentration, i.e., the conversion of a calcium signal to exocytosis. For regulations at these two steps, there are different mechanisms observed experimentally to regulate calcium influx and release machinery. Increase in calcium concentration due to summation of calcium influx and residual calcium occurs during repeated stimulation cause an increase in the vesicular release probability. Broadening the action potential waveform can also increase the calcium influx and thereby potentially increasing the synaptic vesicular release. Presynaptic calcium channels can also be modulated by G-proteins in a voltage dependent and independent manner. GPCRs that are coupled with Gi/o proteins can inhibit both P/Q- and N-type calcium channel. G protein βγ subunits can bind to a cytoplasmic site on the calcium channel subunit, to stabilize the closed conformation of the channel (Herlitze et al., 1996). This inhibition is voltage dependent as it is favored at hyperpolarized potentials and can be relieved by strong membrane depolarizations or rapid trains of action potentials, thus allowing for activity-dependent disinhibition (Bean, 1989; Hille, 1994). In biochemical studies, PKC has been shown to phosphorylate Thr 422 site to increase N-type calcium channel activity (Hamid et al., 1999). PKA has been shown to indirectly increase P/Q-type by interfering with phosphatidylinositol 4,5 biphosphate (PIP2) that inhibit these channels (Zamponi and Snutch, 2002).

Finally, kinases can also directly regulate proteins involved in the release machinery. The Synapsin-1 is one of most studied proteins in different plasticity mechanisms and is synaptic vesicle-associated phosphoprotein that regulates the number of synaptic vesicles available for vesicular exocytosis. PKA has been shown to phosphorylates Synapsin-1 at serine 9 substrate and increase the rate of vesicular exocytosis (Menegon et al., 2006). Synapsin-1 is also phosphorylated by CaMKII and MAPK at different serine substrates to regulate synaptic vesicular docking and release. PKA has also been shown to phosphorylate RIM1α at serine 413, which is an active zone protein and regulates vesicular priming (Lonart et al., 2003). The above evidence comes mostly from biochemistry studies in cultured cells or from non-hippocampal synapses and thus further investigation is required to understand the mechanisms underlying presynaptic plasticity in the hippocampus. The limitation as discussed before is due to the technical challenges of studying functional presynaptic physiology.

### 1.3.1 (c) *Trans-synaptic mechanisms to induce LTP*

Apart from signaling pathways regulating pre and postsynaptic physiology that underlies synaptic plasticity, there is also evidence of some retrograde signaling and trans-synaptic signaling. An example is the retrograde actions of Nitric oxide (NO) that gets released in the postsynaptic cell and affects the presynaptic machinery during LTP. NO has been shown to be required in L-type voltage gated calcium channel dependent LTP at the CA3-CA1 synapses (Pigott and Garthwaite, 2016). PKC has been shown to activate NO synthesis from L-arginine in the postsynaptic cell and can pre-synaptically

regulate proteins at the active zone, the size of readily releasable pool, and vesicular recycling (Hardingham et al., 2013). The cell adhesion molecules, neurexin-neuroligins complexes that play role in synapse formation during development have recently been studied also in the context of synaptic plasticity (Sudhof, 2017). Neurexins and neuroligins are clustered in presynaptic and postsynaptic membranes, respectively, with their extracellular regions adhering to each other in the synaptic cleft. Intracellularly, neurexins and neuroligins interact with several molecular components that play role in baseline synaptic transmission. Pre-synaptically, neurexins bind to the calcium sensor synaptotagmin to regulate vesicular exocytosis (Hata et al., 1993), as well as to the scaffolding proteins, which modulate presynaptic calcium channels and actin filaments (Rui et al., 2017). Postsynaptically, neuroligins interact with glutamate receptors via scaffolding proteins, such as PSD95 (Irie et al., 1997). Experiments in Aplysia used oligonucleotides to deplete the mRNA of different adhesion proteins and found that depleting mRNA of neurexins and neuroligins blocked the serotonin induced potentiation (Choi et al., 2011). It still needs to be explored how exactly these adhesion molecules participate in different mammalian plasticity mechanisms.

### 1.3.2 *Molecular mechanisms underlying neuromodulation*

When it comes to signaling underlying neuromodulation, some of the components are similar, but there are differences as compared to mechanisms underlying LTP. As described above, most neuromodulator receptors are GPCRs and, upon binding of the neuromodulator, lead to the activation of receptor-associated heterotrimeric G proteins

and consequent downstream signaling. Heterotrimeric G proteins are composed of three subunits, α, β and γ. Ligand binding catalyzes the exchange of bound guanosine diphosphate (GDP) on the Gα subunit for guanosine triphosphate (GTP). The exchange in the guanine nucleotides leads to a reduction in the affinity of the Gα subunit for the Gβγ complex and functional dissociation of the heterotrimer. The dissociated G protein subunits can then transmit signals to effector proteins, such as enzymes and ion channels, resulting in rapid changes in the concentration of intracellular secondary messengers (Marinissen and Gutkind, 2001). GPCR signaling can be broadly categorized into 3 subtypes depending on which Gα protein binds to it. $G\alpha_s$ protein activate cAMP signaling. For example, D1 type dopamine receptors activate $G\alpha_s$ of signaling. $G\alpha_q$ activate PLC, PKC, DAG molecular signaling (Sibley and Monsma, 1992). Examples of $G\alpha_q$ type GPCRs are group 1 metabotropic glutamate receptors (Niswender and Conn, 2010). $G\alpha_{i/o}$ protein inhibit cAMP signaling and activates phosphodiesterases and phospholipases. Example of this are D2-type dopamine receptors (Jiang et al., 2001), CB1 receptors (Busquets-Garcia et al., 2018), adenosine type-1 receptors (Cunha, 2001). Activation of GPCRs have been shown to regulate cytosolic calcium levels. One such example is the activation of IP3 that can bind to IPR3 receptors on the internal stores and release calcium. Some GPCRs have also been shown to be physically coupled to ion channels to regulate resting membrane potential. Coimmunoprecipitation study showed that mGlur5 is physically coupled to L-type calcium channel and modulates calcium entry through these channels in the CA1 neurons (Kato et al., 2012). In contrast to only postsynaptic locus of NMDAR-dependent

LTP at CA3-CA1 synapses, the effects of activation of these GPCRs can be both pre and postsynaptic depending on where the receptor is expressed.

1.3.3 *Age dependent differences in molecular mechanisms underlying plasticity*

The synaptic plasticity phenomenon occurs throughout life and can have different roles in in different phases of development. The above described synaptic plasticity and neuromodulation experiments vary widely in the age of animals used. There is now evidence that different signaling components contribute in developing brain versus adults. One piece of evidence comes from studying PKA and CAMKII signaling underlying CA3-CA1 early LTP. Studies found that although in adults CAMKII is required and not PKA in induction of LTP, the roles of these kinases are reversed in neonatal animals, where PKA is required and not CAMKII in induction of LTP (Yasuda et al., 2003). Another age dependent signaling evidence comes from studying cpAMPARs in LTP, where it was found that cpAMPARs are required in expression of high frequency induced LTP in 8-week-old mice but not in a 4 week old mice (Lu et al., 2007). How does different signaling undergo such a developmental switch? One of the possible explanations comes from the observation that in neonatal animals ~50% of the synapses are made on the dendritic shafts and the increase in dendritic spines occur dramatically during P1-P12 (Fiala et al., 1998) thereby increasing the bio compartmentalization of the calcium transients and the specific molecular signaling it activates. Second explanation could be that the protein and receptor expression profiles on the synapse change as the brain develops and this results in activation of different

kind of signaling. Lastly, it could also be due to peripheral variability that affect these signaling; for example, different plasma levels of sex hormones in developing brain versus adult brain. Given the differences in the signaling, it is comprehensible but to be experimentally established that the functional role of LTP could be different between developing animals and adults.

### 1.4 Influence of estrogens on hippocampal physiology

Estrogens are classically categorized as hormones that are produced primarily in the gonads and adrenal cortex of both males and females. Estrogens are a class of steroid and are synthesized from cholesterol in vertebrates. Estrogens are lipophilic and thus, can move easily through cell membranes. However, they are usually carried throughout the body in the blood stream bound to either albumins or sex hormone binding globulins. Their immediate precursor molecules are testosterone and androstenedione, which get converted into estrogens by a process called aromatization and this process is mediated by P-450 aromatase enzyme. Although estradiol, estrone and estriol are different types of estrogens that can by synthesized, 17β-estradiol (E2) is the predominant form of estrogen in adults. Estrone is a significant estrogenic hormone contributor in both postmenopausal women and in men; estriol levels are significantly higher in pregnant women (Greenspan and Gardner, 2004). Apart from different plasma E2 levels across different menstrual/ estrous cycle in adult females, E2 levels have shown to vary across fetus, infants, adolescent and adult animals. Moreover, the levels of E2 vary between sexes in developing as well as adult brain (Rosenfield et al., 2000).

In the following sections diverse actions of E2 will been described mainly in the hippocampus and how some of these actions could be considered as neuromodulatory actions on the hippocampal physiology. For my thesis, it is important to distinguish and categorize the different functions of E2 and their mechanisms. Some acute functions are directly comparable to the E2 effects I observe on hippocampal physiology. Other E2 effects, especially during development could help us hypothesize the reasons behind the mechanistic differences we observe between males and females.

### 1.4.1 *Organizational versus activational effects of E2*

A seminal paper in 1959 proposed for the first time that sex steroids synthesized from the gonads can influence brain functions (Phoenix et al., 1959). This study showed that upon exposure to testosterone at birth, male like sexual behaviors were developed in females. Since then effects of other steroids in the brain has also been studied. E2 levels vary across different phases of life in both sexes and thus perform diverse functions of E2 during development and in adults are described in the body, including the brain. Historically, E2 has been extensively studied as a sex hormone, where it modulates the hypothalamus and other brain regions to influence reproductive physiology. However, over last several years, the non-reproductive functions of E2 have been recognized and studied. Besides affecting brain regions related to reproduction, E2 has now been shown to influence physiology of hippocampus, amygdala, cerebellum, several areas in the cortex and also brain regions that synthesize other

neuromodulators like serotonin and acetylcholine in the system (Woolley, 2007; McEwen et al., 2012).

E2 effects are broadly categorized as organizational and activational effects. Organizational effects mainly refer to the effects during development, where exposure to different E2 levels incurs permanent changes to the brain anatomy and physiology. For example, during development E2 regulates the cell number and connectivity in anteroperiventricular nucleus by increasing cell death via Bax/ Bcl2 apoptotic signaling (Simerly, 2002). Activational effects mainly refer to the E2 effects in adults, where E2 exposure transiently activates/modulates specific neuronal circuitry when synthesized. For example, varying levels of E2 during menstrual cycle influences several behaviors in females. However, it is difficult to categorize these two as separate functions, because the activational E2-effect in adults could be a consequence of the organizational E2-effect during development (McCarthy, 2009). There is now an extensive behavioral, electrophysiology and biochemical literature describing the diverse effects of E2 in adult hippocampus.

### 1.4.2 *Acute versus delayed effects of E2*

Another way of categorizing E2 actions is based on timing - acute or delayed effects. Acute effects of E2 are rapid and occur within minutes whereas the delayed effects take hours-days. Multiple studies have described acute and delayed E2 effects in the hippocampus. Most classical examples of the delayed or slow effects are observed with varying levels of E2 during menstrual cycle. One of the early studies

showed the influence of varying E2 levels in females while measuring local seizures in the limbic system (Terasawa and Timiras, 1968). They found different threshold levels to induce seizures across different estrous phases. Later studies showed that different E2 levels influences the number of excitatory synapses in the hippocampus across different estrous phases. Using Golgi impregnated cells it was found that the density of dendritic spines and of axo-spinous synapses in hippocampal CA1 stratum radiatum and s.lacunosum moleculare varies with plasma estrogen levels in female rats (Woolley et al., 1990; Woolley and McEwen, 1992). When estrogen is high, during proestrus, synapse number is at its highest; and when estrogen is low, during estrus, synapse number is at its lowest. The decline in spine and synapse number is, on average, greater than 30%. The basal synaptic transmission was similar across different phases but the hippocampal LTP mirrored the fluctuations well. The greatest increases in EPSP slope was evident during the proestrus and minimal increases during estrus (Warren et al., 1995). There is some evidence that the cellular effects were matched up with behavioral experiments, where E2 was shown to improve hippocampal dependent memory. Intraperitoneal injections of E2 2-3 days before the water maze task in ovariectomized females showed greater retention of memory (Sandstrom and Williams, 2001).

Acute effects of E2 modulate the synaptic and intrinsic properties of the cell in the hippocampus. For example, in the hippocampal CA1 region, 10 minutes of exposure to E2 depolarized CA1 neurons and caused them to fire spontaneously (Wong and Moss, 1992). In another study, E2 increased excitability of CA1 pyramidal cells within 5–10

min by suppressing the afterhyperpolarization (AHP) that follows an action potential (Kumar and Foster, 2002). Another study in hippocampal slices showed that E2 increased the amplitude of population spike in CA1 pyramidal neurons within 5-10 minutes (Teyler et al., 1980). This increase in the amplitude of population spike could also be due to increased synaptic inputs to the CA1 neurons. In addition to intrinsic properties, E2 can affect baseline synaptic transmission. With evidence from several studies, it is now established that E2 effects both excitatory and inhibitory synaptic transmission. One of the first studies showed that E2 can increase the amplitude of intracellularly recorded EPSPs within minutes in CA1 pyramidal cells in females (Wong and Moss, 1992). Later it was shown that this potentiation does not require NMDAR and EPSPs can be potentiated in the presence of APV, an NMDAR antagonist, but blocked in the presence of CNQX, an antagonist of non-NMDARs (Gu and Moss, 1998). More recent studies have used whole cell voltage clamp recordings in female hippocampal slices and shown that E2 can acutely increase EPSCs at CA3-CA1 synapses (Smejkalova and Woolley, 2010). Interestingly, in females, but not in males, E2 can also acutely suppress inhibitory synaptic transmission in CA1 pyramidal neurons (Huang and Woolley, 2012). Studies using cortical and hippocampal cultures show that E2 can also induce rapid structural changes in the dendritic spines (Soma et al., 2018) or even acutely form new spines. For example, in E2 induces the formation of novel dendritic spines within 30 minutes (Srivastava et al., 2008). Moreover, apart from modulating the basal synaptic transmission, E2 has also been shown to effect other synaptic plasticity mechanisms like LTP. Weak theta burst stimulation did not increase the CA3-CA1

fEPSP slope in control hippocampal slices, however a 30-minute incubation in E2 showed LTP induction at these synapses with the same stimulus (Hasegawa et al., 2015). Intriguingly, most of the studies with intracellular recordings have shown that the effect of E2 occurs in a subset of CA1 pyramidal neurons. Furthermore, evidence from our lab shows that E2-induced synaptic potentiation occurs in an input specific manner. In these experiments, using two stimulating electrodes non-overlapping sets of Schafer Collaterals were stimulated and it was found that even within a cell, E2 potentiated one group of EPSCs but not the other (Smejkalova and Woolley, 2010). Is it possible that the machinery via which E2 exerts its action is present at some synapses but not present at others? To explain these synapse specific effects of E2, we need to understand the mechanisms underlying the E2-induced potentiation. Acute effects of E2 are like the other neuromodulator effects described above and suggest that E2 could also act as a neuromodulator in the brain.

### 1.5 Estrogen receptors mediate different E2 effects

E2 performs its actions through different estrogen receptors (ERs). ERs can mediate the effects of E2 either through genomic mechanisms that underlie the delayed E2 effects and require gene expression, or through non-genomic mechanisms that underlie acute effects of E2 and activate downstream molecular/kinase signaling. The ground-breaking findings in the field were made in late 1950s when Elwood Jensen discovered and characterized an estrogen binding protein, today recognized as ERα. In 1993, nearly three decades later, the first ERα knockout mouse was created, and it was

discovered that animals were viable without this receptor which, at the time, was thought to be the sole mediator of estrogen signaling. Soon after the characterization of the ERα knockout mouse, ERβ was discovered, and this discovery raised the question of whether survival of the ERα knockout mouse was due to ERβ substituting for the functions of ERα. ERβ knockout mice were made, followed by ERαβ double knockouts. These different mouse models revealed that life is possible without either or both ERs but that reproductive functions were severely impaired. In addition, ERα and ERβ were found to have roles in the immune, skeletal, cardiovascular, and central nervous systems (Walker and Korach, 2004).

### 1.5.1 *Specific agonist to study estrogen receptors*

It is now well established that there are many types of ERs expressed in different parts of the brain including the hippocampus. ERα and ERβ protein structure is like the other nuclear receptors. However, these receptor subtypes share less than 60% amino acid sequence identity in the ligand binding domain (LBD). Researchers utilized this difference in the amino acid sequence and based on the LBD of different ERs, developed specific agonists and antagonist to activate or inhibit these receptors respectively. For example, WAY 200070 (Malamas et al., 2004) and diarylpropionitriles DPN, (Meyers et al., 2001) have been designed to activate ERβ specifically. Similarly, PPT is designed to activate specifically ERα (Stauffer et al., 2000). The combination of ER KO animals and selective agonists tools provided evidence for the diverse and distinct functions of ERs.

### 1.5.2 *Genomic functions of estrogen receptors*

Given the structural similarity of ERs to other nuclear receptors and initial radioactive assays showing high level of ERs in the nucleus, its genomic mechanisms were first characterized in detail. For example, it was shown that E2 requires ERα to induce transcription of progesterone receptors (Alves et al., 2000). Also, EREs (Estrogen response elements) have been found at the promoter regions of brain derived neurotrophic factor (BDNF) gene, where ERs bind and increase the BDNF mRNA levels (Sohrabji et al., 1995). Also, some genomic effects are mediated indirectly via activation of other transcription factors. For example, it was shown that increase in the spine density in the hippocampus during different estrous phases described above occurs due to phosphorylation of CREB (Murphy and Segal, 1997). This activation of CREB is shown to be NMDAR dependent and also requires phosphorylation by CAMKII and MAPK (Lee et al., 2004).

### 1.5.3 *Non-genomic functions of estrogen receptors*

In contrast to the delayed genomic effects of E2, the acute effects are thought to occur via non-genomic signaling and are initiated by extra nuclear ERs. Like the kinase signaling that underlies LTP, extra nuclear ERs could also activate similar kinase signaling cascades to show the acute effects of E2. One of the first evidence for non-genomic signaling came from the study done in the uterus, where E2 increased cAMP levels within seconds (Szego and Davis, 1967). In hippocampal cultures, cAMP/PKA signaling was shown to be required for E2-induced potentiation of kainate-evoked

currents (Gu and Moss, 1996). More recently in hippocampus, E2-induced excitatory potentiation has shown to facilitate actin polymerization via activation of Src/ROCK signaling (Kramar et al., 2009). This increase in actin polymerization may underlie E2-induced structural changes on the dendritic spines described above.

Due to lack of good antibodies it is difficult to determine where do the extranuclear ERs localize in the cell, whether they have with extracellular LBD or in the cytoplasm. One indirect way to investigate whether the E2- signaling is membrane initiated, research groups have studied ERs conjugated to heavy proteins like bovine serum albumin (BSA). Although E2-BSA replicated some effects of E2 on physiology, it failed to replicate some other effects suggesting that not all ERs have extracellular LBD to initiate membrane signaling (Wong and Moss, 1992). Apart from the classical estrogen receptors, there is now a new category of ERs, which are structurally similarly to GPCRs, namely the G-protein estrogen receptors -1 (GPER-1). GPER-1 has shown to be expressed on the surface and increase calcium using intracellular calcium stores following E2 application (Filardo et al., 2007).

It is intuitive how these GPCR-type ERs perform non-genomic functions, but it is still not clear how classical ERs mediate non-genomic downstream signaling cascades. Based on biochemical data in peripheral tissues, I describe 3 ways in which classical ER can activate downstream molecular signaling. One, ERs have shown to be regulated by membrane bound scaffolding lipid rafts called calveolins (CaV) that compartmentalize and regulate molecular signaling. Specifically, ERα has been demonstrated to physically interact with CaV1, which is necessary for the trafficking of

ERα to the membrane surface. A subsequent study showed that membrane-localized ERs are localized within distinct caveolae and that CAV1 is necessary to couple ERα to the group I mGluRs and CAV3 is necessary for the association of ERα and ERβ with group II mGluRs (Boulware et al., 2007). However, recent studies on ER-interacting scaffold proteins have demonstrated that other scaffold proteins, such as striatin, might link different ERs with kinases to potentially mediate estrogen-induced kinase signaling (Lu et al., 2004). Second way for classical ERs to activate non-genomic signaling could be due to direct interaction with second messenger signaling. A study reported that transfection of ERα and ERβ into Chinese hamster ovarian cells led to coupling of the ER with second messenger systems that are stimulated by E2 (Razandi et al., 1999). Lastly, there is evidence E2 acutely regulate translocation of ERβ and ERα, to the cell surface to activate specific signaling (Sheldahl et al., 2008; Tabatadze et al., 2013). These mechanisms still need to be investigated during synaptic transmission to fully understand the mechanisms underlying varied non-genomic effects of E2.

### 1.5.3 (a) *ERs in the hippocampus*

Studies using mRNA analysis and specific antibodies provide evidence of expression of nuclear and extra-nuclear ERα and ERβ in different parts of the hippocampus. Ultrastructural evidence in separate studies show localization of both ERα and ERβ in different part of CA1 neurons including in the spines, dendrites and axonal terminals (Milner et al., 2001; Milner et al., 2005). However, one study using immuno-staining argues that there is differential expression of ERs in the hippocampus and these ERs

are regulated differently during different estrous phases (Mendoza-Garces et al., 2011). Although the expression patterns are debatable in the hippocampus, the non-redundant function of ERα and ERβ are described by many pharmacological, electrophysiological and behavioral studies in the brain. Previous studies from our lab showed that DPN, ERβ selective agonist mimicked E2-induced potentiation in females (Smejkalova and Woolley, 2010). In another study PPT, an ERα selective agonist suppressed inhibitory synaptic transmission in the hippocampus (Huang and Woolley, 2012). Specific functions of ERs have also been observed at the behavioral level. One study using open field testing showed that female ERβKO mice had increased anxiety-like behaviors (Krezel et al., 2001), suggesting that ERβ might transmit an anxiolytic signal. Furthermore, the selective ERβ agonist DPN administered to ovariectomized female rats had anxiolytic activity when animals were tested in thee elevated plus maze, open field arena and light dark box (Oyola et al., 2012). Distinct roles of ERs give E2 the capacity to modulate hippocampal physiology in many ways.

1.5.3 (b) *Gonadal E2 levels can't explain acute E2 effects in the brain*

Most of the acute effects of E2 described above were examined using exogenous application of E2. These studies have used a wide range of E2 concentration from 100 pM – 1 μM. In females, the plasma E2 levels peak at around 100 pM during the proestrous phase, however some acute E2-effects were observed at the minimum concentration that is higher than the peak plasma E2 levels. For example, studies show at minimum 10nM E2 concentration, it can potentiate kainate-evoked currents (Gu and

Moss, 1996). Similarly, our lab previously showed that E2-suppressed inhibitory synaptic transmission at only 10nM E2 concentration but not 1 nM in females (Huang and Woolley, 2012). If gonadally synthesized E2 levels only reach up to 100 pM in females and much lower in males, do all these *in-vitro* studies on acute E2-effects have any physiological relevance? Moreover, given how peak E2 levels change over days in different estrous phases, it is difficult to conceptualize how gonadal E2, if synthesized upon stimulus, could have acute and specific effects on few synapses the hippocampus? Interestingly, recent studies indicate that E2 should be added to the growing list of steroids termed “neurosteroids”, and that E2 can be locally synthesized within the hippocampus of both females and males.

## 1.6 Neurosteorid E2 as a neuromodulator

### 1.6.1 *Overview of neurosteroids*

The term ‘neurosteroids’, originally coined by the French physiologist Etienne Baulieu, is now widely used to refer to steroids that are synthesized in the brain (Baulieu, 1991). Based on structural features, neurosteroids can be classified as pregnane neurosteroids, such as allopregnanolone and allotetrahydrodeoxycorticosterone (THDOC), androstane neurosteroids, such as androstanediol and etiocholanone, and sulfated neurosteroids, such as pregnenolone sulfate (PS) and dehydroepiandrosterone sulfate (DHEAS). The steroids are believed to be synthesized from precursor steroids produced in the peripheral tissues. Progesterone and deoxycorticosterone serve as precursors for the endogenous

neurosteroids allopregnanolone (5α-pregnane-3α-ol-20-one) and THDOC (5α-pregnane-3α,21-diol-20-one), respectively (Reddy, 2003). Testosterone-derived androgens such as androstenediol (5α-androstane-3α,17β-diol) are also considered as neurosteroids (Reddy, 2008; Reddy and Jian, 2010). However, there is recent evidence that some enzymes are produced in certain parts of the brain like pineal gland, cortex and hippocampus, where steroids can be directly synthesized from cholesterol. Four key enzymes that are required to synthesize neurosteroids are 17β-hydroxysteroid dehydrogenase (17β-HSD), 5α -reductase (5α -R), 3α -hydroxysteroid dehydrogenase (3α -HSD), cytochrome P450 aromatase (P450arom).

Neurosteroids exert a large array of biological activities in the brain either through direct interaction with membrane receptors or by indirectly activating secondary messenger signaling. In particular, neurosteroids have been found to act as allosteric modulators of the GABAA/central-type benzodiazepine receptor complex, NMDAR and AMPARs (Ratner et al., 2019). The effects of various neurosteroids on different subunits of GABAR have been studied by many groups. Neurosteroids can modulate GABAa receptors both positively and negatively. For example, it has been shown in different studies that allopregnanolone increases the activational kinetics and decay time of spontaneous inhibitory postsynaptic currents (Haage and Johansson, 1999), which likely depends on faster binding to, and reduced GABA unbinding from the receptor. In addition to directly modulating the channel properties, some neurosteroids have shown to effect GABAA receptor mediated synaptic transmission as well. For example, a study in pre-optic nucleus shows that allopregnanolone increases the frequency of GABAAR

miniature currents (Haage et al., 2002). Pregnanolone sulfate on the other hand is a negative regular of GABAA receptors and NMDAR (Park-Chung et al., 1997; Park-Chung et al., 1999).

### 1.6.2 *E2 as a neurosteroid in the hippocampus*

There is now evidence that E2 act as neurosteroids and can be locally synthesized in the hippocampus. Different studies using mRNA analysis and immunostaining have shown that hippocampus contains the enzymatic machinery to synthesize E2 (Hojo et al., 2004; Tabatadze et al., 2014). Moreover, *in-vitro* studies in primary hippocampal cultures (Prange-Kiel et al., 2003), slice cultures (Kretz et al., 2004) and acute slices (Hojo et al., 2004) have shown that the hippocampus can synthesize E2. For example, 30 min application of NMDA increased E2 levels in the hippocampal slices (Hojo et al., 2004). NMDA induced synthesis of E2 suggests that E2 synthesis is activity dependent. Direct evidence came from our lab when we showed acute local synthesis of E2 in the hippocampus of adult gonadectomized male and female brains. Using *in-vivo* microdialysis approach, upon infusion of precursor molecule androstenedione in the dorsal hippocampus, E2 levels increased almost 3-fold within 45 mins of infusion (Sato and Woolley, 2016). The local synthesis of E2 was similar between sexes and interestingly, this study estimated the levels of locally synthesized E2 to be higher than blood plasma levels of E2. Moreover, this study showed that E2 synthesis increased during kainic acid induced seizures. Although, seizures are a pathological phenomenon where there is a disbalance between excitation and inhibition, it still suggests that

increased excitation results in higher E2 levels in the hippocampus Taken together, these studies show an activity/stimulus-dependent local synthesis of E2 at a concentration that provides physiological relevance to the acute effects of E2 described earlier. Although the acute effects of E2 on cellular excitability and synaptic transmission have been described in the past, the underlying molecular mechanisms are still unclear. Furthermore, as hippocampal E2 synthesis occurs in both males and females, it becomes important to compare the molecular mechanisms in both sexes.

### 1.6.3 *Regulation of aromatase activity and E2 synthesis*

E2 is synthesized in the gonads of both males and females, however the peripheral levels of E2 differ between sexes as well as changes across different estrous/menstrual cycle in females. In contrast to this peripheral difference, E2 synthesis in the hippocampus occurs at a very similar level in both sexes (Sato and Woolley, 2016). Moreover, our lab using invivo microdialysis measured the peak concentration of E2 in the hippocampus to be around 300 pM (Sato and Woolley, 2016) and would be even higher at the site of synthesis. This range of E2 concentration is much higher (~100 nM) than the peripheral concentration of E2 (~1-100 pM).

Gonadal E2 is synthesized with the concerted action of gonadotropin releasing hormones (GnRH), follicular stimulating hormones (FSH) and luteinizing hormones (LH) (Stocco, 2008). In the ovarian granulosa cells, FSH/LH through cAMP/CREB signaling activates Cyp19 gene to express aromatase. Cyp19 gene expression is regulated by two promoters- a proximal promoter, which controls aromatase expression in peripheral

cells (Fitzpatrick et al., 1997), and a distal promoter, which drives aromatase expression in the brain (Kato et al., 1997). It is also believed that in the gonads E2 acts in an autocrine manner and activates further aromatase expression. For example, studies show that E2 enhanced FSH-induced aromatase expression in a dose dependent way (Adashi and Hsueh, 1982). Moreover, this feed-forward effect of E2 is believed to be mediated by the activation of ERβ (Wang et al., 2000). One intriguing characteristic of the effect of FSH on aromatase expression is that it takes a relatively long time (24 to 48 h) for FSH to induce aromatase mRNA (Fitzpatrick and Richards, 1991). Since FSH stimulates cAMP production very rapidly, it has been proposed that proteins synthesized as a consequence of PKA activation may be required for aromatase induction (Fitzpatrick and Richards, 1991). This slow expression of aromatase and the synthesis of E2 does not match up with our observation of acute increase in E2 levels in the hippocampus and thus, suggests some distinctions in the mechanisms of E2 synthesis in the brain/hippocampus.

The expression of aromatase gene, Cyp19, in various tissue is regulated using tissue-specific promoters, that are in turn regulated by different transcription factors and signaling pathways. Furthermore, regulation of aromatase expression in the brain is region-specific. In the preoptic area and hypothalamus, gonadal steroids, mainly androgens, regulate aromatase expression whereas in the other brain regions like the amygdala and hippocampus, Cyp19 lacks androgen responsive element on its gene (Abdelgadir et al., 1994). Interestingly, in this study E2 application increased aromatase activity without changing the mRNA levels. This observation suggests that aromatase

protein activity can be regulated by some post-translational mechanisms. A more recent evidence shows that aromatase enzyme can be acutely regulated by direct phosphorylation. In Quail explants it was shown that there is rapid regulation of aromatase enzyme by direct phosphorylation and this reduces the enzymatic activity (Balthazart et al., 2003). In separate experiments, they showed that either increasing intracellular calcium or activating glutamate receptors also reduces aromatase activity (Balthazart et al., 2006; Charlier et al., 2013). In contrast to avian literature, in mammalian hippocampus, a study showed that 30 min application NMDA activates E2 synthesis in hippocampal slices (Hojo et al., 2004). Additionally, experiments from our group demonstrated that kainic acid induced seizures promotes E2-synthesis (Sato and Woolley, 2016). Thus, although this provides evidence of acute regulation of aromatase, it suggests that there could be either species-specific and/or brain region-specific aromatase regulation, as well as that there are multiple mechanisms to activate aromatase activity. Further tools using animals where aromatase is tagged to different fluorescent proteins would provide a better understanding of where aromatase is localized within neurons.

## 1.7 Sex differences in the mechanisms of synaptic plasticity

### 1.7.1 *Evidence of sex differences in the hippocampus*

Most of the neuromodulation and synaptic plasticity questions have been addressed only in males. Initially, the male and female brain was compared only in a small subset of studies to address questions on reproductive physiology. Thus, sex difference was

limited to the context of reproductive behaviors and the physiology of the hypothalamic nuclei, which is known to release/stimulate hormones involved in these behaviors, was found to be different between males and females. One of the first studies to find sexual dimorphism in vertebrate brain was by Art Arnold lab. In this study in zebra finches and canaries he found the vocal areas in the brain to be bigger in males than in females (Nottebohm and Arnold, 1976). The first sex difference in rats was observed in the medial pre optic nuclei, being bigger in males than in females (Gorski et al., 1978). Now several groups have identified anatomical and neurophysiological sex differences in other brain regions like preoptic area, arcuate nucleus, locus coeruleus, corpus callosum and even hippocampus. Studies have now demonstrated behavioral, physiological and molecular sex differences in the hippocampus and hippocampal-dependent behaviors.

Behavioral sex differences have been found in studies investigating hippocampal dependent spatial and object location tasks. For example, when naive rats are trained in the Morris water maze tasks, male rats show faster acquisition than female rats (Perrot-Sinal et al., 1996).

A variety of factors could influence the performance of male and female rodents on hippocampal-dependent learning. For example; stressful stimuli influence hippocampal dependent learning and studies have found sex differences in the effects of stress. For instance, it was found that tail shock and restraint as a stressor had opposite effects on eye blink conditioning in the two sexes. While in female learning was impaired following stress stimulus, in males learning was actually improved (Wood and Shors, 1998).

Many forms of synaptic plasticity are hypothesized to underlie the above described learning paradigms. The same group later showed that the same stressors could induce structural plasticity in a sex specific manner. The study showed that 24 hours following tail shock stimulus there was an increase in dendritic spine density in males whereas a decrease in dendritic spine density in females (Shors et al., 2001).

Few studies have compared the different forms of hippocampal LTP in males versus females and shows sex differences in forms of hippocampal LTP at CA1 synapses. Using standard LTP induction protocols, a study showed that LTP induction in males was higher than females at temporoammonic-CA1 synapses (Qi et al., 2016).

The sex difference in the molecular mechanisms underlying different synaptic plasticity forms are just beginning to be recognized. Studies using genetic knockouts of Calcium/ calmodulin kinase B protein show impaired LTP at CA3-CA1 synapses in males but not in females (Mizuno et al., 2007). A more recent study from our lab showed sex difference in the endocannabinoid metabolism in the hippocampus. In these experiments, blocking the breakdown of anandamide, suppresses inhibitory synaptic transmission in females but not in males (Tabatadze et al., 2015). Despite such robust sex differences observed at behavioral, synaptic and molecular levels in the hippocampus, most researchers use only males to describe the mechanisms of plasticity.

### 1.7.2 *Mechanisms and types of sex differences in the brain*

How do these sex differences occur in the brain? Experimental evidence across several species have led to a general theory of sexual differentiation which suggests that “all the biological sex differences in gonadal and nongonadal tissues are seen downstream of the inherent sexual inequality in the sex chromosomes” (Arnold, 2017). Y chromosome in males have Sry gene that initiates development of masculine gonads. In the absence of Sry, in females, X-linked or autosomal genes, which (unlike Sry) are not inherently sexually different in their representation in the genome, initiate ovarian development. Different gonads set up lifelong sex differences in the plasma levels of steroid hormones such as testosterone, E2, and progesterone, which act throughout the body at multiple life stages to make tissues of one sex different from the other. It is thought that these hormonal factors cause the majority of sex differences in the brain throughout one’s life, like the organizational versus activational effects of E2 mentioned earlier.

In the brain, it is important to differentiate different types of sex differences. For example, an overall sex difference could be either due to a preexisting sex difference that led to differential activation of neuronal circuits upon stimulus (organizational) or there could be different levels of stimulus that then differentially activate similar neural signaling/circuits in the two sexes. Margaret M. McCarthy has nicely classified different types of sex differences in a review article (McCarthy et al., 2012). Type 1 sex difference is sexual dimorphism where there are two functional endpoints, one more common in males and the other more common in females. Absolute sexual

dimorphisms are mostly associated with reproductive behaviors. Type 2 sex difference can be a range of functional endpoints and the sex difference is observed at a population level. Such a sex difference is seen in threshold for pain sensitivities in males versus females. Type 3 sex difference is convergence or divergence of differences, like when there are different neural mechanisms underlying a similar outcome and vice versa. Convergent sex difference is also called latent sex difference. An example of divergent sex difference is the effect of a stressful experience/stimulus on learning, which was described above.

Despite sex differences observed at many different levels in the brain, males are still predominantly used in many biological fields including neuroscience. Many preclinical studies avoid females considering higher variability due to cyclical changes. Recent data suggest that it is a misconception that the cyclical changes in females produce more variability in data as compared to males. Meta-analysis of gene expression in various tissues of mice and humans show no difference between sexes (Itoh and Arnold, 2015). Just as age and circadian rhythms are considered as biological variables, sex should also be considered as one, to explain a biological phenomenon as fundamental as synaptic plasticity. Ignoring sex as a biological variable could lead to the lack of reproducibility/consensus in the basic science literature. For example, there is discrepancy in which ERs initiate E2-induced excitatory synaptic potentiation in the hippocampus. While studies from our lab shows that ERβ initiates E2-potentiation (Smejkalova and Woolley, 2010), other group shows that ERα initiates E2-potentiation (Kramar et al., 2009). We now know and will describe in the following chapters that this

is due each lab using different sex. While Smejkelova and Woolley (2010) used females, Kramer et al., (2009) used males and there is a sex difference in the requirement of different ERs in E2-potentiation. Moreover, studying both sexes has even larger clinical relevance. Indeed, synaptic function is altered in a number of neurological conditions, such as neurodegenerative diseases (Alzheimer's, Parkinson's, and Huntington's disease),(Selkoe, 2002; Picconi et al., 2012; Raymond, 2017), as well as neuropsychiatric conditions such as autism and major depression (Nanou and Catterall, 2018; Hansel, 2019). Thus, to treat these disorders, drugs are designed to target specific proteins/molecules that are involved in these synaptic mechanisms. For example, ketamine is used as an antidepressant, that antagonizes NMDARs function (Sattar et al., 2018). However, most of these preclinical studies are done in males. It is incomprehensible how drugs developed based on studies only done in male models, can be cleared for the FDA approved trials, where both human males and females participate. Furthermore, comparing the synaptic mechanisms in both sexes and identifying sex differences, would give a wider range of therapeutic strategies, including some that could be different between sexes.

*Overview of my thesis work*

My thesis work in combination with related work done by other members of the lab contributes to three fundamental aspects of synaptic neurophysiology. 1) To understand the role of neurosteroid E2 as a neuromodulator and investigate the molecular mechanisms that underlie acute-E2 effects on the excitatory synaptic transmission in

the hippocampus. 2) To investigate E2-synaptic mechanisms systematically in both females and males and compare how similar or different these signaling mechanisms are between sexes. 3) To investigate whether the sex differences observed in the mechanisms that underlie E2-plasticity are generalizable to other forms of plasticity. In the following section, I will outline three data chapters that addresses all the above points.

*Chapter 2: 17β-Estradiol acutely potentiates glutamatergic synaptic transmission in the hippocampus through distinct mechanisms in males and females.*

In this chapter, we investigated whether E2-induced synaptic potentiation occurs via presynaptic and/or postsynaptic mechanisms and which estrogen receptors (ERs) mediate E2's effects. Moreover, we performed these experiments in both sexes. We found that E2-induced synaptic potentiation occurs largely via independent pre or postsynaptic components different similarly in both sexes. Additional experiments using ER-selective agonists showed that although the overall effect of E2 is similar between sexes, distinct ER subtypes mediate pre or postsynaptic components of E2-potentiation and these requirements are different between sexes. This is the evidence of the latent sex difference downstream of E2 that underlie E2-potentiation. This work was done in collaboration with senior postdoctoral trainee Guang Zhe Huang who performed some miniature EPSC agonists and E2 experiments. The work has been published as a part of the research article in the *Journal of Neuroscience* in 2017 (Oberlander and Woolley, 2016).

*Chapter 3: Latent sex differences in molecular signaling that underlies excitatory synaptic potentiation in the hippocampus.*

In this study, we further examined the molecular mechanisms, potentially downstream of different ERs, that underlie E2-potentiation. We specifically investigated kinases and calcium sources that have been previously shown to be important in other forms of plasticity in both sexes. This study revealed further sex differences in the requirement of PKA, calcium release from internal calcium stores and L-type calcium channel in the initiation of E2-potentiation. Not all signaling components were different. The requirements of Src, ROCK and MAPK in initiation of E2-potentiation were similar in both sexes. CaMKII was found to be required for expression/maintenance of E2-potentiation in both sexes. Interestingly, we found that the sex difference in the requirement of PKA is generalizable to different forms of LTP. These results add to the evidence of latent sex differences in mechanisms of synaptic potentiation. This work was done in collaboration with Guang Zhe Huang, who performed all the experiments with Src, ROCK and MAPK inhibitors. The work has been published in the *Journal of Neuroscience* in 2019 (Jain et al., 2019).

*Chapter 4: Sex differences in AMPAR modulation that underlie 17β-estradiol-induced potentiation in the hippocampus.*

In this study using electrical stimulation experiments and two-photon glutamate uncaging experiments, we investigated the postsynaptic mechanisms of E2-potentiation

in both sexes. The main aim of this study was to understand if the sex differences persist even downstream of kinase signaling or do they converge, leading to an overall similar magnitude of potentiation in both sexes. We found that even the downstream mechanisms underlying the postsynaptic component of potentiation appear to be different. Using noise analysis on two-photon evoked currents, we found that either an increase in conductance or number can underlie E2-potentiation. However, the proportion of requirement of these mechanisms differed between sexes. Further in electrical stimulation experiments, we found a sex difference in the requirement of calcium permeable AMPARs in the stabilization of E2-potentiation.

# Chapter 2: 17β-Estradiol acutely potentiates glutamatergic synaptic transmission in the hippocampus through distinct mechanisms in males and females

## 2.1 Abstract

Estradiol (E2) acutely potentiates glutamatergic synaptic transmission in the hippocampus of both male and female rats. Here, we investigated whether E2-induced synaptic potentiation occurs via presynaptic and/or postsynaptic mechanisms and which estrogen receptors (ERs) mediate E2's effects in each sex. Whole-cell voltage-clamp recordings of mEPSCs in CA1 pyramidal neurons showed that E2 increases both mEPSC frequency and amplitude within minutes, but often in different cells. This indicated that both presynaptic and postsynaptic mechanisms are involved, but that they occur largely at different synapses. All these results were essentially the same in males and females. However, additional experiments using ER-selective agonists indicated sex differences in the mechanisms underlying E2-induced potentiation. In males, an ERβ agonist mimicked the postsynaptic effects of E2 to increase mEPSC, amplitude, whereas in females, these effects were mimicked by an agonist of G protein-coupled ER-1. The presynaptic effect of E2, increased mEPSC frequency, was mimicked by an ERα agonist in males, whereas in females, an ERβ agonist increased mEPSC frequency. Thus, E2 acutely potentiates glutamatergic synapses similarly in both sexes, but distinct ER subtypes mediate the presynaptic and postsynaptic aspects of potentiation in each sex. This indicates a latent sex difference in which different molecular mechanisms converge to the same functional endpoint in males versus females.

## 2.2 Significance Statement

Some sex differences in the brain may be latent differences, in which the same functional endpoint is achieved through distinct underlying mechanisms in males versus females. Here we report a latent sex difference in molecular regulation of excitatory synapses in the hippocampus. The steroid 17β-estradiol is known to acutely potentiate glutamatergic synaptic transmission in both sexes. We find that this occurs through a combination of increased presynaptic glutamate release probability and increased postsynaptic sensitivity to glutamate in both sexes, but that distinct estrogen receptor subtypes underlie each aspect of potentiation in each sex. These results indicate that therapeutics targeting a specific estrogen receptor subtype or its downstream signaling would likely affect synaptic transmission differently in the hippocampus of each sex.

## 2.3 Introduction

It has been known for decades that estrogens, such as 17β-estradiol (E2), potentiate excitatory synapses in the hippocampus within minutes, and in both sexes(Teyler et al., 1980; Wong and Moss, 1992). Although the physiological relevance of this effect initially was unclear, there is now compelling evidence that the hippocampus can synthesize estrogens as neurosteroids, which could provide a source of estrogens that fluctuate on the rapid time scale of E2-induced synaptic potentiation. For example, both the male and female hippocampus express the E2-synthesizing enzyme, P450 aromatase (Roselli et al., 1985; MacLusky et al., 1994; Hojo et al., 2004; Tabatadze et al., 2014), and hippocampal cultures (Prange-Kiel et al., 2003), and acute slices (Hojo et al., 2004) produce E2 *in vitro*. These observations have motivated efforts to understand the cellular mechanism(s) that underlie acute E2-induced synaptic potentiation.

Results from separate studies provide conflicting evidence as to whether presynaptic versus postsynaptic changes underlie E2-induced synaptic potentiation. Early experiments showed that E2 rapidly increases the amplitude of intracellularly recorded excitatory potentials evoked by glutamate application to CA1 pyramidal cells in slices (Wong and Moss, 1992) or after acute dissociation (Gu and Moss, 1996), indicating that E2 increases postsynaptic sensitivity to glutamate (and/or sensitivity of extrasynaptic receptors). Subsequent studies supported a postsynaptic effect of E2. For example, Kramar et al. (2009) found that E2 potentiates extracellularly recorded dendritic field potentials in CA1 without affecting paired-pulse ratio. On the other hand,

whole-cell voltage-clamp recordings in slices have shown that E2 potentiates EPSCs in CA1 pyramidal cells, at least in part, through a presynaptic mechanism. E2 potentiation of EPSCs occurred selectively at inputs with low initial glutamate release probability and was paralleled by decreased paired-pulse ratio, increased individual vesicle release probability, and greater cleft glutamate concentration (Smejkalova and Woolley, 2010).

One consistent finding, even among studies that have reached different conclusions about presynaptic versus postsynaptic mechanisms of E2-induced synaptic potentiation, is that estrogen receptor (ER) β (ERβ) plays an important role. The ERβ-selective agonists WAY200070 (Kramar et al., 2009) and DPN (Smejkalova and Woolley, 2010) each were shown to mimic the effects of E2, whereas ERα agonists were not effective. Other studies, however, indicate involvement of ERα and G-protein coupled ER-1 (GPER1) (Lebesgue et al., 2010; Kumar et al., 2015). Thus, which ERs participate in E2-induced synaptic potentiation is unresolved. Another consistent finding in studies recording from individual neurons is that only a subset of cells (Wong and Moss, 1992) or inputs to cells (Smejkalova and Woolley, 2010) responds to E2, indicating that the effects of E2 at synapses are likely to be heterogeneous.

One complication in understanding the mechanism(s) by which E2 acutely potentiates synapses in the hippocampus is that most studies have been done in only one sex. For example, Kramar et al. (2009) studied exclusively males, whereas Smejkalova and Woolley (2010) and Kumar et al. (2015) studied exclusively females. To address this, we used whole-cell voltage-clamp recording in both sexes to: (1) investigate whether E2-induced synaptic potentiation occurs through presynaptic and/or

postsynaptic mechanisms in males and/or in females; and (2) identify which ERs underlie E2-induced synaptic potentiation in each sex. The results show that E2 acts via both presynaptic and postsynaptic mechanisms in both sexes, but that each component of E2's actions is mediated by a different ER in each sex. Thus, the mechanisms of E2-induced synaptic potentiation reflect latent sex differences in which the same endpoint is achieved through distinct mechanisms in males versus females.

## 2.4 Materials and Methods

### 2.4.1 *Animals*

All procedures were performed in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Northwestern University Animal Care and Use Committee. Young adult male and female Sprague Dawley rats (Harlan) were group-housed on a 12:12 h light/dark cycle with phytoestrogen-free chow and water given *ad libitum*. Rats were either gonadally intact or gonadectomized. For gonadectomy, females were ovariectomized and males were castrated under ketamine (85 mg/kg, i.p., Bioniche Pharma) and xylazine (13 mg/kg, i.p., Lloyd Laboratories) anesthesia using standard aseptic procedures. Gonadectomized animals were used for experiments 5–8 d after surgery.

### 2.4.2 *Electrophysiological recording*

Rats were deeply anesthetized with sodium pentobarbital (100–125 mg/kg, i.p., Virbac) and transcardially perfused with oxygenated (95%$O_2$/5% $CO_2$) ice-cold sucrose aCSF

containing the following (in mM): 75 NaCl, 25 $NaHCO_3$, 15 dextrose, 75 sucrose, 1.25 $NaH_2PO_4$, 2 KCl, 2.4 Na pyruvate, 1.3 ascorbic acid, 0.5 $CaCl_2$, 3 $MgCl_2$, pH7.4. Following dissection, 300 µm transverse slices of the dorsal hippocampus were cut on a vibrating tissue slicer (VT1000S, Leica), incubated at 33°C in oxygenated regular aCSF containing the following (in mM): 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 1.25 $NaH_2PO_4$, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, pH 7.4, 310–315 mOsm, for 30–35 min, then allowed to recover at room temperature in oxygenated aCSF for 1–6 h before recording.

Slices were transferred to a recording chamber mounted to a Zeiss Axioskop equipped with a video camera (MTI NC-70) and bathed in oxygenated aCSF containing the following: TTX (1 µM, Tocris Bioscience), blockers of $GABA_A$ (SR-95531, 2 µM, Tocris Bioscience), and $GABA_B$ (CGP46381, 10 µM, Tocris Bioscience) receptors and 0.01% (v/v) DMSO (Sigma) at room temperature. Whole cell voltage-clamp recordings ($V_{hold}$= -70 mV) were made from CA1 pyramidal cells using borosilicate glass pipettes with resistances of 4–7 MΩ containing the following (in mM): 115 K-gluconate, 20 KCl, 10 HEPES, 10 Na creatine phosphate, 2 Mg-ATP, 0.3 Na-ATP, pH 7.3, 290–295 mOsm. Recordings were acquired using an Axopatch 200B amplifier with pClamp 9.2 and digitized using a Digidata 1322A, or with a MultiClamp 700B amplifier with pClamp 10.4 and digitized using a Digidata 1440A. Recordings were filtered with a 2 kHz low pass filter, and digitized at 20 kHz. Series resistance (10–40 MΩ) was monitored throughout each experiment with 5 mV, 10 ms voltage steps, and did not fluctuate by >10% in recordings used for analysis. Miniature EPSCs (mEPSCs) were recorded for 10–20 min, which was followed by bath application of E2 (100 nM, Sigma), the ERβ

agonist WAY200070 (WAY, 10 nM, Tocris Bioscience), the ERα agonist PPT (100 nM, Tocris Bioscience), or the GPER1 agonist G1 (100 nM, Cayman Chemical), dissolved in 0.01% (v/v) DMSO for 10 min. Thus, aCSF contained an equivalent concentration of DMSO in all phases of each experiment. In most recordings with ER-selective agonists, E2 alone was applied for an additional 10 min beginning 5–20 min after agonist washout to assess E2 responsiveness of each measured parameter.

2.4.3 *Data analysis*

Electrophysiological analyses were performed offline using Clampfit (version 10.4.06), Minianalysis software (version 6.0, Synaptosoft Inc), Neuromatic (Igor Pro version 2.0) and/or custom scripts in MATLAB (The MathWorks, version R2014b). Statistical tests were performed in Graphpad PRISM software (version 7.0b), Statview (version 5.0.1) or MATLAB, and α was set at 0.05. The minimum threshold for accepted EPSC amplitudes was determined individually for each recording and was set at the average noise plus 3× the SD (typically 3-7 pA).

Unpaired two-tailed Student's *t* tests were used to determine whether individual cells were responsive to E2 or an ER-selective agonist. Baseline values for each parameter were averaged per minute during 10-15 min of recording in aCSF, and values for each treatment were averaged per minute from data recorded 5–15 min after its application. The magnitude of E2 or ER-selective agonist effect on each cell was then calculated by comparing each measure after treatment with the same measure immediately preceding that treatment. Group effects on mEPSCs were determined from

individual cell measurements using paired, two-tailed *t* tests or by ANOVA on normalized effects as indicated in Results. $\chi^2$ tests or Fisher's exact tests (when contingency table contained fewer than 10 observations) were used to determine whether the fraction of E2 or ER-agonist responsive cells differed between males and females or among treatments.

## 2.5 Results

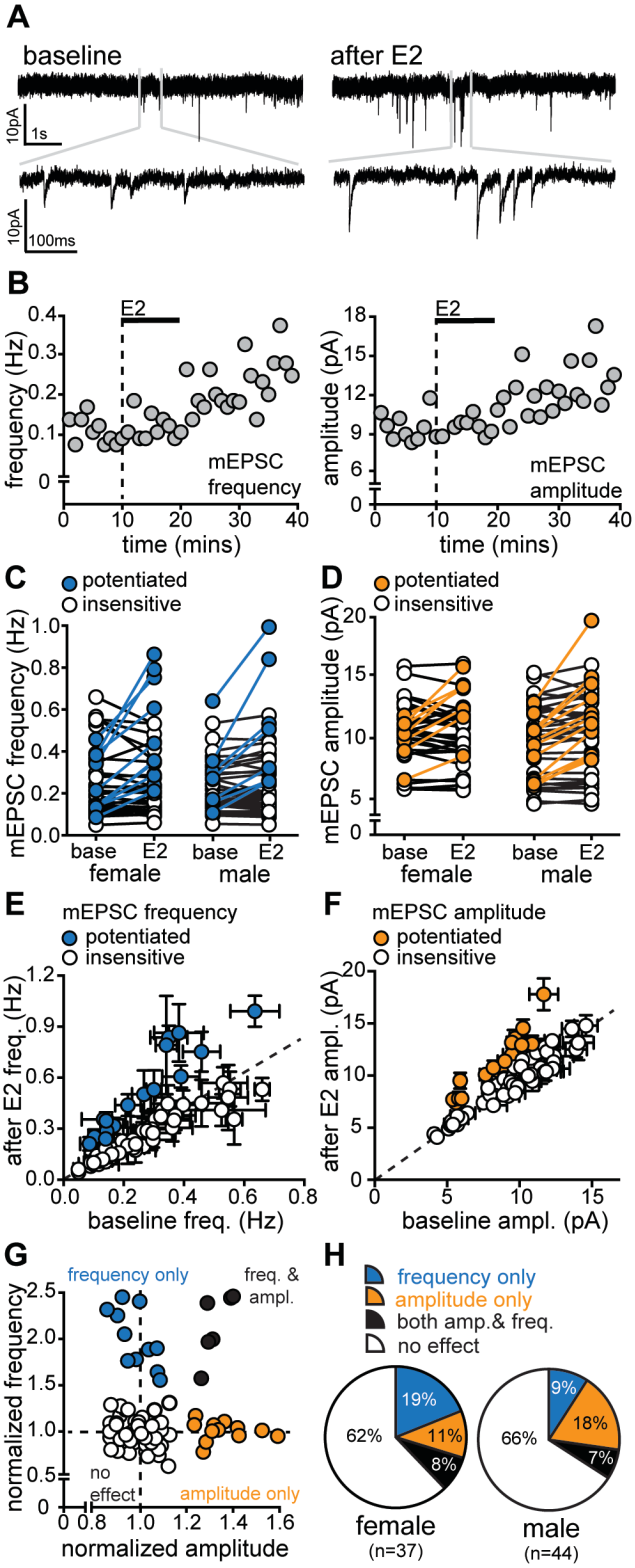
### 2.5.1 Both presynaptic and postsynaptic mechanisms contribute to acute E2-induced excitatory synaptic potentiation

We first investigated presynaptic versus postsynaptic mechanisms of E2-induced synaptic potentiation by recording mEPSCs in CA1 pyramidal cells before, during, and after 10 min application of E2 (100 nM) to hippocampal slices from adult gonadectomized male and female rats (Fig. 2.1A,B). All recordings were made in TTX to block action potential-dependent glutamate release. These experiments showed that E2 increased both mEPSC frequency and mEPSC amplitude, but often in different cells. As has been shown before in females (Smejkalova and Woolley, 2010), only a subset of CA1 pyramidal cells was responsive to E2. In the current study, E2 increased mEPSC frequency by 27 ± 9% overall in females ($t(36) = 2.16$, $p = 0.037$) and 16 ± 6% overall in males ($t(43) = 2.79$, $p = 0.0078$), driven by statistically significant within-cell increases ranging from 56% to 145% in 10 of 37 cells in females and from 56% to 146% in 7 of 44 cells in males (Fig. 2.1C). Neither the fraction of responsive cells (10 of 37 in females, 7 of 44 in males, Fisher's exact test, $p = 0.28$) nor the magnitude of E2 effect in

responsive cells (109 ± 11% females, 98 ± 12% males; $F(1,30) = 0.514$, $p = 0.47$) differed by sex.

E2 also increased mEPSC amplitude. The overall effect of E2 was small, only 3 ± 3% in females ($t(36) = 0.81$, $p = 0.41$) and 10 ± 3% in males ($t(43) = 3.39$, $p = 0.0015$), but included substantial statistically significant within-cell increases in subsets of cells in each sex. Within-cell analyses showed that E2 increased mEPSC amplitude by 20% to 42% in 7 of 37 cells in females, and by 23% to 59% in 11 of 44 cells in males (Fig. 2.1D). As with mEPSC frequency, there were no sex differences in the fraction of cells that responded to E2 (7 of 37 in females, 11 of 44 in males, Fisher's exact test, $p = 0.60$) or the magnitude of response among cells that showed a significant increase (31 ± 3% females, 37 ± 3% males; $F(1,32) = 1.46$, $p = 0.23$).

None of the 81 cells recorded showed a statistically significant decrease in either mEPSC frequency or amplitude after E2, and E2-responsive cells included those with both high and low initial mEPSC frequency (Fig. 2.1E) and mEPSC amplitude (Fig. 2.1F). This indicates that E2 responsiveness of mEPSCs and our ability to detect effects of E2 on mEPSCs were not influenced by baseline values. Together, mEPSC recordings indicated that E2-induced excitatory synaptic potentiation involves both presynaptic and postsynaptic mechanisms in both sexes. The increase in mEPSC frequency likely reflects increased presynaptic glutamate release probability, shown previously in females (Smejkalova and Woolley, 2010) and the increase in mEPSC amplitude likely reflects increased postsynaptic sensitivity to glutamate, which has been inferred previously in studies using males (Kramar et al., 2009).


A
baseline
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frequency (Hz)
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D
potentiated
insensitive
mEPSC amplitude (pA)
base E2 base E2
female male
E
mEPSC frequency
potentiated
insensitive
after E2 freq. (Hz)
baseline freq. (Hz)
F
mEPSC amplitude
potentiated
insensitive
after E2 ampl. (pA)
baseline ampl. (pA)
G
frequency only
freq. & ampl.
normalized frequency
no effect
amplitude only
normalized amplitude
H
frequency only
amplitude only
both amp.& freq.
no effect
19%
11%
8%
62%
female
(n=37)
9%
18%
7%
66%
male
(n=44)

**Figure 2.1**: **E2 acutely potentiates mEPSC frequency and mEPSC amplitude in both sexes.**

**A**, **B**, Sample experiment showing (**A**) mEPSC recording during baseline and after E2 and (**B**) the time course of the E2-induced increase in instantaneous mEPSC frequency and mEPSC amplitude in the same cell. **C**, Plots showing mean mEPSC frequency during baseline and after E2 for all cells in both females and males. Connected symbols represent data from an individual cell. Colored symbols represent the subset of cells in which within-cell t tests showed a significant effect of E2. White symbols represent cells with no significant effect of E2 (also in **D–G**). **D**, Plots showing mean mEPSC amplitude during baseline and after E2 for the same cells as in **C**. **E**, **F**, Plotting mean (±SEM) mEPSC frequency (**E**) or amplitude (**F**) after E2 versus during baseline for each cell shows that E2 potentiated mEPSC frequency and/or amplitude in cells that began with a wide range of baseline values. **G**, Plotting the normalized change in mEPSC frequency versus amplitude for each cell shows that E2 rarely increased both mEPSC frequency and amplitude in the same cells (black); more often, E2 increased mEPSC frequency only (blue), mEPSC amplitude only (orange), or had no effect on mEPSCs (white). **H**, The proportion of cells in each category of mEPSC response to E2 is similar in females and males.

Interestingly, although E2 increased both mEPSC frequency and amplitude in both sexes, these effects typically occurred in different subsets of cells within each sex. Plotting the normalized effects of E2 on mEPSC frequency and amplitude for all cells individually (Fig. 2.1G) showed that only 6 of 81 cells responded to E2 with increases in both mEPSC frequency and amplitude. There were no sex differences in the fractions of cells showing increased mEPSC frequency, amplitude, or both (Fisher's exact test, $p > 0.99$; Fig. 2.1H). That mEPSC frequency and amplitude were affected primarily in separate cells indicates that the presynaptic and postsynaptic components of E2-induced excitatory synaptic potentiation likely occur through independent mechanisms operating at distinct subsets of synapses. The relative timing of E2-induced increases in mEPSC frequency and amplitude support this idea. In the 6 cells in which both mEPSC frequency and amplitude were increased by E2, 2 showed the increase in frequency before the increase in amplitude, 2 showed the opposite, and 2 showed the increase in frequency and amplitude concurrently.

### 2.5.2 ERβ activation potentiates excitatory synapses through distinct presynaptic versus postsynaptic mechanisms in females versus males

Previous studies have pointed to ERβ as mediating acute E2-induced potentiation of excitatory synapses in both females (Smejkalova and Woolley, 2010) and males (Kramar et al., 2009), albeit using different approaches and coming to different conclusions about the involvement of presynaptic versus postsynaptic mechanisms. To resolve this issue, we compared the effects of the ERβ agonist,

WAY200070 (WAY, 10 nM), on mEPSC frequency and amplitude in identical experiments done in females (Fig. 2.2A) and males (Fig. 2.2B). When recordings lasted long enough, E2 was applied after washout of WAY to confirm E2 responsiveness of mEPSCs and/or test for non–ERβ- mediated effects of E2.

Within-cell statistical tests showed that, in females, a 10 min application of WAY increased mEPSC frequency in 6 of 24 cells, by 91 ± 15%; in these 6 cells in which E2 was applied after WAY, E2 had no further effect on mEPSC frequency (Fig. 2.2C). The fraction of cells in which WAY increased mEPSC frequency (6 of 24) was similar to the fraction of cells in which E2 increased mEPSC frequency (10 of 37) (Fisher's exact test, $p > 0.99$; Fig. 2.2C vs Fig. 1H). Thus, WAY mimicked and occluded the E2-induced increase in mEPSC frequency in females. In contrast, in males, WAY failed to affect mEPSC frequency in any of 32 cells, but E2 applied after WAY washout increased mEPSC frequency in 1 of the 32 cells tested, by 57% (Fig. 2.2D), confirming its E2 responsiveness. Figure 2.2E summarizes the different effects of WAY and E2 on mEPSC frequency in both sexes.

Measurements of mEPSC amplitude in the same recordings showed the converse results for females and males. In females, WAY had no effect on mEPSC amplitude in any of 24 cells, but E2 applied after WAY washout increased mEPSC amplitude in 3 cells, by 41 ± 15% (Fig. 2.2F). In males, WAY increased mEPSC amplitude in 5 of 32 cells, by 35 ± 5%, and this occluded a further increase in mEPSC amplitude when E2 was applied after WAY (Fig. 2.2G). The fraction of male cells in which WAY increased mEPSC amplitude (5 of 32) was statistically similar to the fraction

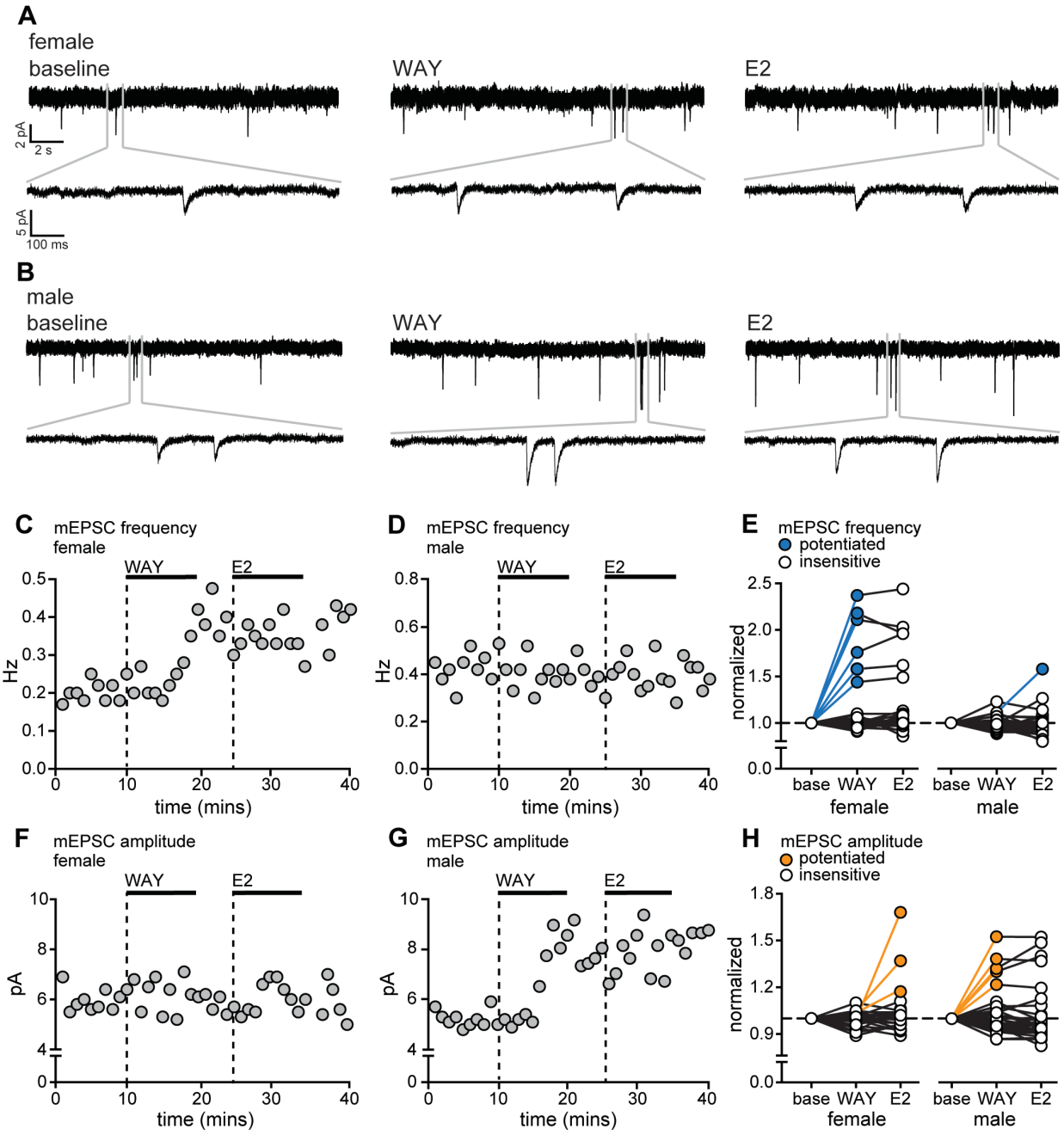



**Figure 2.2**: **The ERβ agonist WAY200070 acutely potentiates mEPSC frequency in females and mEPSC amplitude in males.**

**A**, **B**, Sample mEPSC recordings during baseline, after WAY, and after E2 in a female (**A**) and a male (**B**) cell. **C**, Time course of mEPSC frequency changes for the same

female cell as in **A** showing that WAY acutely increased mEPSC frequency and that E2 after WAY had no further effect. **D**, Time course of mEPSC frequency changes for the same male cell as in **B** showing that WAY had no effect on mEPSC frequency. **E**, Summary of mEPSC frequency analysis in female and male experiments with WAY. Colored symbols represent points in the experiment in which within-cell t tests indicated a significant difference from the preceding condition (also in **H**). **F**, Time course of mEPSC amplitude changes for the same female cell as in **A** showing that WAY had no effect on mEPSC amplitude. **G**, Time course of mEPSC amplitude changes for the same male cell as in **B** showing that WAY acutely increased mEPSC amplitude and that E2 after WAY had no further effect. **H**, Summary of mEPSC amplitude analysis in female and male experiments with WAY. There were no female cells tested with WAY that showed both a WAY-induced increase in mEPSC frequency and an E2-induced increase in mEPSC amplitude. Similarly, no male cells showed both a WAY-induced increase in mEPSC amplitude and an E2-induced increase in mEPSC frequency.

of male cells in which E2 increased mEPSC amplitude (11 of 44) (Fisher's exact test, p = 0..40; Fig. 2.2G vs Fig. 2.1H). Figure 2.2H summarizes different effects of WAY and E2 on mEPSC amplitude in both sexes.

Together, these findings confirm that ERβ activation acutely potentiates excitatory synapses in both sexes but indicate that this potentiation occurs through distinct mechanisms in each sex. The WAY-induced increase in mEPSC frequency in females suggests that ERβ activation increases presynaptic glutamate release probability as reported by Smejkalova and Woolley (2010), whereas the WAY induced increase in mEPSC amplitude in males suggests that ERβ activation increases postsynaptic glutamate sensitivity as reported by Kramar et al. (2009). At the same time, however, that E2 applied after WAY was able to produce the corresponding increase in mEPSC amplitude in females and frequency in males indicates that ERβ activation, alone, does not account for all aspects of E2-induced synaptic potentiation in either sex. We therefore tested how agonists of other known estrogen receptors, ERα and GPER1, affect mEPSCs in females and males.

### 2.5.3 ERα activation potentiates excitatory synapses through a presynaptic mechanism in males that is absent in females

To investigate whether ERα plays a role in E2-induced synaptic potentiation, we tested the effect of the ERα agonist, PPT (100 nM), on mEPSC frequency and amplitude in females (Fig. 2.3A) and males (Fig. 2.3B). As with experiments using WAY, when the recording lasted long enough, E2 was applied after PPT washout to confirm

E2 responsiveness and/or test for non–ERα-mediated effects of E2.

Within-cell statistical comparisons showed that a 10 min application of PPT had no effect on mEPSC frequency in any of 20 cells from females. When E2 was applied to these cells after PPT washout, mEPSC frequency increased in 6 cells, by 69 ± 12% (Fig. 2.3C). The lack of PPT effect on mEPSC frequency in cells that subsequently responded to E2 argues against ERα as being involved in the E2-induced increase glutamate release probability in females, corroborating results of a previous study (Smejkalova and Woolley, 2010). In contrast, PPT robustly increased mEPSC frequency in males. In 7 of 33 recordings in males, PPT increased mEPSC frequency, by 96 ± 13%. In contrast to the effect of WAY on mEPSC frequency in females, however, the effect of PPT on mEPSC frequency in males was transient; mEPSC frequency returned to baseline during PPT washout. When E2 was applied after PPT, mEPSC frequency increased again in 5 of the cells that had previously shown an increase in PPT and had no effect in any of the cells that had not responded to PPT (Fig. 2.3D). The fraction of male cells in which PPT increased mEPSC frequency (7 of 33) was statistically similar to the fraction of male cells in which E2 increased mEPSC frequency (7 of 44) (Fisher's exact test, $p = 0.57$; Fig. 2.3E vs Fig. 1H). Thus, PPT mimicked the E2-induced increase in mEPSC frequency in males, indicating that ERα mediates the E2-induced increase in glutamate release probability in males. Figure 2.3E summarizes mEPSC frequency results from experiments with PPT and E2 in both sexes.

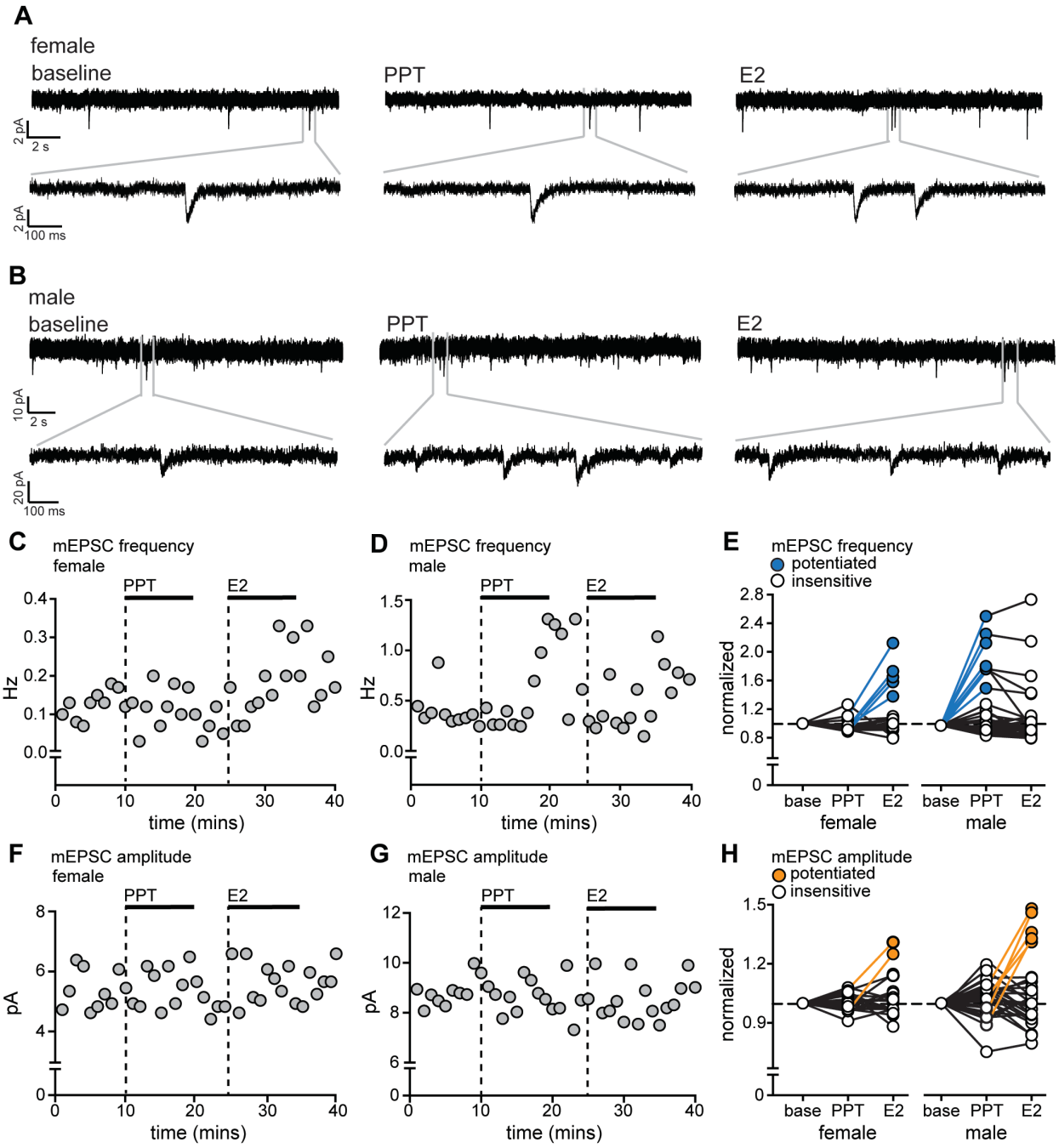



**Figure 2.3**: **The ERα agonist PPT has no effect on mEPSCs in females but acutely potentiates mEPSC frequency in males.**

**A**, **B**, Sample mEPSC recordings during baseline, after PPT, and after E2 in a female (**A**) and a male (**B**) cell. **C**, Time course of mEPSC frequency changes for the same

female cell as in **A** showing that PPT had no effect on mEPSC frequency but that E2 applied after PPT increased mEPSC frequency, confirming that mEPSC frequency in this cell was responsive to E2. **D**, Time course of mEPSC frequency changes for the same male cell as in **B** showing that PPT acutely (and transiently) increased mEPSC frequency and that E2 after PPT increased mEPSC frequency similarly to PPT. **E**, Summary of mEPSC frequency analysis in female and male experiments with PPT. Colored symbols represent points in the experiment in which within-cell t tests indicated a significant difference from the preceding condition (also in **H**). **F**, Time course of mEPSC amplitude changes for the same female cell as in **A** showing that PPT had no effect on mEPSC amplitude. **G**, Time course of mEPSC amplitude changes for the same male cell as in **B** showing that PPT had no effect on mEPSC amplitude. **H**, Summary of mEPSC amplitude analysis in female and male experiments with PPT. There were no female cells tested with PPT that showed an E2-induced increase in both mEPSC frequency and amplitude, and only one male cell showed both a PPT-induced increase in mEPSC frequency and an E2-induced increase in mEPSC amplitude.

frequency in males. In 7 of 33 recordings in males, PPT increased mEPSC frequency, by 96 ± 13%. In contrast to the effect of WAY on mEPSC frequency in females, however, the effect of PPT on mEPSC frequency in males was transient; mEPSC frequency returned to baseline during PPT washout. When E2 was applied after PPT, mEPSC frequency increased again in 5 of the cells that had previously shown an increase in PPT and had no effect in any of the cells that had not responded to PPT (Fig. 2.3D). The fraction of male cells in which PPT increased mEPSC frequency (7 of 33) was statistically similar to the fraction of male cells in which E2 increased mEPSC frequency (7 of 44) (Fisher's exact test, $p = 0.57$; Fig. 2.3E vs Fig. 1H). Thus, PPT mimicked the E2-induced increase in mEPSC frequency in males, indicating that ERα mediates the E2-induced increase in glutamate release probability in males. Figure 2.3E summarizes mEPSC frequency results from experiments with PPT and E2 in both sexes.

Measurements of mEPSC amplitude in the same recordings showed no effect of PPT in any of the 20 cells in females (Fig. 2.3F) or 33 cells in males (Fig. 2.3G). When E2 was applied after PPT washout, mEPSC amplitude increased significantly in 2 of 20 cells from females, by 25 ± 5%, and 5 of 32 cells from males, by 39 ± 3%. Thus, mEPSC amplitude was responsive to E2 in cells that showed no effect of PPT on mEPSC amplitude. Figure 2.3H summarizes mEPSC amplitude results from experiments with PPT and E2 in both sexes. The absence of any effect of PPT on mEPSC amplitude in either sex argues against a role for ERα in the effect of E2 to increase postsynaptic sensitivity to glutamate.

Together, results from experiments with WAY and PPT showed that activation of ERβ and ERα together can account for most, but not all, components of E2-induced excitatory synaptic potentiation. The E2-induced increase in mEPSC frequency can be replicated by ERβ activation in females and by ERα activation in males; the E2-induced increase in mEPSC amplitude can be replicated by ERβ activation in males, but not in females. This leaves open the question of what ER subtype accounts for the E2-induced increase in mEPSC amplitude in females. One possibility is GPER1. G1, a GPER1 agonist, has been shown to increase field EPSP amplitude (Lebesgue et al., 2010) or slope (Kumar et al., 2015) in extracellular recordings in CA1 and to increase evoked EPSC amplitude in a subset of whole-cell recordings of CA1 pyramidal cells in females (Lebesgue et al., 2009; Smejkalova and Woolley, 2010).

### 2.5.4 GPER1 activation potentiates excitatory synapses through a postsynaptic mechanism in females that is absent in males

To investigate the role of GPER1 in E2-induced synaptic potentiation, we tested the effects of G1 (100 nM) on mEPSCs in females (Fig. 2.4A) and males (Fig. 2.4B). As in experiments with WAY and PPT, E2 was applied after G1 washout to confirm E2 responsiveness and/or to test for non–GPER1-mediated effects of E2.

Within-cell statistical comparisons showed that 10 min application of G1 had no effect on mEPSC frequency in any of 23 cells from females (Fig. 2.4C) or 25 cells from males (Fig. 2.4D). When E2 was applied following G1 washout, mEPSC frequency increased in 6 of 22 cells from females, by 92 ± 19%, and in 1 of 25 cells from males, by

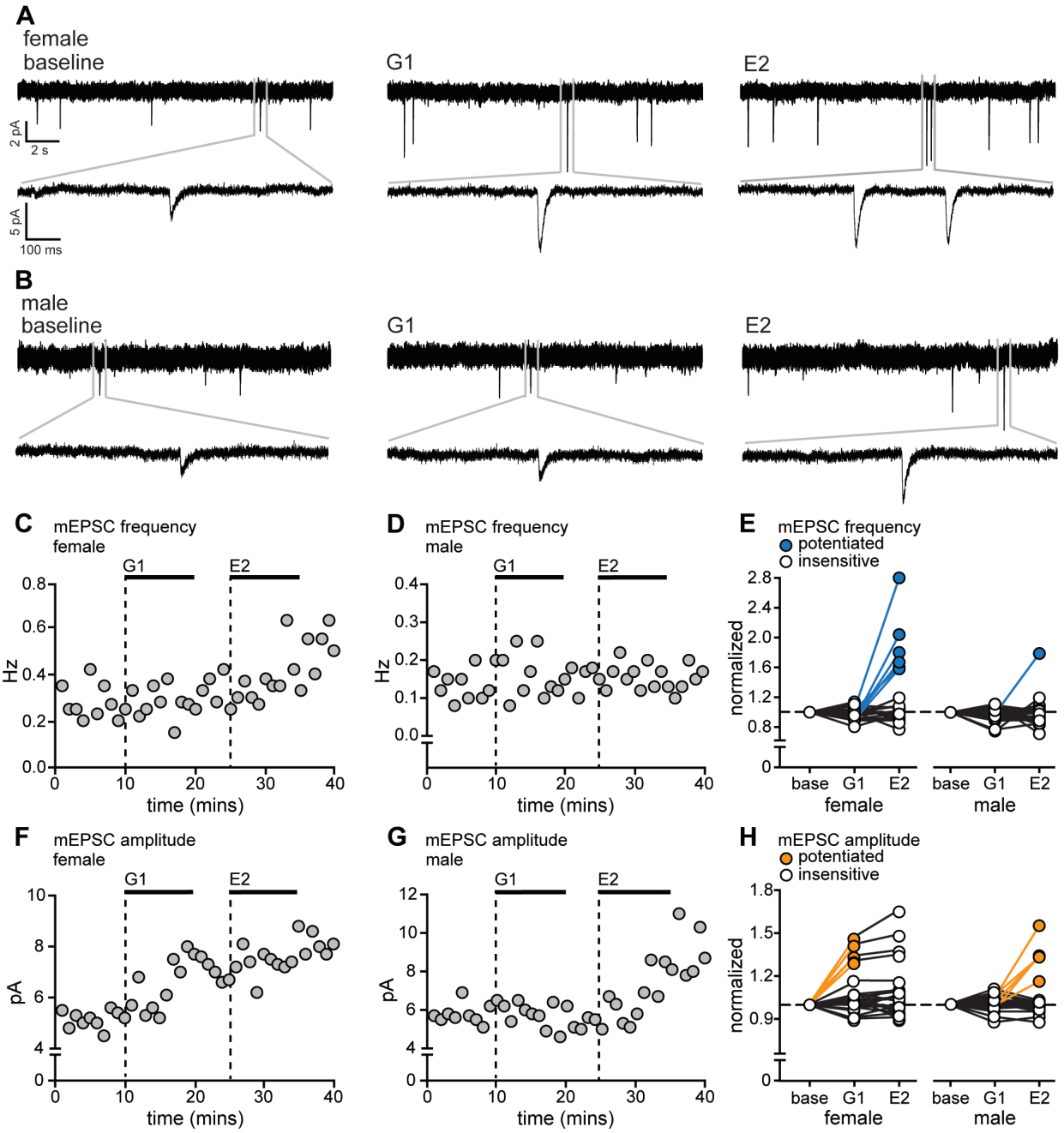



**Figure 2.4**: **The GPER1 agonist G1 acutely potentiates mEPSC amplitude in females but has no effect on mEPSCs in males.**

**A**, **B**, Sample mEPSC recordings during baseline, after G1, and after E2 in a female (**A**) and a male (**B**) cell. **C**, Time course of mEPSC frequency changes for the same female

cell as in **A** showing that G1 had no effect on mEPSC frequency but that E2 applied after G1 increased mEPSC frequency, confirming that mEPSC frequency in this cell was responsive to E2. **D**, Time course of mEPSC frequency changes for the same male cell as in **B** showing that G1 had no effect on mEPSC frequency. **E**, Summary of mEPSC frequency analysis in female and male cells with G1. Colored symbols represent points in the experiment in which within-cell t tests indicated a significant difference from the preceding condition (also in **H**). **F**, Time course of mEPSC amplitude changes for the same female cell as in **A** showing that G1 acutely increased mEPSC amplitude and that E2 after G1 had no further effect. **G**, Time course of mEPSC amplitude changes in the same male cell as in **B**. Like all other male cells recorded with G1, this cell showed no effect of G1 on mEPSC amplitude; E2 applied after G1 increased mEPSC amplitude, confirming that mEPSC amplitude in this cell was responsive to E2. There were no male cells tested with G1 that showed E2-induced increases in both mEPSC frequency and amplitude. **H**, Summary of mEPSC amplitude analysis in female and male cells with G1.

79% Thus, G1 failed to mimic the E2-induced increase in mEPSC frequency in cells in which mEPSC frequency did respond to E2, indicating that GPER1 does not modulate presynaptic glutamate release probability in either sex. Figure 6E summarizes the effects of G1 and E2 on mEPSC frequency in both sexes.

Measurements of mEPSC amplitude in the same recordings showed that G1 increased mEPSC amplitude in 5 of 23 cells from females, by 36 ± 3% (Fig. 2.4F); in the 22 of these cells in which E2 was applied after G1, there was no further effect on mEPSC amplitude. The fraction of G1-responsive cells in females (5 of 23) was statistically similar to the fraction of cells in which E2 increased mEPSC amplitude (7 of 37) (Fisher's exact test, $p > 0.99$; Fig. 2.4F vs Fig. 1H). Thus, G1 is sufficient to mimic and occlude the E2-induced increase of mEPSC amplitude in females, indicating that GPER1 activation increases postsynaptic sensitivity to glutamate in females. In contrast, and as expected from experiments in which ERβ activation was able to fully mimic and occlude the E2-induced increase in mEPSC amplitude in males, G1 failed to affect mEPSC amplitude in any of 25 cells from males (Fig. 2.4G). E2 increased mEPSC amplitude, by 34 ± 6%, in 5 of the 25 male cells in which E2 was applied after G1 washout confirming E2 responsiveness of mEPSC amplitude in these cells. Figure 2.4H summarizes the effects of G1 and E2 on mEPSC amplitude in both sexes. By demonstrating that GPER1 activation increases mEPSC amplitude specifically in females, the results of experiments with G1 provided the last piece of the puzzle to account for each presynaptic and postsynaptic component of E2-induced synaptic potentiation in both females and males.

## 2.6 Discussion

The results of this study demonstrate a latent sex difference in the mechanisms by which E2 acutely potentiates excitatory synaptic transmission in the hippocampus. We found that E2 increases both presynaptic glutamate release probability and postsynaptic sensitivity to glutamate in both sexes, but through distinct mechanisms that operate largely at separate synapses within each sex (Fig. 2.5). In females, E2 acts via ERβ to increase presynaptic glutamate release probability and through GPER1 to increase postsynaptic sensitivity to glutamate. In males, E2 acts via ERα to increase glutamate release probability and through ERβ to increase glutamate sensitivity. Remarkably, each presynaptic and postsynaptic component of E2-induced potentiation appears to be mediated exclusively by one ER subtype in each sex: ERα, ERβ, or GPER1 agonists each fully recapitulated one presynaptic or postsynaptic component of E2's effects in each sex and occluded any further effect of E2 on that component in that sex; applying E2 after an ER-selective agonist often produced the corresponding presynaptic or postsynaptic component that had not been activated by the ER agonist. This indicates a latent sex difference in which a particular stimulus produces the same functional endpoints through different mechanisms in each sex.

### 2.6.1 Sex differences in mechanisms of acute E2-induced synaptic potentiation

Our study builds on previous work that has investigated mechanisms involved in acute E2-induced synaptic potentiation. Most previous studies have been done in only one sex, however, which has led to contradictions in the literature. For example, Kramar

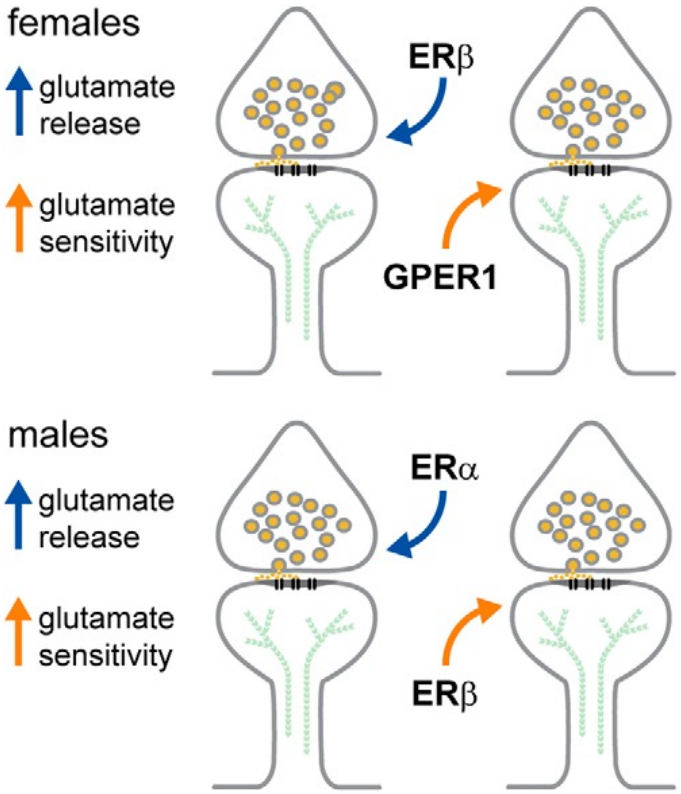



**Figure 2.5: A latent sex difference in the mechanisms of E2-induced synaptic potentiation in the hippocampus.**

Our results support a model in which E2-induced synaptic potentiation is due to both an increase in presynaptic glutamate release probability and postsynaptic sensitivity to glutamate in each sex. In both sexes, the presynaptic and postsynaptic effects of E2 occur largely at separate groups of synapses. Despite these commonalities, however, a distinct combination of ER subtypes mediates E2's effects in each sex. In females, the presynaptic increase in glutamate release probability is mediated by ERβ and the postsynaptic increase in glutamate sensitivity is mediated by GPER1. In males, the presynaptic increase in glutamate release probability is mediated by ERα and the postsynaptic increase in glutamate sensitivity is mediated by ERβ.

et al. (2009) studied males and concluded that E2- induced synaptic potentiation depends primarily on ERβ and occurs through a postsynaptic mechanism involving structural plasticity of the dendritic spine cytoskeleton. In contrast, Smejkalova and Woolley (2010) studied females, also found that ERβ mediates E2-induced synaptic potentiation but showed that this is due to an increase in presynaptic glutamate release probability. The current findings explain these apparently conflicting results by demonstrating a sex difference: ERβ activation increases postsynaptic sensitivity to glutamate in males and increases presynaptic glutamate release probability in females.

Other studies have implicated ERα and/or GPER1 in E2-induced synaptic potentiation, again typically in one or the other sex. For example, Kumar et al. (2015) investigated effects of agonists for ERβ, ERα, and GPER1 using extracellular recordings from ovariectomized female mice. These authors found that the GPER1 agonist G1 produced robust potentiation of synaptic responses, whereas ERβ and ERα agonists each produced only modest potentiation. We found that the same concentration of G1 (100 nM) increased mEPSC amplitude in females, without affecting mEPSC frequency, indicating that the G1 effect measured by Kumar et al. (2015) was likely due to increased postsynaptic glutamate sensitivity. Based on our results, the effect of an ERβ agonist (1μM DPN) in the Kumar et al. (2015) study most likely resulted from increased glutamate release probability. The source of modest potentiation induced by an ERα agonist is unresolved, however, as we found no effect of the same ERα agonist (100 nM PPT) in females. This could reflect a species difference and/or ERα-dependent suppression of GABAergic inhibition, which occurs

only in females (in rats, (Huang and Woolley, 2012; Tabatadze et al., 2015), and may have influenced the recordings in Kumar et al. (2015). Further studies will be necessary to test these possibilities.

### 2.6.2 Synapse specificity in acute E2-induced synaptic potentiation

Our results indicate that, even within one sex, distinct ER-driven mechanisms involved in the presynaptic versus postsynaptic aspects of synaptic potentiation operate largely at separate synapses. E2 increased both mEPSC frequency and amplitude in only 7-8% of recorded cells; in most cells, E2 increased either mEPSC frequency (9-19%) or mEPSC amplitude (11-18%) or had neither effect (62-66%). The synapse specificity of E2's postsynaptic effects parallels results from a previous study in which stimulation of multiple non-overlapping inputs to individual cells showed that the presynaptic component of potentiation is also input-specific (in females, (Smejkalova and Woolley, 2010).

Synapse specificity of E2-induced synaptic potentiation is likely to be related, at least partly, to the heterogeneous distribution of extranuclear ERs that mediate rapid actions of E2. All three ERs studied here, ERα (Milner et al., 2001), ERβ (Milner et al., 2005), and GPER1 (Waters et al., 2015), have been localized to subsets of dendritic spines, excitatory axonal boutons, and glial processes within the CA1 region of the hippocampus using immunoelectron microscopy. Although the fraction of spines, boutons, or glia containing immunoreactivity for each extranuclear ER has not been reported, the heterogeneous distribution of ERs predicts that effects mediated by any

one ER subtype should occur at only a subset of synapses, as we found. In the several studies that have investigated the subcellular localization of extranuclear ERs in both sexes (e.g., (Waters et al., 2015) for GPER1; (Mitterling et al., 2010) for ERβ, in mice), some quantitative sex differences have been observed, but no qualitative differences have been reported that would account for the profound sex differences we observed in the sensitivity of presynaptic versus postsynaptic components of synaptic potentiation to ER-selective agonists. Thus, it is likely that the E2 responsiveness of a synapse requires both the relevant ER subtype and downstream signaling machinery that links ER activation to increased synaptic strength. This indicates that circulating gonadal hormones do not affect acute postsynaptic E2 sensitivity of synapses, for example, by regulating extrasynaptic ERs, at least over the short time period tested in these experiments (~1 week).

Most studies that have investigated downstream mechanisms of extranuclear ER signaling have been done in cell culture (Srivastava et al., 2013) and have focused either on coupling of membrane-associated ERα or ERβ to second messenger signaling cascades through interactions with G protein-coupled receptors or signaling downstream of GPER1, which is itself a G protein-coupled receptor. One consistent finding across multiple studies of acute E2 modulation of synapses from both sexes is involvement of the mitogen-activated protein kinase pathway (Lebesgue et al., 2009; Zadran et al., 2009; Kumar et al., 2015); beyond this, however, it is unclear which of the many pathways that are acutely activated by E2 contribute to acute E2-induced synaptic potentiation.

One important question to address in future studies will be to determine where males and females converge in the pathway(s) that link specific ERs with increased glutamate release probability and sensitivity. For example, it will be useful to know whether activation of ERβ in males and GPER1 in females, both of which lead to increased postsynaptic sensitivity to glutamate, produce the same or different effects on AMPA receptor number and/or conductance, and whether through common or distinct signaling pathways. In males, E2 has been shown to increase surface expression of AMPA receptors (Zadran et al., 2009) as well as to promote postsynaptic actin polymerization through a Rho kinase-dependent increase in phosphorylated cofilin (Kramar et al., 2009), likely acting through ERβ (Babayan and Kramar, 2013). In females, GPER1 has been shown to interact with the postsynaptic scaffolding protein PSD95 (Akama et al., 2013), which could influence AMPA receptor dynamics at synapses (Nair et al., 2013). Whether GPER1 links to a similar Rho kinase/cofilin-dependent mechanism in females is not yet known, however.

### 2.6.3 Potential significance of latent sex differences in mechanisms of rapid E2 signaling

The observation of latent sex differences in mechanisms of synaptic modulation is reminiscent of De Vries' description of compensatory sex differences (De Vries, 2004), which posits that the significance of some sex differences may be to compensate for other sex differences, making males and females more similar at the behavioral level rather than more different. Our findings could be viewed in this way, in that the

combination of ER-dependent synaptic strengthening mechanisms in each sex compensates for lack of the complementary ER-dependent synaptic strengthening mechanisms in the opposite sex.

For both sexes, understanding the significance of E2's acute actions will depend on identifying the circumstances under which estrogens are produced as neurosteroids and may therefore activate acute signaling endogenously. This is an active research area in our laboratory and others. Additionally, however, latent sex differences in mechanisms of acute estrogen actions in the brain are relevant to the development and testing of therapeutics that target ER subtypes selectively, such as ERβ agonists for Alzheimer's disease (Zhao et al., 2015) and other neurological or neuropsychiatric conditions (www.clinicaltrials.gov). Given our findings, it can be anticipated that ER subtype-selective compounds may have different effects in the brains of men and women.

# Chapter 3: Latent sex differences in molecular signaling that underlies excitatory synaptic potentiation in the hippocampus

## 3.1 Abstract

Excitatory synapses can be potentiated by chemical neuromodulators, including 17β-estradiol (E2), or patterns of synaptic activation, as in long-term potentiation (LTP). Here, we investigated kinases and calcium sources required for acute E2-induced synaptic potentiation in the hippocampus of each sex and tested whether sex differences in kinase signaling extend to LTP. We recorded excitatory postsynaptic currents from CA1 pyramidal cells in hippocampal slices from adult rats and used specific inhibitors of kinases and calcium sources. This revealed that although E2 potentiates synapses to the same degree in each sex, cAMP-activated protein kinase (PKA) is required to initiate potentiation only in females. In contrast, mitogen-activated protein kinase, Src tyrosine kinase, and rho-associated kinase are required for initiation in both sexes; similarly, Ca2+/Calmodulin-activated kinase II is required for expression/maintenance of E2-induced potentiation in both sexes. Calcium source experiments showed that L-type calcium channels and calcium release from internal stores are both required for E2-induced potentiation in females, whereas in males, either L-type calcium channel activation or calcium release from stores is sufficient to permit potentiation. To investigate the generalizability of a sex difference in the requirement for PKA in synaptic potentiation, we tested how PKA inhibition affects LTP. This showed that although the magnitude of both high frequency stimulation-induced

and pairing-induced LTP is the same between sexes, PKA is required for LTP in females but not males. These results demonstrate latent sex differences in mechanisms of synaptic potentiation in which distinct molecular signaling converges to common functional endpoints in males and females.

### 3.2 Significance Statement

Chemical and activity-dependent neuromodulation alters synaptic strength in both male and female brains, yet few studies have compared mechanisms of neuromodulation between the sexes. Here, we studied molecular signaling that underlies estrogen-induced and activity-dependent potentiation of excitatory synapses in the hippocampus. We found that despite similar magnitude increases in synaptic strength in males and females, the roles of cAMP-regulated protein kinase, internal calcium stores, and L-type calcium channels differ between the sexes. Thus, latent sex differences in which the same outcome is achieved through distinct underlying mechanisms in males and females include kinase and calcium signaling involved in synaptic potentiation, demonstrating that sex is an important factor in identification of molecular targets for therapeutic development based on mechanisms of neuromodulation.

### 3.3 Introduction

There is compelling evidence that the hippocampus can synthesize estrogens as neurosteroids. In vitro studies initially showed that neural synthesis of 17β-estradiol (E2) is possible, both in cell culture (Prange-Kiel et al., 2003) and in acute hippocampal slices (Hojo et al., 2004). More recently, studies from our lab (Sato and Woolley, 2016) and others (Tuscher et al., 2016) have shown that hippocampal neurosteroid E2 synthesis also occurs in vivo. These observations motivate efforts to understand the mechanisms by which E2 synthesized in the hippocampus could influence hippocampal neurophysiology.

One likely action of neurosteroid E2 is to acutely modulate synaptic transmission. It has been known for decades that E2 can potentiate excitatory synapses in the hippocampus on a time scale of minutes and in both sexes (Teyler et al., 1980; Wong and Moss, 1992; Kramar et al., 2009; Smejkalova and Woolley, 2010). E2 has also been shown to suppress perisomatic inhibitory synapses on a similar acute time scale, although this occurs only in females (Huang and Woolley, 2012).

The possibility that acute E2-induced excitatory synaptic potentiation shares underlying mechanisms with other forms of synaptic plasticity, such as long-term potentiation (LTP), suggests molecular signaling that could be involved in E2-induced synaptic potentiation. Indeed, multiple kinases known to be important in LTP, including Src tyrosine kinase (Bi et al., 2000), mitogen-activated protein kinase (MAPK) (Zadran et al., 2009), rho-associated kinase (ROCK) (Kramar et al., 2009), and Ca2+/Calmodulin-activated kinase II (CaMKII) (Hasegawa et al., 2015), have been

indicated in E2-induced synaptic potentiation in one or the other sex. In addition, acute E2 potentiation of kainate-evoked currents in female hippocampal neurons depends on cyclic AMP-activated protein kinase (PKA) (Gu and Moss, 1996), which is implicated in some (Blitzer et al., 1998; Otmakhova et al., 2000; Yasuda et al., 2003; Wu et al., 2006) but not other (Huang and Kandel, 1994; Abel et al., 1997; Park et al., 2014) forms of LTP. Despite this extensive literature, however, no study has directly compared the involvement of specific kinases in E2-induced synaptic potentiation in males versus females to investigate the possibility of sex differences.

Thus, the first aim of the current study was to test the requirement for five kinases known to be involved in LTP: PKA, MAPK, ROCK, Src, and CaMKII, in the initiation and expression/maintenance of E2-induced potentiation of excitatory synaptic transmission in the hippocampus of each sex. The results showed that each kinase is involved either in initiation or expression/maintenance. Further, while most of the kinases tested were similarly required in males and females, we found that PKA plays a sex-specific role in initiation, being required only in females. Given this sex difference, we then investigated whether mechanisms that underlie E2-induced synaptic potentiation also involve distinct sources of increased intracellular calcium in males and females, namely L-type calcium channels and calcium release from internal stores. These experiments showed that whereas both L-type calcium channels and calcium release from stores are required for E2- induced potentiation in females, in males, either of these calcium sources appears to be able to compensate for the other. Finally, to test the generalizability of a sex difference in the involvement of PKA in synaptic plasticity,

we tested how PKA inhibition affects LTP in each sex. This showed that multiple forms of LTP require PKA specifically in females, and not in males. Thus, sex differences in molecular signaling that underlies synaptic plasticity extend beyond neurosteroid estrogen actions and may be broadly relevant for the translation of basic mechanisms of neuromodulation to the development of therapeutics appropriate for each sex.

## 3.4 Materials and Methods

### 3.4.1 *Animals*

Young adult female and male Sprague Dawley rats (50–70 days of age, Envigo) were group-housed on a 12 hrs light/dark cycle with ad libitum access to water and phytoestrogen-free chow. All rats were gonadectomized 3-8 days 87 before being used for experiments. Surgeries were performed under ketamine (85 mg/kg, i.p.; Bioniche Pharma) and xylazine (13 mg/kg, i.p.; Lloyd Laboratories) anesthesia using aseptic surgical procedures. All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Northwestern University Animal Care and Use Committee.

### 3.4.2 *Preparation of hippocampal slices*

Rats were deeply anesthetized with sodium pentobarbital (100-125 mg/kg, i.p.; Virbac) and transcardially perfused with oxygenated (95% O2/5% CO2) ice-cold sucrose-containing artificial cerebrospinal fluid (s-aCSF) containing (in mM): 75 NaCl, 25 NaHCO3, 15 dextrose, 75 sucrose, 1.25 NaH2PO4, 2 KCl, 2.4 Na pyruvate, 1.3 L-

ascorbic acid, 0.5 CaCl2, 3 MgCl2; 305-310 mOsm/L, pH 7.4. The brain was quickly removed and 300 μm transverse slices through the dorsal hippocampus were cut into a bath of ice-cold s-aCSF using a vibrating tissue slicer (VT1200S, Leica). The slices were incubated at 33$^{o}$ C in oxygenated regular aCSF containing (in mM): 126 NaCl, 26 NaHCO3, 10 dextrose, 1.25 NaH2PO4, 3 KCl, 2 CaCl2, 1 MgCl2; 305-310 mOsm/L, pH 7.4 for 30 min, then allowed to recover at room temperature for 1-6 hrs until recording.

3.4.3 *Electrophysiological recording*

Slices were transferred to a recording chamber mounted on a Zeiss Axioskop and were perfused with warm (33$^{o\ un}$C) oxygenated regular aCSF at a rate of ~2 ml/min. In most experiments, somatic whole-cell voltage-clamp recordings (Vhold = -70 mV) were obtained from visually identified CA1 pyramidal cells using patch electrodes (4–7 MΩ) filled with intracellular solution containing (in mM): 115 K-gluconate, 20 KCl, 10 HEPES, 10 Na creatine phosphate, 2 Mg-ATP, 0.3 Na-GTP, 0.001 QX-314 chloride salt; 290-295 mOsm/L, pH 7.2. In a subset of experiments, extracellular field excitatory 113 postsynaptic potentials (fEPSPs) were recorded with a glass pipette filled with regular aCSF (1-2 MΩ) and positioned in the CA1 stratum radiatum ~150 μm from the cell body layer. A glass bipolar stimulating electrode (10-50 μm tip diameter) filled with regular aCSF was placed in the stratum radiatum 200-250 μm from the recorded cell in whole-cell recordings or 100-200 μm from the recording pipette in fEPSP recordings. During an experiment, stimulation intensity was fixed at 50% of the maximal response and stimuli were delivered every 15 secs to evoke excitatory postsynaptic currents (EPSCs)

or fEPSPs. For whole cell recordings, series resistance (20-45 MΩ) was monitored throughout each recording and experiments were discontinued if series resistance fluctuated by more than 20%. All E2 experiments were done in the presence of the GABAA and NMDA receptor blockers, SR-95531 (2 μM) and DL-APV (25 μM), respectively, and were terminated by applying DNQX (25 μM) to confirm that the recorded EPSCs were mediated by AMPA receptors. LTP experiments were done in the presence of SR-95531 (2 μM). Data were acquired with a Multiclamp 700B amplifier and pClamp 10.5 software (Molecular Devices), filtered at 1-2 kHz, and digitized at 5 kHz or 20 kHz using a Digidata 1440A data acquisition system (Molecular Devices).

To investigate the roles of specific kinases in E2-induced synaptic potentiation, two types of protocols were used. In the first, baseline EPSCs were recorded for 10-20 min, followed by application of a kinase inhibitor 10-20 min prior to applying E2 (10 min) in the presence of the inhibitor to test the requirement of each kinase in initiation and/or expression of E2-induced potentiation. In the second protocol, kinase inhibitors were applied after E2-induced potentiation was established to determine the requirement of each kinase in the maintenance of potentiation. To investigate the roles of calcium release from internal stores and L-type calcium channels, baseline EPSCs were recorded, followed by application of thapsigargin and/or nifedipine for the remainder of the experiment.

To test the involvement of PKA in LTP, three protocols were used, each with or without the cell-permeant PKA inhibitor myristoylated PKI (mPKI) in the bath. In extracellular recordings, baseline fEPSPs were recorded for 15-20 min until stable.

Then, high frequency stimulation (HFS, 1 sec at 100 Hz) was delivered once or three times with a 10 min interval (Huang and Kandel, 1994) and fEPSPs were recorded for 50-55 min after LTP induction. In whole-cell recordings, baseline EPSCs were recorded for 10-12 min and then LTP was induced by pairing postsynaptic depolarization to 0 mV with 200 presynaptic stimulations delivered at 1.4 Hz (Otmakhova et al., 2000). In most cells, EPSCs were recorded for 40-50 min following induction of LTP, except for four cells (one each in female control and mPKI, and two male mPKI) in which EPSCs could be recorded for only 30-40 mins following LTP induction.

### 3.4.4 *Chemicals*

Chemicals were purchased from Tocris unless otherwise specified. Stock solutions of DL-APV, SR-95531, H89, mPKI, KN93, PD98059, SU6656, Y27632, QX-314, and tatCN21 (Calbiochem) were prepared in ddH2O, while 17β-estradiol (Sigma-Aldrich), nifedipine, thapsigargin, and DNQX were made in DMSO. The bath contained an equivalent concentration of DMSO (0.01% (v/v)) in all phases of each experiment. Stock solutions were stored at -20$^{o}$C and diluted in aCSF on the day of recording to achieve final concentrations.

### 3.4.5 *Experimental Design and Statistical Analysis*

Clampfit 10.5 was used to analyze EPSCs and fEPSPs. IGOR (ver 6.37) and Graphpad Prism (ver 6.0) were used to perform statistical analyses. To determine the E2-responsiveness of each recording individually, unpaired, two-tailed t tests were used to

compare EPSC amplitude during 2 min immediately before E2 application to 2 min beginning 4 min after E2 was removed from the bath. Measured EPSC 165 amplitudes for each E2-responsive recording are shown in the figures and data are discussed in the text as mean ± sem percent change from baseline. Two-tailed Fisher's exact tests were used to compare the proportions of cells that responded to E2 between sexes within an experiment and within-sex between different experiments. Unpaired, two-tailed t tests were used to compare the magnitude of E2-induced potentiation between sexes. To determine whether pharmacological inhibitors affected EPSC amplitude in E2-responsive experiments, unpaired, two-tailed t tests were performed within-cell to compare EPSCs recorded during 2 min immediately before application of the inhibitor to EPSCs recorded during 2 min beginning 10 min after the inhibitor was applied. In experiments to test the role of CaMKII on E2-potentiated EPSCs, in addition to within-cell analyses, paired, two-tailed t-tests were performed to evaluate the effect of tatCN21 within each sex. Measured EPSC amplitudes for each E2-responsive recording in each condition are shown in the figures and data are discussed in the text as mean ± sem percent change between pairs of conditions.

For LTP experiments, the initial slope of fEPSPs or amplitude of EPSCs were measured and the magnitude of potentiation within each recording was determined by comparing fEPSPs or EPSCs recorded during the last 10 min of the baseline period to those recorded from 40 to 50 min after LTP induction (or during the last 10 mins of the four EPSC recordings that lasted less than 50 mins following LTP induction). Paired, two-tailed t tests were used to determine whether each LTP induction protocol produced

significant potentiation within each group (male control, male mPKI, female control, female mPKI). Two-way ANOVA was used to test for an interaction between mPKI and sex in the magnitude of LTP, followed by Bonferroni Multiple Comparisons post hoc tests to evaluate differences in LTP magnitude between groups. To generate LTP plots, data for each slice or cell were normalized to the average fEPSP slope or EPSC amplitude during the baseline period and mean ± sem normalized fEPSP slope or EPSC amplitude are shown per minute in the figures. Results are discussed in the text as mean ± sem percent increase above baseline in fEPSP slope or EPSC amplitude following LTP induction.

All statistics were calculated with n as the number of cells for whole-cell recordings or number of slices for fEPSP recordings. We recorded 1-4 cells or slices per animal with 4-8 animals per experiment, except for one control tatCN21 experiment in which two cells from two animals were used. Significance for statistical tests was defined as $p < 0.05$. Full results of all statistically significant comparisons are included in the text.

## 3.5 Results

### 3.5.1 PKA is required for initiation of acute E2-induced potentiation of excitatory synapses in females but not in males

Previously, we found that E2 acutely potentiates EPSCs in a subset of CA1 pyramidal cells of adult female rats (Smejkalova and Woolley, 2010). To determine whether this effect of E2 is similar between the sexes, we performed whole-cell recordings from CA1

neurons and recorded EPSCs evoked by Schaffer collateral stimulation before, during, and after 10 min exposure to E2 (100 nM) in acute hippocampal slices from males and females. This showed that E2 increased EPSC amplitude within minutes in a subset of recordings in both sexes. Within-cell t tests showed that E2 significantly increased EPSC amplitude in 9 of 16 cells from females (Fig. 3.1A, B), by 83±16% (range: 35%-172%; Fig. 3.1C). Similarly, in males, E2 significantly increased EPSC amplitude in 11 of 18 cells (Fig. 3.1D, E), by 89±16% (range: 26%-175%, Fig. 3.1F). In both sexes, EPSC amplitude began to increase within 5-8 minutes of E2 application and remained elevated following E2 washout. Neither the proportion of cells that responded to E2 (Fishers exact test, $p > 0.1$) nor the magnitude of EPS potentiation in responsive cells (unpaired t test, $p > 0.1$) differed between the sexes. Thus, there is no apparent sex difference in the acute effect of E2 to potentiate evoked EPSCs.

Early studies in dissociated CA1 neurons showed that cAMP/ PKA signaling is required for E2-induced potentiation of kainate-evoked currents in females (Gu and Moss, 1996). To test whether PKA is also required for E2 potentiation of synaptic transmission, we inhibited PKA activity by bath application of either membrane-permeant myristoylated PKI (mPKI, 0.5 μM) or H89 (1 μM), which have different mechanisms of action. PKA inhibitors were applied after establishing baseline values for EPSC amplitude, E2 was applied for 10 min in the presence of the inhibitor and then, because ~40% of recordings are not responsive to E2, E2 was applied a second time after inhibitor washout to test for E2-responsiveness of EPSCs. Within-cell t tests were

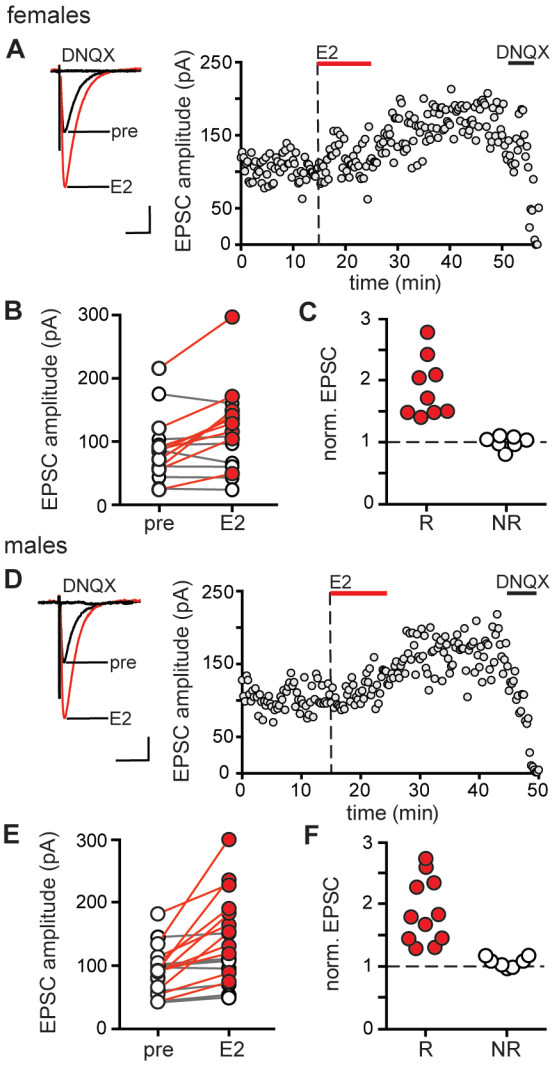



**Figure 3.1: E2 potentiates excitatory synaptic transmission in both females and males.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment in females in which E2 (100 nM) was applied for 10 min. Each point is an individual sweep and DNQX (25 μM) applied at the end of the experiment eliminated EPSCs (also in **D**). **B**, Group EPSC amplitude data for experiments in females (n=16) showing that E2 potentiated EPSC amplitude in a subset of experiments in females. Points in red represent experiments that showed a significant difference in EPSC

amplitude following E2 (n=9, unpaired t test, $p < 0.05$) and non-responsive experiments are in white (n=7). **C**, Normalized group EPSC amplitude data for E2 experiments in females where the magnitude of potentiation is shown separately for E2-responsive (R, red) and non-responsive (NR, white) experiments. **D**, Individual traces and time course of synaptic potentiation in a representative experiment in males in which E2 was applied for 10 min. **E**, Group EPSC amplitude data for experiments in males (n=18) showing that E2 potentiated EPSC amplitude in a subset of experiments in males. Points in red represent experiments that showed a significant difference in EPSC amplitude following E2 (n=11, unpaired t test, $p < 0.05$) and non-responsive experiments are in white (n=7). **F**, Normalized group EPSC amplitude data for E2 experiments in males as in C. Scales indicate 50 pA, 25 ms.

used to evaluate significant differences between each condition. Identical experiments were done in females and males.

In females, inhibiting PKA with mPKI blocked E2-induced EPSC potentiation (Fig. 3.2A). Applying mPKI itself had no effect on EPSC amplitude (5±5%) and E2 applied in the presence of mPKI failed to increase EPSC amplitude in any of 7 E2-responsive cells (Fig. 3.2B) in 11 recordings. The magnitude of potentiation induced by E2 following mPKI washout, 86±12% (range: 42%-129%), was the same as that with E2 alone (unpaired t test, $p > 0.1$). Identical experiments done with H89 confirmed that inhibiting PKA blocked E2-induced potentiation of EPSCs in females (6 E2-responsive cells in 10 recordings; Fig. 3.2C).

In contrast to results in females, mPKI failed to block E2 potentiation of EPSCs in males (Fig. 3.2D). E2 applied in the presence of mPKI increased EPSC amplitude by 115±23% (range: 76%-224%; Fig. 3.2E) in 6 E2-responsive cells from 10 recordings in males. EPSC amplitude remained elevated following mPKI+E2 washout and a second application of E2 had no further effect (Fig. 3.2E). The magnitude of E2-induced EPSC potentiation in the presence of mPKI was not different from that with E2 alone (unpaired t test, $p > 0.1$). As in females, mPKI alone had no effect on EPSC amplitude in males (3±4%). Experiments with H89 also showed that inhibiting PKA failed to block E2-induced potentiation of EPSCs in males (9 E2-responsive cells in 13 recordings; Fig. 3.2F).

We performed two additional experiments to confirm that PKA inhibition does not block EPSC potentiation in males. First, to test whether the apparent E2-induced

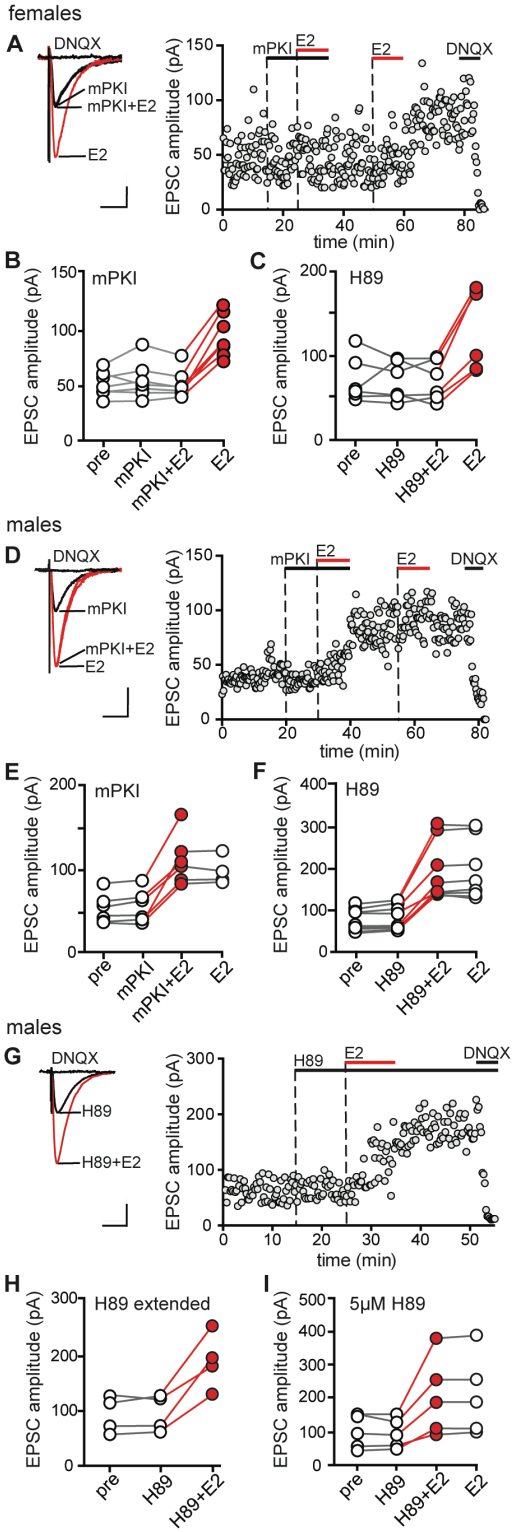

females
A
DNQX
mPKI
mPKI+E2
E2
EPSC amplitude (pA)
mPKI
E2
E2
DNQX
time (min)
B
mPKI
C
H89
pre
mPKI
mPKI+E2
E2
H89
H89+E2
males
D
E
F
G
H89
H89+E2
H
H89 extended
I
5μM H89

**Figure 3.2: PKA is required for initiation of E2-induced synaptic potentiation in females but not in males.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment in females in which mPKI (0.5 μM) was applied before applying E2 in the presence of mPKI and a 2nd application of E2 confirmed E2-responsiveness. Each point is an individual sweep and DNQX (25 μM) applied at the end of the experiment eliminated EPSCs (also in **D**, **G**). **B**, Group EPSC amplitude data for E2-responsive experiments done with mPKI in females (n=7) showing that mPKI blocked E2-induced synaptic potentiation in females. Points in red represent a significant difference in EPSC amplitude compared to the preceding condition (unpaired t test, $p < 0.05$; also in **C**, **E**, **F**, **H**, **I**). **C**, Group EPSC amplitude data for E2-responsive experiments done with H89 in females (n=6), which confirmed mPKI results. **D**, Individual traces and time course of synaptic potentiation in a representative experiment in which mPKI (0.5 μM) was applied before applying E2 in the presence of mPKI in males. **E**, Group EPSC amplitude data for E2-responsive experiments done with mPKI in males (n=6) showing that mPKI failed to block E2-induced potentiation in males. **F**, Group EPSC amplitude data for E2-responsive experiments done with H89 in males (n=9), which confirmed mPKI results. **G**, Individual traces and time course of E2-induced synaptic potentiation in a representative experiment in males in which H89 was applied until the end of the recording. **H**, Group EPSC amplitude data for E2-responsive experiments done in males with extended application of H89 (n=4). **I**, Group EPSC amplitude data for E2-

responsive experiments done in males with a higher concentration of H89 (5 μM, n=5). Scales indicate 25 pA, 25 ms.

increase in EPSC amplitude in males was related to washout of the PKA inhibitor, we extended H89 application for the duration of an experiment (Fig. 3.2G). This showed that E2 increased EPSC amplitude even in the continued presence of H89 (4 E2-responsive cells in 5 recordings; Fig. 3.2H). Second, we tested a higher concentration of H89 (5 μM), and found this also failed to block E2 potentiation of EPSCs in males (5 E2-responsive cells in 7 recordings; Fig. 3.2I). Thus, the results of experiments with PKA inhibitors showed that PKA activity is required for initiation of E2-induced EPSC potentiation in females, but not in males.

### 3.5.2 MAPK, Src, and ROCK are each required for initiation of E2-induced potentiation of excitatory synapses in both sexes

Previous studies have implicated ROCK (Murakoshi et al., 2011; Briz et al., 2015) Src (Lu et al., 1998) and MAPK (English and Sweatt, 1997) in potentiation of excitatory synapses in CA1, including a role for ROCK in E2-induced synaptic potentiation in males (Kramar et al., 2009). Therefore, we tested whether each of these kinases is required for acute E2 potentiation of synaptic transmission, and we investigated sex differences. As in experiments with PKA inhibitors, MAPK, Src, or ROCK inhibitors were applied after establishing baseline values for EPSC amplitude, E2 was applied for 10 min in the presence of the inhibitor and then again after inhibitor washout to check for E2-responsiveness in each recorded cell. Within-cell t tests were used to determine whether a recording was E2-responsive.

Inhibiting MAPK with PD98059 (PD, 50 μM) blocked E2-induced EPSC potentiation in all 6 E2-responsive cells among 10 recordings from females (Fig. 3.3A, B) and all 6 E2-responsive cells among 11 recordings from males (Fig. 3.3C). The magnitude of potentiation by E2 following PD washout (females: 65±10%, males: 69±5%) was not different from that with E2 alone (unpaired t tests, $p > 0.10$). Similarly, inhibiting Src kinase with SU6656 (SU, 10 μM) blocked E2-induced EPSC potentiation in the 6 E2-responsive cells among 13 recordings from females (Fig. 3.3D, E) and the 6 E2-responsive cells among 11 recordings from males (Fig. 3.3F), and the magnitude of potentiation by E2 following SU washout (females: 80±4%, males: 79±3%) was not different from that with E2 alone (unpaired t tests, $p > 0.10$). Finally, inhibiting ROCK with Y27632 (Y27, 30 μM) also blocked E2-induced EPSC potentiation in the 6 E2-responsive cells among 11 recordings from females (Fig. 3.3G, H) and the 6 E2-responsive cells among 12 recordings from males (Fig. 3.3I), and the magnitude of potentiation after Y27 washout (females: 83±6%, males: 81±6%) was also not different from that with E2 alone (unpaired t tests, $p > 0.10$). None of these inhibitors had any effect on EPSC amplitude on their own (PD: 1±3%, SU: -1±2%, Y27: 1±2%). Together, these experiments demonstrated that MAPK, Src, and ROCK are each required for initiation of E2-induced EPSC potentiation in both sexes.

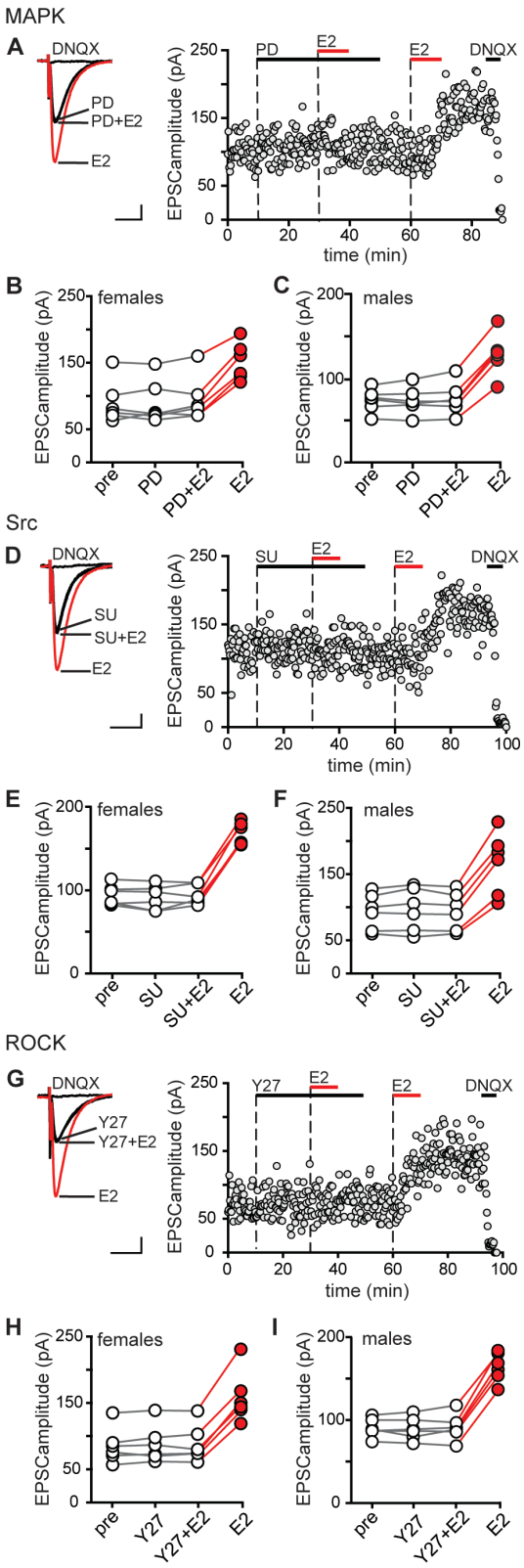

MAPK
A
DNQX
PD
PD+E2
E2
EPSCamplitude (pA)
PD
E2
E2
DNQX
time (min)
B
females
C
males
pre
PD
PD+E2
E2
Src
D
DNQX
SU
SU+E2
E2
SU
E2
E2
DNQX
time (min)
E
females
F
males
pre
SU
SU+E2
E2
ROCK
G
DNQX
Y27
Y27+E2
E2
Y27
E2
E2
DNQX
time (min)
H
females
I
males
pre
Y27
Y27+E2
E2

**Figure 3.3: MAPK, Src and ROCK are each required for initiation of E2-induced synaptic potentiation in females and males.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment where the MAPK inhibitor PD98059 (PD, 50 μM) was applied prior to E2 in females and a 2nd application of E2 confirmed E2- responsiveness. Each point is an individual sweep and DNQX (25 μM) applied at the end of the experiment eliminated EPSCs (also in **D**, **G**). **B**, Group EPSC amplitude data for E2-responsive experiments done with PD98059 in females (n=6). Points in red represent a significant difference in EPSC amplitude compared to the preceding condition (unpaired t test, $p < 0.05$; also in **C**, **E**, **F**, **H**, **I**). **C**, Group EPSC amplitude data for E2-responsive experiments done with PD98059 in males (n=6). PD98059 blocked E2-induced potentiation in both sexes. **D**, Individual traces and time course of synaptic potentiation in a representative experiment where the Src inhibitor SU6656 (SU, 803 10 μM) was applied prior to E2 in females. **E**, Group EPSC amplitude data for E2-responsive experiments done with SU6656 in females (n=6). **F**, Group EPSC amplitude data for E2-responsive experiments done with SU6656 in males (n=6). SU6656 blocked E2-induced potentiation in both sexes. **G**, Individual traces and time course of synaptic potentiation in a representative experiment where the ROCK inhibitor Y27632 (Y27, 30 μM) was applied prior to E2 in females. **H**, Group EPSC amplitude data for E2-responsive experiments done with Y27632 in females (n=6). **I**, Group EPSC amplitude data for E2-responsive experiments done with Y27632 in males (n=6). Y27632 blocked E2-induced potentiation in both sexes. Scales indicate 25 pA, 25 ms.

### 3.5.3 PKA, MAPK, Src and ROCK are not required for maintenance of E2-induced potentiation in either sex

To study mechanisms underlying the maintenance of E2-induced synaptic potentiation, we investigated whether inhibitors of PKA, MAPK, Src, or ROCK affect E2-induced EPSC potentiation after it was established. In separate experiments, we applied the same kinase inhibitors as above following stabilization of E2-induced potentiation in responsive cells from both sexes. This showed that inhibitors of PKA, MAPK, Src, or ROCK each failed to affect potentiated EPSCs in either females or males (H89: females: 7 cells Fig. 3.4A, B, males: 6 cells Fig. 3.4C; PD: females: 6 cells Fig. 3.4D, E, males: 5 cells Fig. 3.4F; SU: females: 4 cells Fig. 3.4G, H, males: 5 cells Fig. 3.4I; Y27: females: 5 cells Fig 3.4J, K, males: 6 cells Fig. 3.4L). Thus, ongoing activities of PKA, MAPK, Src, and ROCK are not required to maintain E2-induced potentiation once it has been established.

### 3.5.4 CaMKII is required for the expression and maintenance, but not for initiation, of E2-induced potentiation of excitatory synapses in both sexes

CaMKII is one of the most extensively studied kinases in the context of synaptic potentiation. CaMKII has been shown to translocate and immobilize AMPARs at the postsynaptic density (Opazo et al., 2010) and to phosphorylate Ser 831 of the AMPAR subunit GluA1 to increase single-channel conductance (Poncer et al., 2002), both of which can contribute to synaptic potentiation.

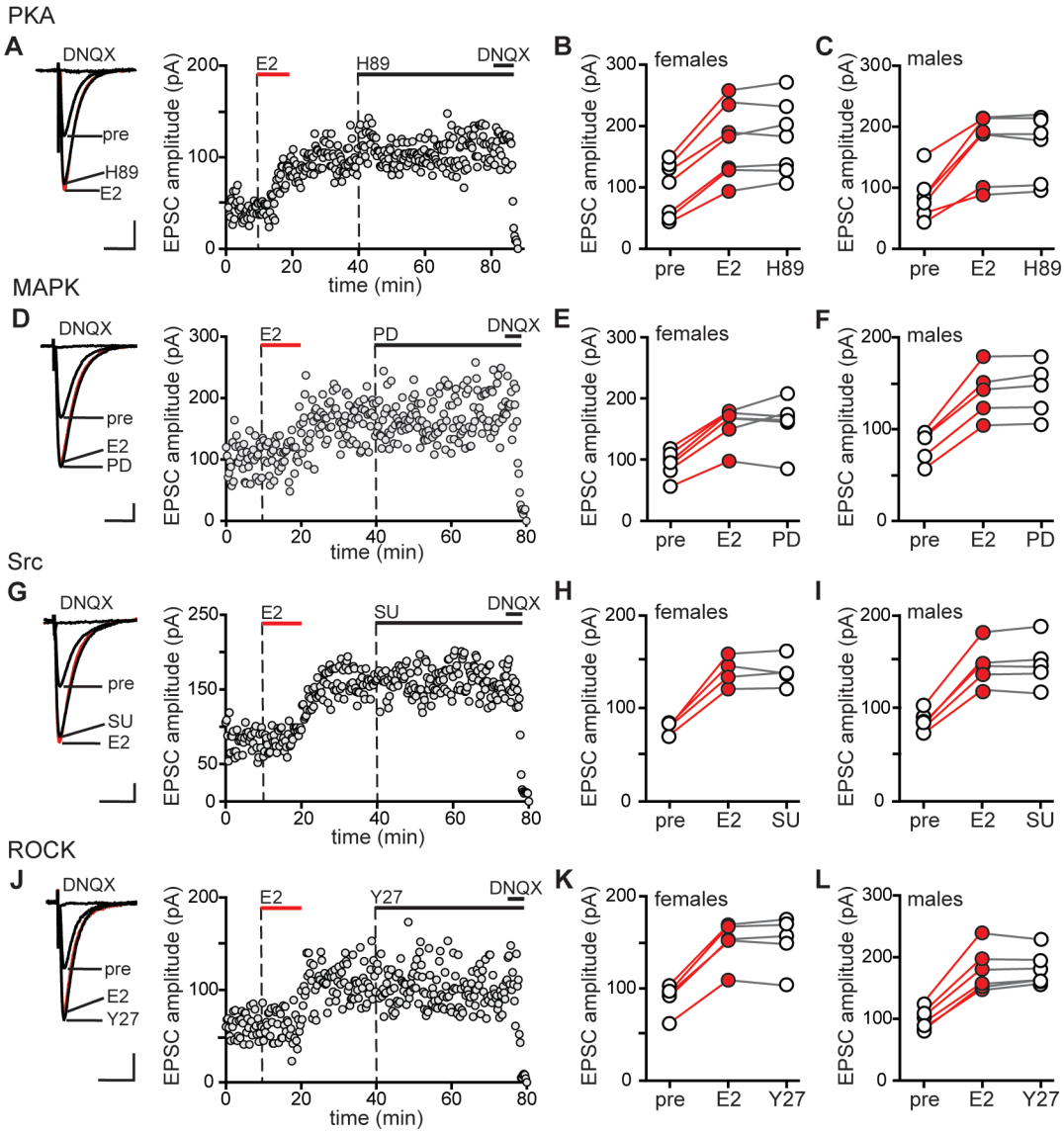


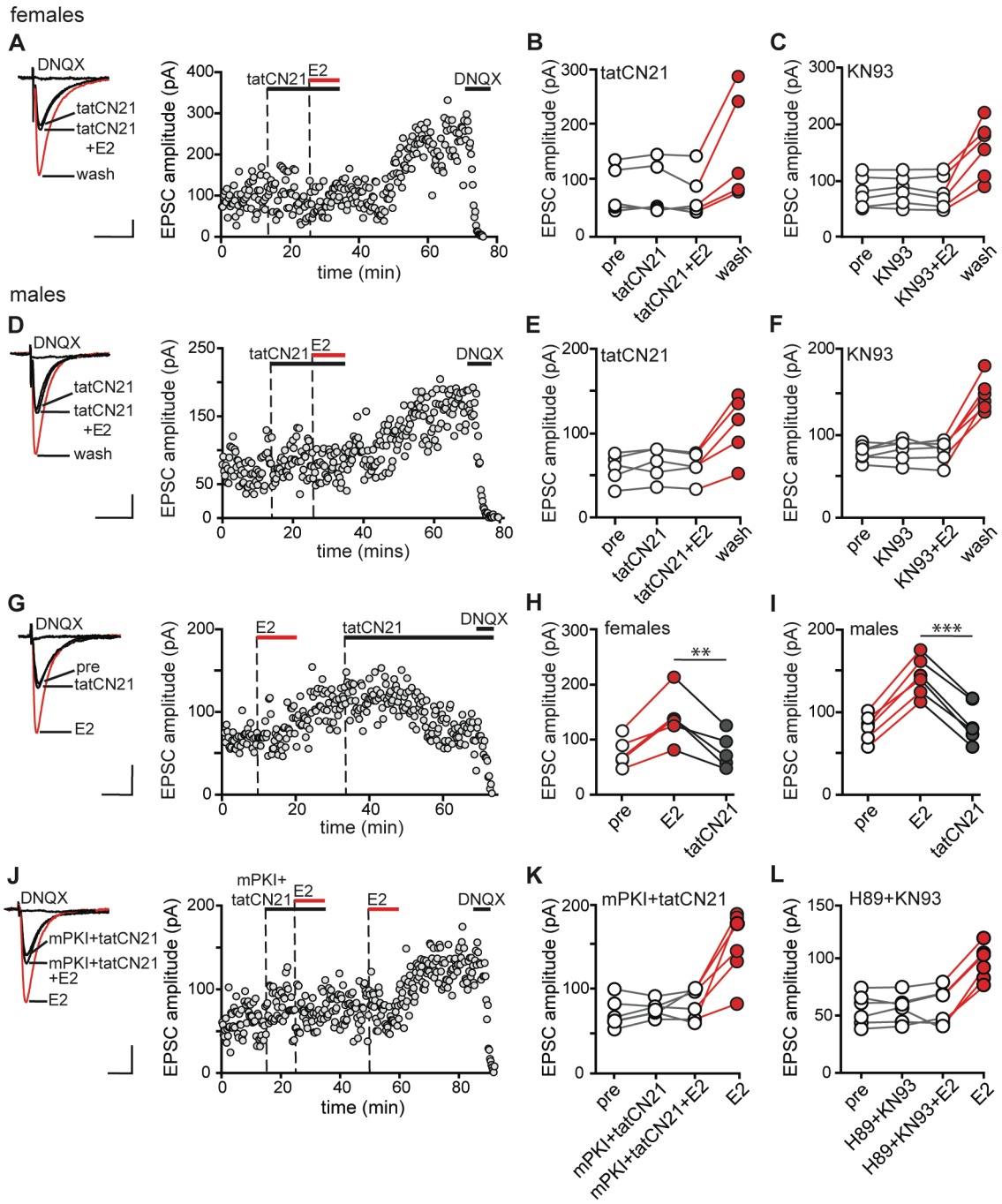

**Figure 3.4: PKA, MAPK, Src and ROCK are not required for maintenance of E2-induced synaptic potentiation in either sex.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment where the PKA inhibitor H89 (1 μM) was applied after E2-induced potentiation was established. Each point is an individual sweep and DNQX (25 μM)

applied at the end of the experiment eliminated EPSCs (also in **D**, **G**, **J**). **B**, Group EPSC amplitude data for E2-responsive experiments done with H89 in females (n=7). Points in red represent a significant difference in EPSC amplitude following E2 (unpaired t test, $p < 0.05$; also in **C**, **E**, **F**, **H**, **I**, **K**, **L**). **C**, Group EPSC amplitude data for E2-responsive experiments done with H89 in males (n=6). H89 had no effect on potentiated EPSCs in either sex. **D**, Individual traces and time course of synaptic potentiation in a representative experiment where the MAPK inhibitor PD98059 (PD, 50 μM) was applied after E2-induced potentiation was established. **E**, Group EPSC amplitude data for E2-responsive experiments done with PD98059 in females (n=6). **F**, Group EPSC amplitude data for E2-responsive experiments done with PD98059 in males (n=5). PD98059 had no effect on potentiated EPSCs in either sex. **G**, Individual traces and time course of synaptic potentiation in a representative experiment where the Src 829 inhibitor SU6656 (SU, 10 μM) was applied after E2-induced potentiation was established. **H**, Group EPSC amplitude data for E2-responsive experiments done with SU6656 in females (n=4). **I**, Group EPSC amplitude data for E2-responsive experiments done with SU6656 in males (n=5). SU6656 had no effect on potentiated EPSCs in either sex. **J**, Individual traces and time course of synaptic potentiation in a representative experiment where ROCK inhibitor Y27632 (Y27, 30 μM) was applied after E2-induced potentiation was established. **K**, Group EPSC amplitude data for E2-responsive experiments done with Y27632 in females (n=5). **L**, Group EPSC amplitude data for E2-responsive experiments done with Y27632 in males (n=6). Y27 had no effect on potentiated EPSCs in either sex. Scales indicate 25 pA, 25 ms.

We tested the involvement of CaMKII in E2-induced potentiation using the membrane-permeant CaMKII inhibitors, tatCN21 (1 μM) and KN93 (1 μM), which inhibit CaMKII activity through different mechanisms. In one set of experiments, we applied CaMKII inhibitors prior to applying E2 in the presence of the inhibitor. In other experiments, tatCN21 was applied after E2-induced potentiation was established. Identical experiments were performed in males and females.

In females, tatCN21 by itself had no effect on baseline EPSCs and E2 applied in the presence of tatCN21 failed to increase EPSC amplitude. However, in 5 of 7 recordings, EPSC amplitude measured 10-15 minutes after tatCN21 and E2 were washed out increased by 97±9%, without a second application of E2 (Fig. 3.5A, B). Identical experiments with KN93 confirmed tatCN21 results. E2 failed to potentiate EPSCs in the presence of KN93, but 10-15 minutes after KN93 and E2 were washed out, EPSC amplitude increased by 101±16% in 6 of 10 recordings in females (Fig. 3.5C). Likewise, in males, tatCN21 appeared to block E2-induced EPSC potentiation, but 10-15 min after tatCN21 and E2 washout, EPSCs increased by 81±13% in 5 of 8 recordings, without a second E2 application (Fig. 3.5D, E). KN93 also appeared to block


females
A
DNQX
tatCN21
tatCN21 +E2
wash
EPSC amplitude (pA)
tatCN21
E2
DNQX
time (min)
B
tatCN21
pre
tatCN21
tatCN21+E2
wash
C
KN93
pre
KN93
KN93+E2
wash
males
D
DNQX
tatCN21
tatCN21 +E2
wash
time (mins)
E
tatCN21
F
KN93
G
DNQX
pre tatCN21
E2
time (min)
H
females
**
I
males
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J
DNQX
mPKI+tatCN21
mPKI+tatCN21 +E2
E2
mPKI+ tatCN21
K
mPKI+tatCN21
mPKI+tatCN21+E2
L
H89+KN93
H89+KN93+E2

**Figure 3.5: CaMKII is required for expression and maintenance of E2-induced potentiation in both sexes.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment where the CaMKII inhibitor tatCN21 (1 μM) was applied prior to applying E2 in females. Each point is an individual sweep and DNQX (25 μM) applied at the end of the experiment eliminated EPSCs (also in **D**, **G**, **J**). **B**, Group EPSC amplitude data for E2-responsive experiments done with tatCN21 in females (n=5). Points in red represent a significant difference in EPSC amplitude compared to the preceding condition (unpaired t test, $p < 0.05$; also in **C**, **E**, **F**, **H**, **I**, **K**, **L**). **C**, Group EPSC amplitude data for E2-responsive experiments done with KN93 in females (n=6). **D**, Individual traces and time course of synaptic potentiation in a representative experiment where tatCN21 (1 μM) was applied prior to applying E2 in males. **E**, Group EPSC amplitude data for E2-responsive experiments done with tatCN21 in males (n=5). **F**, Group EPSC amplitude data for E2-responsive experiments done with KN93 (1 μM) in males (n=6). In both sexes, inhibiting CaMKII masked rather than blocked E2-induced potentiation. **G**, Individual traces and time course of synaptic potentiation in a representative experiment where tatCN21 (1μM) was applied after E2-induced potentiation was established. **H**, Group EPSC amplitude data for E2-responsive experiments done with tatCN21 in females (n=5). **I**, Group EPSC amplitude data for E2-responsive experiments done with tatCN21 in males (n=5). Points in gray indicate a significant difference in EPSC amplitude following tatCN21. In both sexes, inhibiting CaMKII reversed E2-induced potentiation of EPSCs (paired t test, *** indicates $p < 0.001$). **J**, Individual traces and

time course of synaptic potentiation in a representative experiment in males where mPKI (0.5 μM) and tatCN21 (1 μM) were applied together prior to applying E2. **K**, Group EPSC amplitude data for E2-responsive experiments done with mPKI and tatCN21 (n=6). **L**, Group EPSC amplitude data for E2-responsive experiments done with KN93 (1 μM) and H89 (1μM) (n=6). In males, inhibiting PKA and CaMKII together blocked E2-induced potentiation. Scales indicate 25 pA, 25 ms.

E2-induced EPSC potentiation in males, but EPSC amplitude increased by 73±3% in 6 of 12 recordings after KN93 and E2 washout (Fig. 3.5F). In both sexes and with both inhibitors, the proportion of cells that responded to E2 (Fishers exact tests, all p values > 0.1) and the magnitude of EPSC potentiation (unpaired t tests, all p values > 0.1) were similar to those obtained in experiments with E2 alone.

The results of experiments with CaMKII inhibitors suggested that inhibiting CaMKII masked EPSC potentiation rather than inhibited it. To rule out alternative possibilities, we did 3 additional experiments. First, we first modified the protocol to extend tatCN21 for an additional 10 min after washing out E2. Consistent with previous results, EPSC amplitude increased only 10-15 min after washing out tatCN21, in both females (3 of 5 recordings) and males (3 of 5 recordings) (data not shown). In a second set of experiments, we continued KN93 application after E2 (for up to 40 min) and never observed EPSC potentiation in either females (6 recordings) or males (6 recordings) (data not shown). In the last of these control experiments, we applied tatCN21 alone for 20-25 mins and then washed it out, which had no effect on EPSC amplitude (2 recordings in males, data not shown). Thus, the increase in EPSC amplitude observed following washout of E2 plus a CaMKII inhibitor was not an artifact of inhibitor washout, and instead indicates that CaMKII activity is required for the expression and not initiation of E2-induced synaptic potentiation.

Next, to test whether CaMKII is required for maintenance of E2-induced synaptic potentiation, we applied tatCN21 after potentiation was established (Fig. 3.5G). Consistent with a role for CaMKII in maintaining potentiated EPSCs, tatCN21 reversed

E2-induced potentiation in both sexes. In females, tatCN21 decreased EPSC amplitude from 83±13% above baseline to 5±3% above baseline in 5 of 5 E2-responsive recordings (paired t test, $t(4) = 4.9$, $p = 0.0075$) (Fig. 3.5H). Results were similar in males, where tatCN21 decreased EPSC amplitude from 76±7% above baseline to 8±8% above baseline in 6 of 6 E2-responsive recordings (paired t test, $t(5) = 13.78$, $p < 0.0001$) (Fig. 3.5I). These results showed that, in both sexes, CaMKII activity is required not only for initial expression but also ongoing maintenance of E2-induced synaptic potentiation.

### 3.5.5 PKA and CaMKII cooperate to initiate E2- induced potentiation of excitatory synapses in males

Experiments with mPKI and H89 indicated that PKA by itself is not required for initiation of E2-induced synaptic potentiation in males (Fig 3.2E, F). Previous studies have shown that although PKA is not required for many forms of early LTP induction, it can facilitate the activity of other kinases, including CaMKII (Blitzer et al., 1998), by inhibiting protein phosphatase 1 and thereby indirectly contribute to LTP. To investigate whether PKA might play a similar role in E2-induced synaptic potentiation in males, we tested whether inhibiting both PKA and CaMKII simultaneously could block E2-induced potentiation.

In contrast to the results obtained with mPKI or tatCN21 alone, which each failed to block E2-induced synaptic potentiation in males (Fig. 3.2E, F and Fig. 3.5D, E), co-application of these inhibitors blocked initiation of potentiation in males; a second

application of E2 after inhibitor washout potentiated EPSCs in 6 out of 10 recordings (Fig. 3.5J, K) confirming that they were E2-responsive. Identical experiments with H89 (PKA inhibitor) and KN93 (CaMKII inhibitor) showed the same results. Co-application of H89 and KN93 in males also blocked E2-induced potentiation in the 6 E2-responsive cells out of 8 recordings (Fig. 3.5L). These experiments indicate that while PKA is not absolutely required for E2-induced synaptic potentiation in males, it does play a role in cooperation with, and possibly by facilitating activity of, CaMKII.

### 3.5.6 Sex differences in the requirement of internal calcium stores and L-type calcium channels in E2-induced synaptic potentiation

CaMKII activation likely requires an increase in intracellular calcium. Because NMDA receptors were blocked in our experiments, we focused on two alternative calcium sources: internal stores and L-type calcium channels. In separate experiments, we used either thapsigargin (1 μM) to deplete internal calcium stores or nifedipine (10 μM) to block L-type calcium channels. Thapsigargin by itself produced a transient (10-30 min) increase in EPSC amplitude, which was similar in magnitude in both females (70±18%) and males (74±23%). E2 was applied in the continued presence of thapsigargin after EPSCs returned to baseline. Nifedipine by itself had no effect on baseline EPSCs and E2 was also applied in the presence of the inhibitor. Identical experiments were done in females and males.

Experiments with thapsigargin showed that in females but not in males, depletion of internal calcium stores blocked the initiation of E2-induced synaptic potentiation. In

females, E2 applied in the presence of thapsigargin failed to increase EPSC amplitude in any of 9 recordings (Fig. 3.6A, B). In contrast, in males, E2 applied in the presence of thapsigargin potentiated EPSC amplitude in 6 of 9 cells, by 62±6% (Fig. 3.6C, D). Similarly, inhibition of L-type calcium channels blocked E2-induced potentiation in females but not in males. In females, E2 failed to potentiate EPSCs in the presence of nifedipine in any of 9 recordings (Fig. 3.6E, F) whereas, in males, even in the presence of nifedipine, E2 increased EPSC amplitude in 6 of 9 cells, by 66±11% (Fig. 3.6G, H). Parameters of E2-induced potentiation in thapsigargin or in nifedipine in males were statistically similar to potentiation with E2 alone (89±16%, 11 of 18 cells) (unpaired t tests, p values > 0.10).

We hypothesized that, similar to the cooperative action we observed between PKA and CaMKII during E2-induced synaptic potentiation in males (Fig. 3.5J, K, L), calcium release from internal stores and L-type calcium channels might also cooperate during E2-induced potentiation in males. To test this possibility, we blocked both calcium release from internal stores and L-type calcium channels simultaneously. Similar to results with thapsigargin alone, applying nifedipine and thapsigargin together produced a transient increase in EPSC amplitude (75±13%) and when EPSC amplitude stabilized, E2 was applied in the continued presence of both inhibitors.

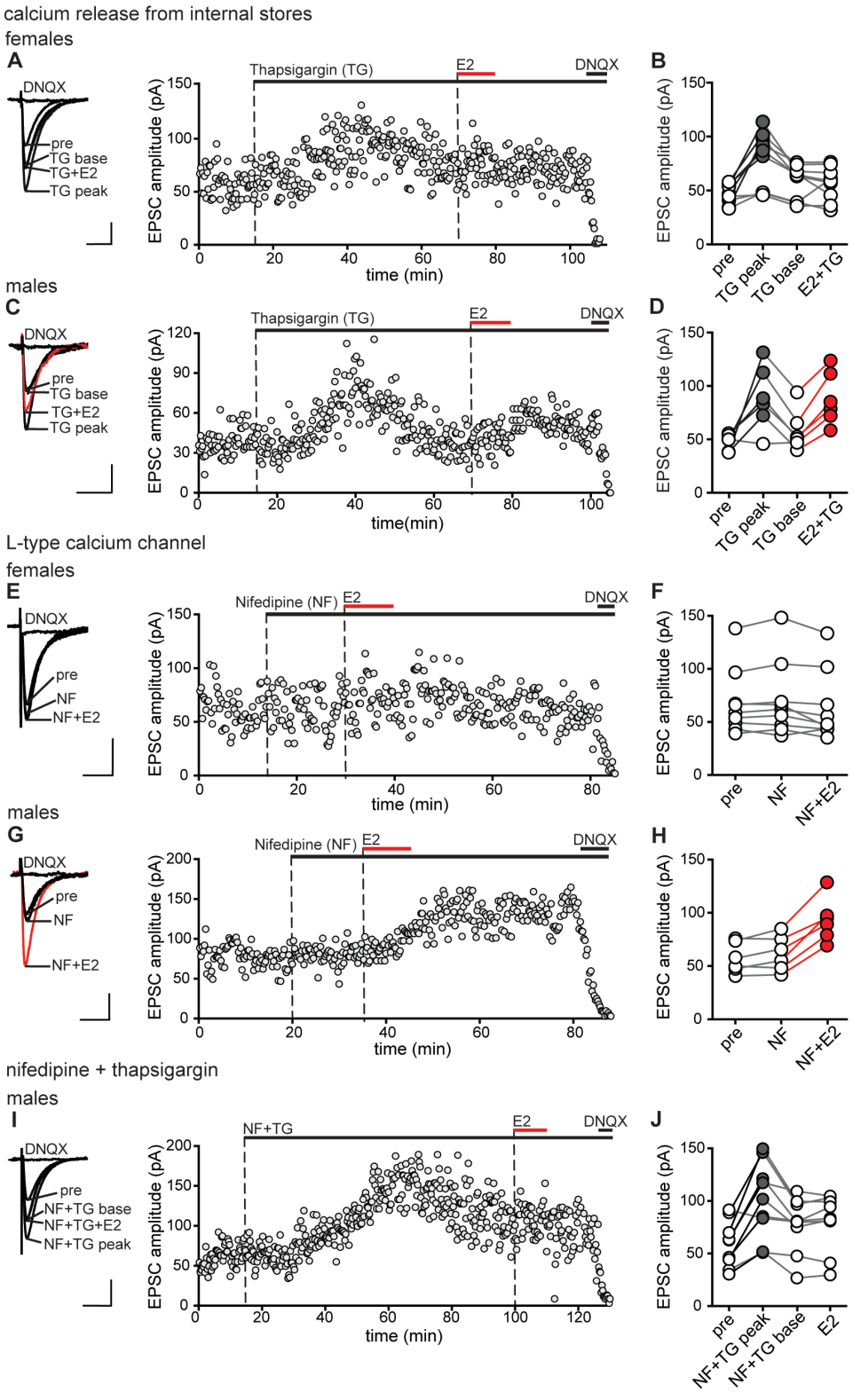

calcium release from internal stores
females
A
DNQX
pre
TG base
TG+E2
TG peak
Thapsigargin (TG)
E2
DNQX
EPSC amplitude (pA)
time (min)
B
EPSC amplitude (pA)
pre
TG peak
TG base
E2+TG
males
C
DNQX
pre
TG base
TG+E2
TG peak
Thapsigargin (TG)
E2
DNQX
EPSC amplitude (pA)
time(min)
D
EPSC amplitude (pA)
pre
TG peak
TG base
E2+TG
L-type calcium channel
females
E
DNQX
pre
NF
NF+E2
Nifedipine (NF)
E2
DNQX
EPSC amplitude (pA)
time (min)
F
EPSC amplitude (pA)
pre
NF
NF+E2
males
G
DNQX
pre
NF
NF+E2
Nifedipine (NF)
E2
DNQX
EPSC amplitude (pA)
time (min)
H
EPSC amplitude (pA)
pre
NF
NF+E2
nifedipine + thapsigargin
males
I
DNQX
pre
NF+TG base
NF+TG+E2
NF+TG peak
NF+TG
E2
DNQX
EPSC amplitude (pA)
time (min)
J
EPSC amplitude (pA)
pre
NF+TG peak
NF+TG base
E2

**Figure 3.6: Sex differences in the requirement of calcium release from internal stores and L-type calcium channels during E2-induced EPSC potentiation.**

**A**, Individual traces and time course of synaptic potentiation in a representative experiment where thapsigargin (TG, 1 μM) was applied prior to E2 in females. Each point is an individual sweep and DNQX (25 μM) applied at the end of the experiment eliminated EPSCs (also in **C**, **E**, **G**, **I**). **B**, Group EPSC amplitude data for E2 experiments done with TG (n=9) showing that E2 failed to potentiate EPSC amplitude in the presence of TG in females. TG alone transiently increased EPSC amplitude in most cells (also in males, **C**), indicated by gray points (unpaired t test, $p < 0.05$; also in **D**). **C**, Individual traces and time course of synaptic potentiation in a representative experiment where thapsigargin (TG) was applied prior to E2 in males. **D**, Group EPSC amplitude data for E2-responsive experiments done with TG (n=6) showing that E2 potentiates EPSCs in the presence of TG in males. Points in red indicate a significant difference from the preceding condition (unpaired t test, $p < 0.05$; also in **H**). **E**, Individual traces and time course of synaptic potentiation in a representative experiment where nifedipine (NF, 10 μM) was applied 881 prior to E2 in females. **F**, Group EPSC amplitude data for E2 experiments done with NF (n=9) showing that E2 failed to potentiate EPSC amplitude in the presence of NF in females. **G**, Individual traces and time course of synaptic potentiation in a representative experiment where nifedipine (NF) was applied prior to E2 in males. **H**, Group EPSC amplitude data for E2-responsive experiments done with NF (n=6) showing that E2 potentiates EPSCs in the presence of NF in males. **I**, Individual traces and time course of synaptic potentiation in a representative

experiment in males where NF and TG were applied together prior to E2. Similar to results with TG alone (see **A**, **C**), EPSC amplitude increased transiently in the presence of TG+NF. **J**, Group EPSC amplitude data for E2 experiments done with TG+NF (n=9), showing that E2 failed to potentiate EPSC amplitude in the presence of TG+NF in males. Gray points indicate a significant difference from baseline (unpaired t test, $p < 0.05$). Scales indicate 25 pA, 25 ms.

Although neither depleting internal calcium stores nor blocking L-type calcium channels, alone, was sufficient to inhibit E2 potentiation of EPSCs in males, inhibiting both together did block potentiation. E2 failed to potentiate EPSCs in any of 9 recordings in which thapsigargin and nifedipine were applied together (Fig. 3.6I, J). Thus, in contrast to females in which both calcium release from internal stores and L-type calcium channels are required for E2-induced synaptic potentiation, these two calcium sources may be able to compensate for each other in E2-induced potentiation in males. Together with a similar result in experiments using PKA and CaMKII inhibitors, this suggests a pattern of parallel signaling leading to synaptic potentiation in males that is distinct from signaling leading to the same outcome in females.

### 3.5.7 Sex difference in the involvement of PKA in LTP

Experiments with mPKI and H89 showed a sex difference in the requirement for PKA in initiation of E2-induced synaptic potentiation (Fig. 3.2). This raises the question of whether a sex difference in involvement of PKA is specific to E2-potentiation or could generalize to other forms of synaptic plasticity. The involvement of PKA in LTP of CA3-CA1 synapses has been studied extensively, and depends on the LTP induction protocol and/or age of animals used. No studies have compared the role of PKA in both sexes, however. Thus, we investigated the possibility of a sex difference in PKA involvement in LTP. We used three protocols: HFS-induced LTP using 1x or 3x 100 Hz stimulation for 1 sec, the early component of which is widely reported to be insensitive to inhibition of PKA (Frey et al., 1993; Huang and Kandel, 1994; Abel et al., 1997; Woo

et al., 2002; Park et al., 2014) and LTP induced by pairing postsynaptic depolarization to 0 mV with 200 presynaptic stimulations at 1.4 Hz, which is reported to be modestly sensitive to PKA inhibition, based on recordings in males (Otmakhova et al., 2000) Identical experiments were performed in both sexes, with or without mPKI (0.5 μM) in the bath.

Experiments with 1x HFS showed that PKA was required for LTP in females but not in males. In males, 1x HFS increased fEPSP slope by 62±15% above baseline in control recordings (n = 7; paired t test, t(6) = 5.86, p = 0.001) and by 43±15% in mPKI (n = 6; paired t test, t(5) = 5.15, p = 0.004; Fig 3.7A). In females, 1x HFS also increased fEPSP slope in control recordings, by 60±15% above baseline (n = 6; paired t test, t(5) = 4.714, p = 0.005), but failed to significantly increase fEPSP slope in the presence of mPKI (13±11%, n = 6; paired t test, t(5) = 1.62, p > 0.10; Fig. 3.7B). Two-way ANOVA confirmed a significant interaction between sex and mPKI in the magnitude of LTP induced by 1x HFS (F(1,21)  4.6, p = 0.043) and post hoc tests showed that results in female mPKI recordings were significantly different from female controls (p = 0.009, Fig. 3.7C) and both male groups (p values < 0.03), which were not different from each other (all p values > 0.10). To confirm that PKA inhibition does not block 1x HFS-induced LTP in males, we performed additional experiments with 1 μM mPKI, which also failed to block LTP in males (51±17%, n = 3, data not shown).

Similar to results with 1x HFS, 3x HFS also showed a sex difference in the involvement of PKA in LTP; however, in these experiments, mPKI decreased LTP in females but did not block it. In males, 3x HFS significantly increased fEPSP slope by

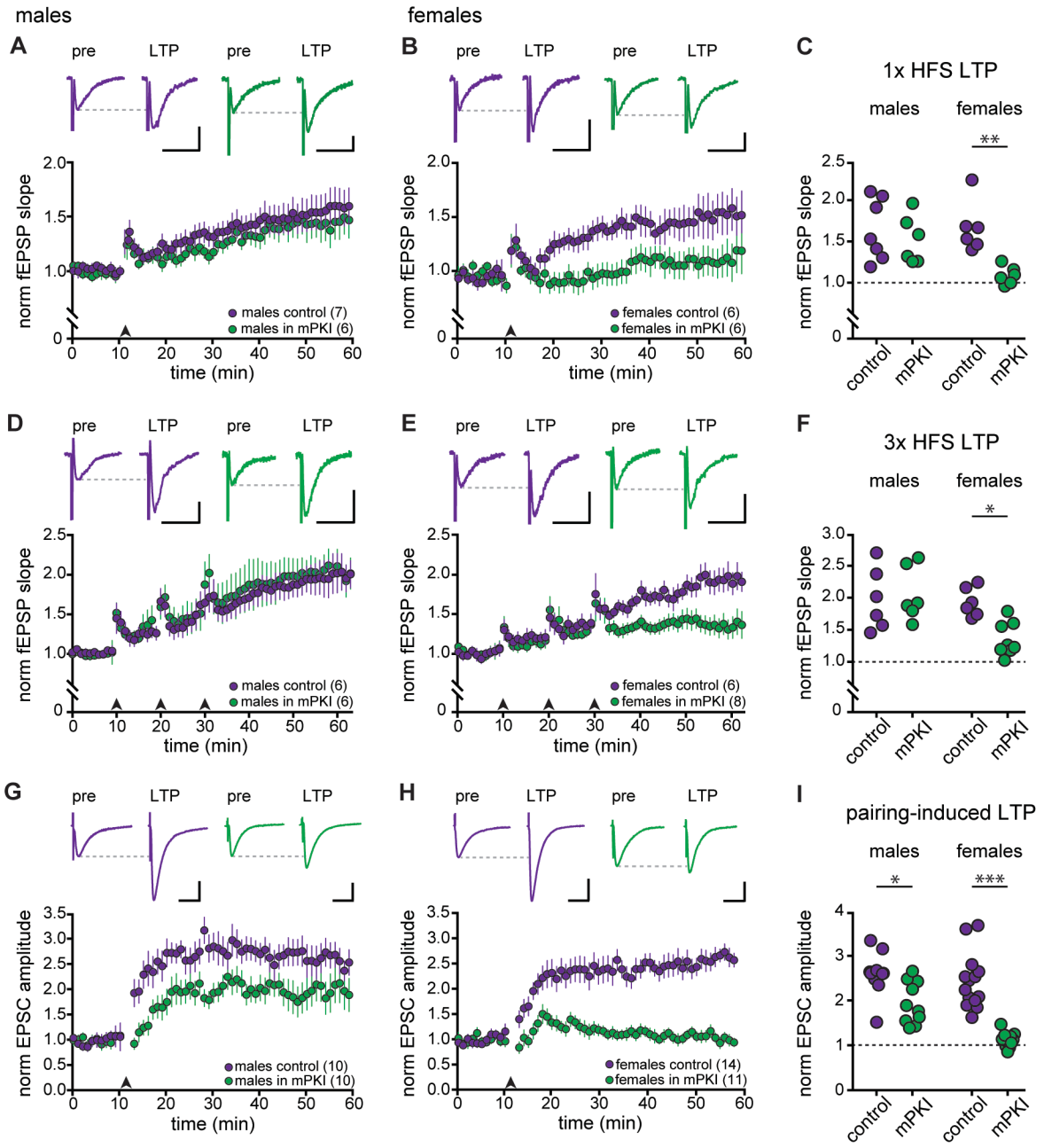



**Figure 3.7: Sex differences in the requirement for PKA in long term potentiation.**

**A,** Representative individual traces and time course of mean±sem normalized fEPSP slope for 1x HFS LTP experiments in males (n=7 control, purple; n=6 mPKI, green). Scales indicate 0.2 mV, 25 ms (also in **B**, **D**, **E**). **B,** Representative individual traces and

time course of mean±sem normalized fEPSP slope for all 1x HFS LTP experiments in females (n=6 control, purple; n=6 mPKI, green). **C,** Normalized increase in fEPSP slope for all 1x HFS LTP experiments where the magnitude of potentiation is shown separately for each control and mPKI experiment in males and females. PKA inhibition had no effect on 1x HFS LTP in males, but blocked 1x HFS LTP in females. ** indicates $p < 0.01$. **D,** Representative individual traces and time course of mean±sem normalized fEPSP slope for all 3x 100Hz LTP experiments in males (n=6 control, purple; n=6 mPKI, green). **E**, Representative individual traces and time course of mean±sem normalized fEPSP slope for all 3x 100Hz LTP experiments in females (n=6 control, purple; n=8 mPKI, green). **F**, Normalized increase in fEPSP slope for all 3x HFS LTP experiments where the magnitude of potentiation is shown separately for each control and mPKI experiment in males and females. PKA inhibition had no effect on 3x HFS LTP in males, but attenuated 3x HFS LTP in females. * indicates $p < 0.05$. **G**, Representative individual traces and time course of mean±sem normalized EPSC amplitude for all pairing-induced LTP experiments in males (n=10 control, purple; n=10 mPKI, green). Scales indicate 50 pA, 25 ms (also in H). **H**, Representative individual traces and time course of mean±sem normalized EPSC amplitude for all pairing- induced LTP experiments in females (n=14 control, purple; n=11 mPKI, green). **I**, Normalized increase in EPSC amplitude for all pairing induced LTP experiments where the magnitude of potentiation is shown separately for each control and mPKI experiment in males and females. PKA inhibition attenuated pairing-induced LTP in males, but blocked pairing-induced LTP in females. * indicates $p < 0.05$, *** indicates $p < 0.001$.

97±19% above baseline in control recordings (n = 6; paired t test, t(5) = 4.76, p = 0.005) and by 101±19% in mPKI (n = 6; paired t test, t(5) = 8.15, p < 0.001; Fig 3.7D). In female controls, 3x HFS increased fEPSP slope by 86±11% (n = 6; paired t test, t(5) = 5.75, p = 0.002), similar to males, but by only 35±8% in the presence of mPKI (n = 8; paired t test, t(7) = 4. 71, p = 0.002; Fig. 3.7E). As in the case of 1x HFS-induced LTP, two-way ANOVA showed a significant interaction between sex and mPKI in the magnitude of LTP induced by 3x HFS (F(1,22) = 5.46, p = 0.028) and post hoc tests showed that female mPKI recordings were significantly different from female controls (p = 0.04, Fig. 3.7F) and both male groups (p values < 0.03), which were not different from each other (all p values > 0.10).

Finally, we tested whether a distinct type of LTP, pairing-induced LTP, also differs by sex in its dependence on PKA. These experiments showed that whereas pairing-induced LTP was modestly attenuated by PKA inhibition in males, consistent with previous reports, it was abolished by PKA inhibition in females. The pairing protocol potentiated EPSCs by 148±19% above baseline in male control recordings (n = 10, paired t test, t(9) = 5.74, p < 0.001), but by only 100±15% above baseline in the presence of mPKI (n = 10, paired t test, t(9) = 5.08, p < 0.001; Fig. 3.7G). In females, pairing potentiated EPSCs by 160 ±17% above baseline in controls (n = 14, paired t test, t(13) = 7.04, p < 0.001), similar to males, but failed to potentiate EPSCs in the presence of mPKI (5±5% above baseline, n = 11, paired t test, t(10) = 1.38, p > 0.10; Fig. 3.7H). Two-way ANOVA indicated a significant interaction between sex and mPKI (F(1,41) = 6.96, p = 0.01). Post hoc tests showed that mPKI decreased LTP in both

sexes (males $p = 0.04$, females $p < 0.001$; Fig. 3.7I), and that female mPKI differed from all other groups (all p values < 0.001). Thus, while PKA contributes to pairing-induced LTP in males, it is required for pairing-induced LTP in females. Together with the effects of PKA inhibition on HFS-induced LTP and E2-induced synaptic potentiation, these results suggest a generalizable sex difference in the requirement for PKA in hippocampal synaptic potentiation.

### 3.6 Discussion

In this study, we found that multiple aspects of the molecular signaling that underlies potentiation of excitatory synapses in the hippocampus differ between the sexes. Specifically, PKA is required for initiation of E2-induced synaptic potentiation in females but not males; and both L-type calcium channels and calcium release from internal stores are required for E2-induced potentiation in females, whereas in males, either of these calcium sources is sufficient. In addition, the sex difference in PKA requirement extends to multiple forms of LTP, suggesting that this difference may be generalizable in mechanisms of synaptic potentiation. In contrast, other aspects of molecular signaling involved in E2-induced synaptic potentiation are similar between the sexes. MAPK, Src, and ROCK are each required for initiation and CaMKII is required for expression and maintenance of potentiation in both sexes. Interestingly, despite sex differences in molecular signaling, the degree of potentiation achieved in LTP or after E2 is essentially identical between the sexes. Thus, these findings extend the concept of latent sex differences (Oberlander and Woolley, 2016, 2017) in which distinct

underlying mechanisms converge to common functional endpoints in males and females.

### 3.6.1 Kinase signaling in E2-induced and activity-dependent synaptic potentiation

Considering parallels between E2-induced potentiation and LTP may give insight into intracellular signaling that leads to synaptic potentiation. In this regard, it is important to note that the male-female differences we observed were specific to synaptic potentiation. None of the kinase inhibitors we used, nor nifedipine, altered baseline synaptic transmission in either sex. Depleting internal calcium stores with thapsigargin transiently increased EPSC amplitude similarly in both sexes.

The three kinases that we found are essential for initiation of E2-induced synaptic potentiation, MAPK, Src, and ROCK, have long been known to play important roles in LTP. For example, MAPK facilitates AMPAR trafficking and insertion at synapses during LTP (Qin et al., 2005; Patterson et al., 2010) and Src activates ROCK to inhibit cofilin, which promotes actin polymerization and increases dendritic spine volume (Koleske, 2013). MAPK, Src, and ROCK are likely to function similarly in E2-induced synaptic potentiation, as has been shown directly for estrogen receptor β activation of ROCK, which promotes actin polymerization in dendritic spines in males (Kramar et al., 2009). Unlike MAPK, Src, and ROCK, CaMKII appears to function differently in LTP and E2-induced potentiation. Inhibiting CaMKII blocks initiation of early LTP (Malinow et al., 1989; Otmakhov et al., 1997; Lisman et al., 2012). However, we found that although E2-induced potentiation was not apparent in the presence of CaMKII inhibitors, EPSC

amplitude began to increase shortly after inhibitor washout, indicating that potentiation had been initiated but could not be expressed. One difference between our E2 experiments and most LTP studies is that we blocked NMDARs to focus exclusively on AMPAR modulation, whereas calcium influx through NMDARs is a critical source of increased intracellular calcium for LTP initiation. Although others have shown that CaMKII interacts with GluN2B to promote maintenance of LTP (Sanhueza et al., 2011; Barcomb et al., 2016), the 1 μM tatCN21 that we used is well below the 20 μM shown to disrupt this interaction. In combination with the fact that NMDARs were blocked in our experiments, it is unlikely that CaMKII interaction with GluN2B is relevant to the tatCN21-induced reversal of E2-induced potentiation that we observed. The requirement for CaMKII in expression of E2-induced potentiation may instead reflect CaMKII's role in trapping newly inserted AMPARs at synapses through phosphorylation of TARPs, such as stargazin (Chen et al., 2000; Tomita et al., 2005; Opazo et al., 2010).

In contrast to other kinases, PKA is more commonly associated with late LTP (Frey et al., 1993; Huang and Kandel, 1994; Abel et al., 1997) although some studies indicate that early LTP can be sensitive to PKA inhibition (Blitzer et al., 1998; Otmakhova et al., 2000; Yasuda et al., 2003; Wu et al., 2006). Most of these previous studies either used males or sex was not noted, raising the possibility that some discrepancies in the literature are related to sex differences.

One potential explanation for the sex differences in PKA requirement that we found derives from the idea that PKA inhibits phosphatases that normally constrain the

activity of other kinases, such as CaMKII, and thereby permits LTP but does not directly cause it (Blitzer et al., 1998). That inhibition of PKA and CaMKII together blocks E2-induced potentiation in males suggests that PKA may play a similar role in E2-induced synaptic potentiation in males. If PKA acts as a gate for E2-induced potentiation, the sex difference in sensitivity of potentiation to PKA inhibition could be related to differences in basal PKA activity. For example, higher basal PKA activity in males might permit activity of other kinases that are essential for potentiation without E2 modulation of PKA. In females, lower basal PKA activity might fail to establish this permissive state and require stimulation by E2 to achieve it. Studies in cardiomyocytes support this possibility. Both basal cAMP levels and PKA activity are lower in female than male cardiomyocytes, due at least in part to females' higher levels of phosphodiesterase (PDE) 4B, which hydrolyzes cAMP (Parks et al., 2014). Whether this sex difference extends to the hippocampus is unknown, however. There is evidence that PDE4B inhibition promotes early LTP in the hippocampus (Titus et al., 2016; Campbell et al., 2017), but this has been tested only in males.

It is also possible that sex differences in the levels of extranuclear estrogen receptors (ERs) that mediate acute E2 signaling, or the coupling of ERs to downstream effectors of synaptic plasticity, differ between the sexes (Wang et al., 2018). Consistent with this idea, we have shown previously that distinct combinations of ERs mediate E2-induced synaptic potentiation in males versus females (Chapter 2). Heterogeneous distributions of extranuclear ERs may also contribute to the consistent finding, here and

previously (Wong and Moss, 1992; Smejkalova and Woolley, 2010), that only a subset of recordings is acutely responsive to E2.

### 3.6.2 Sex differences in the requirement for intracellular calcium sources

Kinases important in LTP initiation are generally activated by calcium, often through NMDAR channels. As noted above, however, NMDARs were blocked in our experiments. When we tested the requirement for two alternative calcium sources, L-type calcium channels and release from internal stores, we found that either of 551 these is sufficient to support E2-induced potentiation in males whereas in females both are required. This difference could reflect parallel signaling in males such that one calcium source can compensate for the other and/or it could arise from differences in regulation of calcium sources. For example, considering the suggestion above that basal cAMP levels and PKA activity might be higher in males, it is possible that males' basal state also includes PKA-dependent phosphorylation of the inositol triphosphate receptor to increase calcium levels (Wagner et al., 2008). In this way, sex differences in the dependence of potentiation on PKA and calcium release from stores may be mechanistically related.

Our results also indicated a role for L-type calcium channels in E2-induced potentiation. Although L-type channels are high-voltage activated and might not be expected to contribute to calcium influx at the relatively negative holding potential used for our experiments (-70 mV), others have shown that L-type channels are active at membrane potentials at or near rest, particularly in adult animals and at elevated

temperatures as in our experiments (Magee et al., 1996; Radzicki et al., 2013). There is literature on acute E2 modulation of L-type channels, focused on activation of kinases involved in neuroprotection, including Src and Erk/MAPK (Wu et al., 2005; Zhao and Brinton, 2007; Vega-Vela et al., 2017). Our observation that L-type calcium channels and calcium release from internal stores are both required for E2-induced potentiation in females suggests that synaptic potentiation in females requires at least two distinct intracellular cascades. The nature of this distinction is unknown, however. It could be that different essential kinases are activated by distinct calcium sources in females, or the distinction could reflect spatial or temporal separation of essential signals. Future studies, for example with calcium imaging, may help to resolve these questions.

### 3.6.3 Implications of latent sex differences in intracellular signaling

Studying acute E2-induced synaptic plasticity is valuable as a model for understanding how estrogens synthesized in the brain, including the hippocampus (Hojo et al., 2004; Tabatadze et al., 2014; Sato and Woolley, 2016), act locally to modulate neural circuits and behavior. For example, recent studies indicate that brain-derived estrogens promote hippocampus-dependent memory, both in female mice (Tuscher et al., 2016) and postmenopausal women (Bayer et al., 2015). Despite evidence that neurosteroid estrogen levels are comparable between the sexes (Sato and Woolley, 2016) or higher in males (Hojo et al., 2004), few studies compare actions of estrogens in males and females. The focus on females in neurosteroid estrogen studies stands in contrast to the over-representation of males in basic neuroscience (Beery and Zucker,

2011), including in studies of LTP. Our findings that males and females differ in the involvement of a well- studied kinase like PKA and intracellular calcium signaling suggest that sex differences in mechanisms related to synaptic plasticity may be more widespread than currently appreciated. Furthermore, the mechanistic differences we observed are latent sex differences in that the degree of potentiation was the same between males and females. Thus, similar outcomes in each sex cannot be presumed to indicate the same underlying mechanisms. This is relevant particularly in the context of therapeutic development because manipulating a specific molecular pathway may have distinct outcomes in each sex, even when males and females do not appear to differ.

# Chapter 4: Sex differences in AMPAR modulation that underlie 17β-estradiol-induced potentiation in the hippocampus

## 4.1 Abstract

17β-estradiol (E2) is locally synthesized in the hippocampus and can acutely potentiate excitatory synaptic transmission in the hippocampus. We previously found that E2-induced synaptic potentiation occurs similarly in both sexes via largely independent pre or postsynaptic mechanisms. Investigating molecular mechanisms that underlie E2-potentiation revealed sex differences in the requirements of estrogen receptors, PKA and different calcium sources. In this study we focused on the postsynaptic components of E2-potentiation and investigated whether downstream functions of the distinct molecular signaling remain different in males versus females or converge to a similar mechanism. Here using electrical stimulation and two-photon uncaging experiments in hippocampal slices from adult gonadectomized females and males, we found a sex difference in the postsynaptic mechanisms. While calcium permeable AMPARs (cpAMPARs) were required for stabilization of E2-induced synaptic potentiation in females, these only contribute to stabilization in males. Non-stationary fluctuation analysis in these two-photon evoked currents showed that while in females E2-potentiation largely occurs via change in conductance, in males E2-potentiation can occur either via a change in number or conductance. This study shows that distinct mechanisms underlie the postsynaptic component of potentiation, most likely due to distinct functions of upstream kinases and calcium sources that get activated. Finding a

sex difference in AMPAR modulation adds to the growing evidence of sex difference in the mechanisms of synaptic plasticity in the hippocampus.

## 4.2 Introduction

The role of 17β-Estradiol (E2) as a neuromodulator of hippocampal physiology has recently been recognized. Our lab and others have shown using both *in vitro* and *in vivo* techniques that in addition to E2 synthesis in the gonads, E2 can also be locally and rapidly synthesized within different brain regions including the hippocampus (Hojo et al., 2004; Sato and Woolley, 2016). Moreover, in contrast to the peripheral E2 levels that are different in adult females and males, the local synthesis of E2 appears to be similar between the sexes (Sato and Woolley, 2016). It is now well established that E2 can acutely modulate both excitatory and inhibitory synaptic transmission in the brain regions like hippocampus and cortex (Teyler et al., 1980; Wong and Moss, 1992; Gu and Moss, 1996; Kramar et al., 2009; Huang and Woolley, 2012; Kumar et al., 2015). Our lab has found that E2 acutely potentiates excitatory synaptic transmission in the hippocampus of both females (Smejkalova and Woolley, 2010) and males (Chapter 2). Moreover, this increase in excitatory synaptic strength is synapse specific and has largely independent pre or postsynaptic components of potentiation (Chapter 2).

Investigating the molecular mechanisms underlying E2-induced excitatory synaptic potentiation revealed latent sex differences in some signaling components. Previous experiments from our lab found that although the overall magnitude of E2-potentiation is similar between the sexes, the requirements of different estrogen receptors (Chapter 2),

PKA and calcium sources (Chapter 3) in E2-induced synaptic potentiation differ between the sexes. All sex differences were observed in the initiation of E2-potentiation. The mechanisms to maintain the potentiation appear to be similar between sexes. We found that CaMKII is required for the maintenance but not for initiation of E2-induced potentiation in both sexes (Chapter 3).

It is intriguing how different signaling cascades in males and females can lead to a similar E2-induced potentiation and it is still unknown if and where these signaling cascades converge to result in the same potentiation in both sexes. In this study, we address this question by focusing on the mechanisms that could underlie the postsynaptic component of E2-potentiation in both females and males. Based on previous LTP experiments, a postsynaptic increase in glutamate sensitivity could occur due to an increase in the number of AMPAR receptors (Andrasfalvy and Magee, 2004) and/or due to an increase in AMPAR conductance (Benke et al., 1998). Moreover, studies show that multiple mechanisms could underlie changes in conductance. 1) CaMKII can increase AMPAR conductance by phosphorylating AMPARs subunits at Ser 841 (Derkach et al., 1999). 2) Calcium permeable AMPARs (cpAMPARs) can replace the calcium impermeable AMPARs (Plant et al., 2006) at the synapse and as cpAMPARs inherently have a higher conductance (Swanson et al., 1997), could increase the overall synaptic conductance (Benke and Traynelis, 2019). A recent LTP study showed that cpAMPARs are required for a PKA-sensitive LTP (Park et al., 2016). To understand the postsynaptic component of E2-potentiation we investigated the role of cpAMPARs in both sexes. Additionally, we performed glutamate uncaging

experiments to isolate the postsynaptic component and performed non-stationary noise analysis on two-photon evoked EPSCs (2pEPSCs) to estimate changes in conductance and/or number in both sexes.

In these experiments we found that calcium influx via cpAMPARs induces signaling that is required for stabilization of E2-induced potentiation in females but not in males. Moreover, this was reflected in the non-stationary fluctuation analysis where we found that in females E2-induced potentiation of 2pEPSCs mainly occurs due to an increase in conductance, while in males 2pEPSC potentiation could occur via either increase in number or conductance. These results demonstrate that there are distinct postsynaptic mechanisms in females and males that underlie surprisingly similar E2-induced potentiation in both sexes.

## 4.3 Materials and Methods

### 4.3.1 *Animals*

Young adult female and male Sprague Dawley rats (50–70 days of age, Envigo) were prepared as discussed in Chapter 3. Briefly, all rats were gonadectomized 3-8 days before being used for experiments using aseptic surgical procedures. All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Northwestern University Animal Care and Use Committee.

4.3.2 *Preparation of hippocampal slices*

Rats were deeply anesthetized with sodium pentobarbital (100-125 mg/kg, i.p.; Virbac) and transcardially perfused with oxygenated (95% $O_2$/5% $CO_2$) ice-cold sucrose-containing artificial cerebrospinal fluid (s-aCSF) containing (in mM): 75 NaCl, 25 $NaHCO_3$, 15 dextrose, 75 sucrose, 1.25 $NaH_2PO_4$, 2 KCl, 2.4 Na pyruvate, 1.3 L-ascorbic acid, 0.5 $CaCl_2$, 3 $MgCl_2$; 305-310 mOsm/L, pH 7.4. The brain was quickly removed and 300 µm transverse slices through the dorsal hippocampus were cut into a bath of ice-cold s-aCSF using a vibrating tissue slicer (VT1200S, Leica). The slices were incubated at 33$^o$ C in oxygenated regular aCSF containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 1.25 $NaH_2PO_4$, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$; 305-310 mOsm/L, pH 7.4 for 30 min, then allowed to recover at room temperature for 1-6 hrs until recording.

4.3.3 *Electrophysiological recording*

Acute hippocampal slices were prepared and whole cell recordings from CA1 pyramidal neurons were obtained as described in Chapter 3. In 2-photon experiments, the bath recirculated in a small volume (~ 8 ml) of aCSF and also contained 2 mM MNI-glutamate. Somatic whole-cell voltage-clamp recordings ($V_{hold}$ = -70 mV) were obtained from CA1 pyramidal cells using patch electrodes (3–6 MΩ) filled with K-gluconate internal solution. In electrical stimulation experiments, a glass bipolar stimulating electrode (10-50 µm tip diameter) filled with regular aCSF was placed in the stratum radiatum 200-250 µm from the recorded cell in whole-cell recordings. All electrical stimulation E2 experiments were done in the presence of the $GABA_A$ and NMDA

receptor blockers, SR-95531 (2 μM) and DL-APV (25 μM), respectively. Separate glutamate uncaging experiments were done in the presence of SR-95531 (2 μM). Data were acquired with a Multiclamp 700B amplifier and pClamp 10.5 software (Molecular Devices), filtered at 1-2 kHz, and digitized at 5 kHz or 20 kHz using a Digidata 1440A data acquisition system (Molecular Devices).

Two types of experiments with electrical stimulation were performed in this study. In the first experiment, to investigate whether synaptic activity is required for expression of E2-potentiation, electrical stimulation was stopped during E2 application and resumed after washing off the E2. In second experiment, NASPM, a drug that specifically blocks calcium permeable AMPARs, was applied in the E2-responsive cells, either immediately after E2-washout or 10-15 mins after E2-washout and E2-potentiation was stabilized.

### 4.3.4 *Two-photon evoked glutamate uncaging experiments*

Dendritic spines were visualized using a dual galvanometer-based 2p laser scanning system (Ultima, Prairie Technologies) using a 40× objective with 8-10× digital zoom. Two pulsed laser beams (Chameleon Ultra II, Coherent) were used: one split at 840 nm to image Alexa-594, and one at 720 nm to uncage glutamate. Laser beam intensity was controlled with electro-optical modulators (Conoptics, model 350–50) with an uncaging dwell time of 0.5 or 1.0 ms at the minimum power required to evoke a 2pEPSC (10–50 mW, as measured at the back aperture of the microscope). Uncaging was focused at the edge of targeted spines. Each spine received an uncaging pulse 1-3 times per minute with a 2 s interval between uncaging pulses at different spines. The laser power

of both lasers was measured and the uncaging laser was calibrated at 1x-12x digital zoom every week. For all our experiments, we used 2mM MNI concentration and uncaged at 2 or 4 ms laser pulse width at laser power 15-50 mW. These values were based on the previous work that characterized amounts of glutamate released by uncaging MNI glutamate at different concentrations (Matsuzaki et al., 2001). The peak glutamate concentration at the center of irradiation was found to be 0.6 mM, at a laser power of 7 mW, MNI- glutamate concentration (MNI) of 8 mM, the duration of irradiation (td) of 0.5 ms.

4.3.5 *Non-stationary fluctuation analysis on 2-photon evoked EPSCs*

To estimate the changes in the single channel properties that underlie E2-induced 2pEPSC potentiation, conventional and peak scaled non-stationary fluctuation analysis (NSFA and psNSFA respectively) were performed on the decay phase of 2pEPSCs using a published protocol (Hartveit and Veruki, 2007). Using a representative spine, different steps of the analysis are described in Figure 4.1. First, data were acquired at 50 kHz digitization frequency. Then the 2pEPSC waveforms were exported to Neuromatic IGOR based software. Here, for each spine, events in baseline or E2 condition were aligned and tested for time stability of peak amplitude and 10-90% rise time using a Spearman rank correlation test (Fig 4.1A, B). Then the waveforms were divided into pre- and E2- conditions and exported as a 2D matrix. NSFA and psNSFA was then performed in two ways. First we used Mini Analysis software (Synaptosoft Inc.) where the scripts to perform the analysis are prewritten. One disadvantage of

using this software is that we have less control over the time window of the 2pEPSC decay phase that we can choose to do the analysis. Therefore, we also built our custom MATLAB generated code to perform NSFA. The decay phase of the stable events (18-41 per condition) were divided into 100 amplitude bins, and within each bin the variance around the scaled average was computed (Fig 4.1C, D). psNSFA was performed in parallel with NSFA in which the events were scaled to peak amplitude (Fig 4.1E, F) as described and characterized previously (Traynelis et al., 1993). Although in psNSFA analysis the absolute values of conductance and number are lost, we can still reliable estimate the relative E2-induced changes in these single channel properties as compared to baseline. The NSFA analysis was performed in both softwares and the results were similar.

The single-channel current (i) and the number of channels (N) were calculated by fitting the theoretical relationship between the variance (σ2) or scaled variance and the current amplitude (I) after subtraction of the background variance ($\sigma_b2$), as follows:

$$\sigma^2(\mathrm{I}) = \mathrm{iI} - \frac{\mathrm{I}^2}{\mathrm{N}} + \sigma_{\mathrm{b}}^2 \qquad \textbf{(1)}$$

where (i) is the single-channel current and (N) total number of ion channels available for activation. The single-channel (unitary) chord conductance ($\gamma$) can then be calculated as:

$$\gamma = i/(V_m - E_{rev}) \qquad \textbf{(2)}$$

from the known membrane holding potential ($V_m$ = -70 mV) and assumed AMPAR reversal potential ($E_{rev}$= 0 mV).

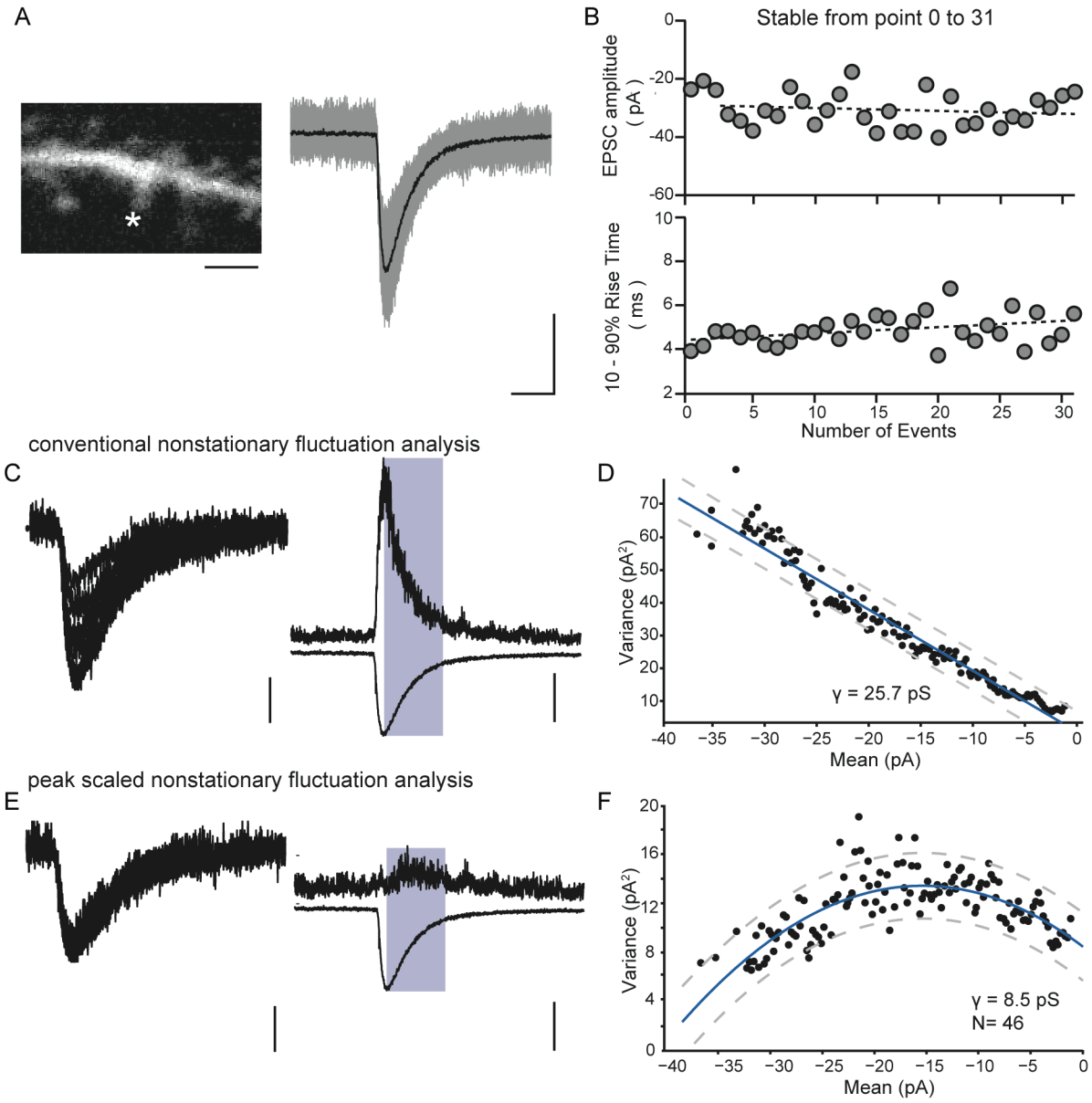



**Figure 4.1: Steps to perform non-stationary fluctuation analysis on 2pEPSCs.**

**A)** Representative dendritic shaft and the spine (*) on which the control uncaging experiment was performed along with representative traces. The 2pEPSCs were recorded once every minute. Scale 5 pA, 5 ms. **B)** Plots of individual 2pEPSC amplitude (top) and rise time (bottom). Spearman rank correlation test showed stable events from 0 to 31. **C)** Representative traces from the same spine shown in **A**, on which the

conventional non-stationary fluctuation analysis is performed. Mean and variance calculated for the 31 events using custom MATLAB code is shown on the right. The purple shaded region is the portion of decay phase used for NSFA. **D)** Mean amplitude (pA) vs variance ($pA^2$) plot of the binned points (100 bins) from the shaded region of the decay phase in **C**. Eq (1) was used to fit the data. As the fitting is linear, with the help of eq (2), only conductance(γ) was estimated to be 25.7pS. This value is closer to the absolute AMPAR conductance at the synapses **E)** Same traces as **D** but peak scaled to the mean amplitude, and peak scaled non-stationary fluctuation analysis was performed on these traces. Mean and variance calculated of the 31 peak scaled events using custom MATLAB code is shown on the right. As all the events were scaled, the variance at the peak was minimum. **F)** Mean amplitude (pA) vs variance ($pA^2$) plot of the binned points (100 bins) from the shaded region of the decay phase in **F**. Eq (1) was used to fit the data. As the fitting is more parabolic, with the help of eq (2), the conductance (γ) was estimated to be 8.5 pS and receptor number (N) was estimated to be 46. As the variance is artificially constrained to be minimum at the peak, these values are not the absolute AMPAR conductance and AMPAR number values. Scale for the raw traces in **E**, **G** is 5pA. Scale for mean and variance plot is 10 pA and $10pA^2$.

4.3.6 *Statistics and Experimental design*

*Electrical stimulation EPSC experiments and two-photon glutamate uncaging experiments*

5 min window in pre, early E2 and late E2 were chosen for within cell or spine unpaired ttest. Statistical significance of this test showed whether a cell or spine is E2-responsive. Unpaired ttest was also performed between sexes or across experiments to test the difference in the magnitude of potentiation. Fisher's exact test was used to identify any difference in the frequency of E2-responsive cells or spines between sexes and across experiments. In NASPM experiments, to test the sensitivity of NASPM, one-way ANOVA and multiple comparison's analysis was performed within an E2-responsive cell. $p<0.05$ was considered significant.

*Non-stationary fluctuation analysis*

The variance was fitted with the scaled average using equation (2) in the curve fitting tool in MALTAB. The reliability of the single channel current and numbers were determined using Goodness of fit $R^2$ values and root mean square error (RMSE) values. Th $R^2$ values of the NSFA fit of all spines are reported in Fig 4.2A, B. In E2-responsive spines, to determine whether there are changes in the conductance, based on the distribution, E2 conductance values which were >20% change over baseline values were chosen to be statistically significant (Fig 4.2C, D). The changes in the rise time and decay time in the E2-responsive spines were statistically measured within spines using unpaired ttest. Pearson correlation analysis was performed between changes in decay constant tau and AMPAR number change (both male and female data were

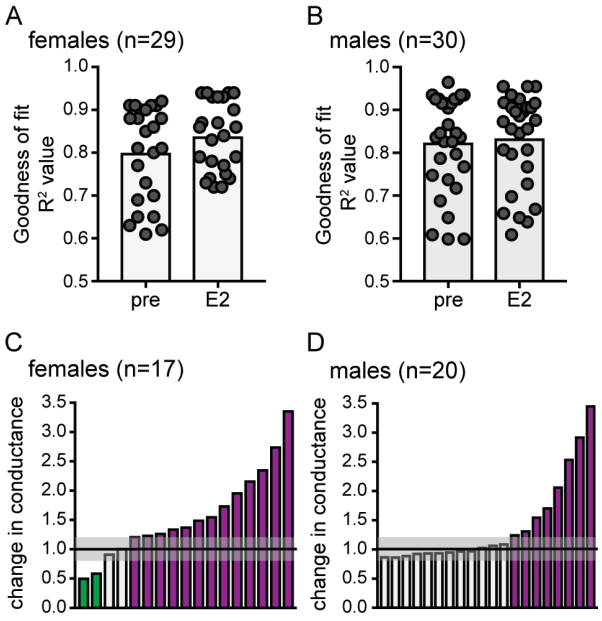



**Figure 4.2: Parameters to test significant increase in conductance.**

**A)** $R^2$ values in females that reflect the goodness of fit obtained while fitting the mean-variance data points of the decay phase of pre or E2 2pEPSCs with eq 1). $R^2$ values of both responsive and non-responsive spines are combined in the plot. An ideal fit would give $R^2$ value equal to 1 (Also in **B**). Female values ranged from 0.61- 0.95 and were similar in pre and E2 mean-variance plots. **B)** $R^2$ values in males that reflect the goodness of fit. **C)** Normalized changed in conductance in 17 female E2-responsive spines. Grey shaded region shows 20% E2-induced change in conductance as compared to pre E2 condition. With this cutoff, 2 spines showed a decrease in conductance (green), 2 spines showed no change in conductance (white) and 13 spines showed an increase in conductance (purple). **D)** Normalized changed in conductance in 20 male E2-responsive spines. Same as **C**, grey shaded region shows 20% E2-induced

change in conductance as compared to pre E2 condition. With this cutoff, 12 spines showed no change in conductance (white) and 8 spines showed an increase in conductance (purple).

pooled together). Separate Pearson's' correlation analyses was performed between magnitude of potentiation and either conductance or number in Graphpad PRISM software. A p value <0.05 was considered significant.

## 4.4 Results

### 4.4.1 Synaptic activity is required for E2-induced synaptic potentiation in females but not in males

We have previously performed electrical stimulation (Smejkalova and Woolley, 2010) and miniature EPSC experiments (Chapter 2) to show that E2 potentiates excitatory synaptic transmission via largely independent pre or post synaptic mechanisms in both sexes. While in electrical stimulation experiments, E2 potentiated EPSC amplitude in 60% of the CA1 cells, in miniature experiments E2 potentiated mEPSC amplitude and/or frequency in ~45% of the recordings. LTP studies suggest that synaptic activity is required for the activation of some molecular signaling pathways. For example, it has been noted that calcium permeable AMPARs require synaptic activity to initiate downstream signaling that is required for the expression of LTP (Plant et al., 2006). Thus, to investigate whether synaptic activation is required for E2-induced potentiation, we performed electrical stimulation EPSC experiments in gonadectomized females and males. In these experiments, after recording baseline EPSCs for 10-15 mins, E2 was applied for 10 mins. Stimulation was stopped during E2 application and was resumed 5 mins after E2 washout and continued till the end of the recording. Identical experiments were done in females and males.

In females, E2 failed to potentiate EPSCs when synapses were not stimulated during E2 application. We saw no change in the EPSC amplitude in any of the cells immediately after stimulation was resumed (-2 ± 5% above baseline) (Fig 4.3A, B). However, in 7 out of 13 cells, 15 - 20 mins of stimulation following E2 washout resulted in an increase in the EPSCs by 78 ± 7% above baseline (Fig. 4.3A, B). The other 6 cells had no change in the EPSC amplitude throughout the recording (Fig. 4.3C). This E2-induced EPSC potentiation was similar to control E2 experiments in females as described previously (Chapter 3, 83±16% above baseline in 9 of 16 cells). These experiments suggest that synaptic activation is required for E2-induced synaptic potentiation in females. In contrast in males, even with no synaptic stimulation, E2-potentiated EPSCs. In 7 out 15 cells, potentiated EPSCs were observed immediately after stimulation was resumed (by 90 ±15% above baseline, Fig. 4.3D, E). In a responsive cell the increase in EPSC amplitude was similar and sustained till the end of the recording (85 ± 11%, Fig. 4.3E, F). The magnitude and frequency of responsiveness of E2-induced potentiation in males is similar to the control E2 experiments in males as described previously (Chapter 3, 89±16% above baseline in 11 of 18 cells). Similar to females, in males, the non-responsive cells had no change in the EPSC amplitude throughout the recording (Fig 4.3F). These experiments show that synaptic activation is not required for an E2-induced synaptic potentiation in males. Together, these results

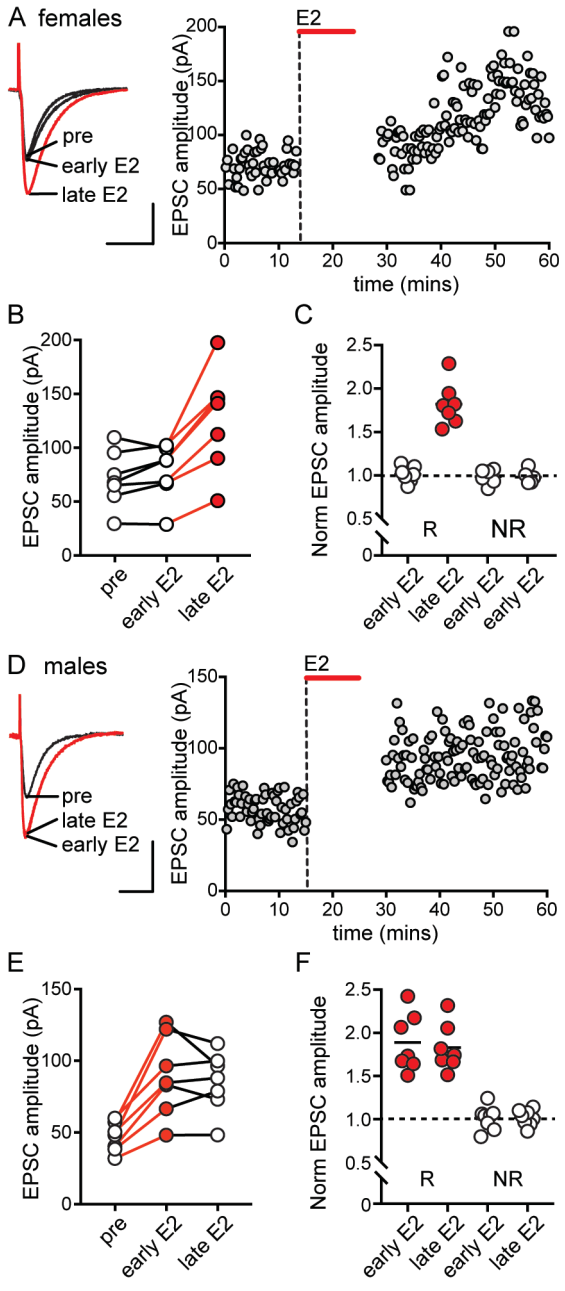



**Figure 4.3: Synaptic activity is required for E2-induced EPSC potentiation in females but not in males.**

**A)** Raw traces and EPSC time course of a representative E2-responsive cell in females, where 10 mins of stimulation was required to observe an increase in EPSC amplitude

after E2-application in an E2-responsive cell. Early E2 refers to the first 10 min window when stimulation was resumed after E2-washout. Late E2 refers to the last 10 min window (15-20 mins after the stimulation was resumed) of the recording **B)** Group EPSC amplitude data in females where E2-induced EPSC amplitude was increased only in the late E2 phase of the recording. Significant EPSC increase as compared to baseline is represented with red circles. **C)** Responsive and non-responsive E2-cells in females, where change in EPSCs of early and late phase after E2-washout is normalized to baseline. Red circles represent significant change in EPSC amplitude. In female responsive cells, EPSC amplitude increase only in the late E2. **D)** Raw traces and EPSC time course of a representative E2-responsive cell in males, where potentiated EPSCs were observed immediately after stimulation was resumed following E2 washout. **E)** Group EPSC amplitude data in males where E2-induced EPSC amplitude was increased immediately after stimulation was resumed. Significant EPSC increase as compared to baseline is represented with red circles. **F)** Responsive and non-responsive E2-cells in males, where change in EPSCs of early and late phase after E2-washout is normalized to baseline. Red circles represent significant change in EPSC amplitude. In male responsive cells, EPSC amplitude increase in early phase and remained potentiated in the late phase. Scale bar 50 pA, 25 ms.

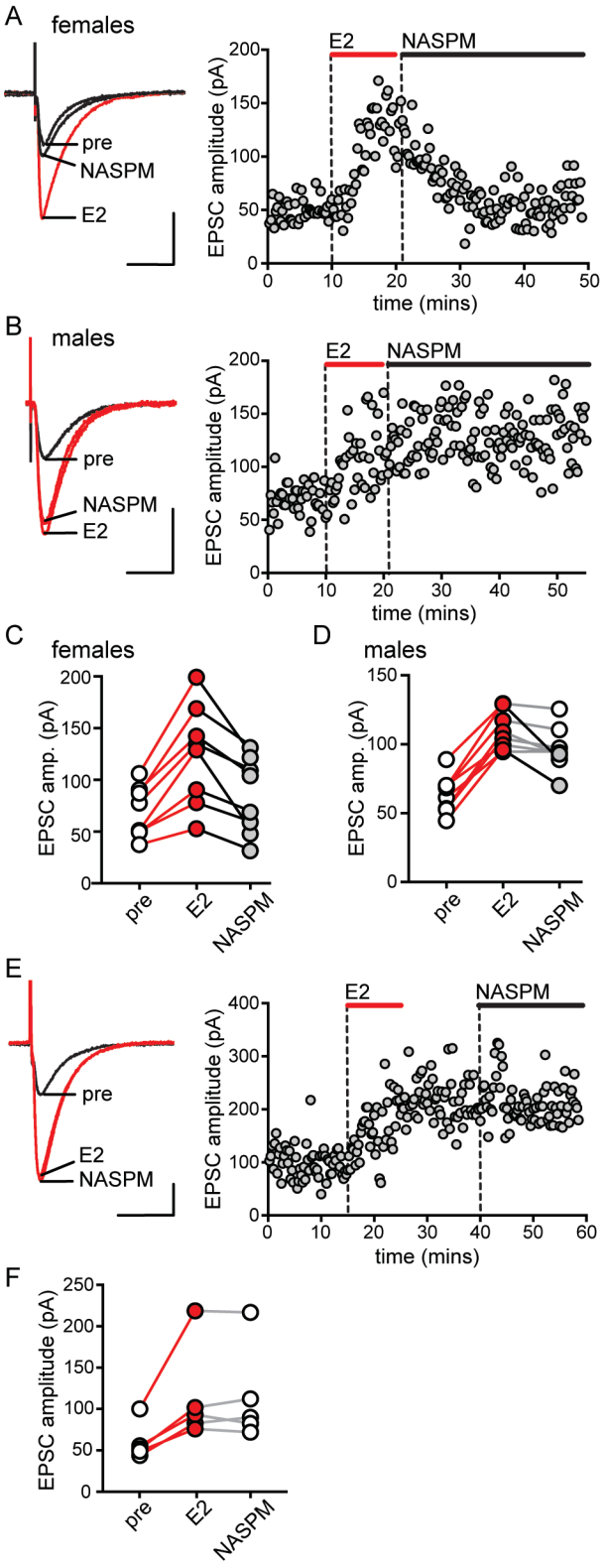
show that whether synaptic activity is required during E2 application differs between males and females.

### 4.4.2 Sex difference in the requirement of calcium permeable AMPARs in the expression of E2-induced EPSC potentiation

It has been previously shown that calcium permeable AMPARs (cpAMPARs) are transiently incorporated and require synaptic activation to activate downstream signaling (Plant et al., 2006). Moreover, the requirement of cpAMPARs for stabilization of LTP has also been shown (Plant et al., 2006). We wondered whether differential requirement of synaptic activity could be due to incorporation of calcium permeable AMPARs. Therefore, next we directly investigated the requirement of cpAMPARs in E2-induced synaptic potentiation using NASPM (40μM), a drug that specifically blocks cpAMPARs. In these experiments, after recording baseline EPSCs for 10-15 mins, E2 was applied to determine the responsiveness of the cell. In E2-responsive cells, in separate experiments, NASPM was added either 1) during stabilization of E2-potentiation (after 10 min E2-application) or 2) after stabilization of potentiated EPSCs (10-15 mins after E2-application). The same protocols were used in both females and males.

In females, NASPM applied immediately after E2 application reversed the E2-induced EPSC potentiation (Fig 4.4A), while in males NASPM did not reverse the potentiated EPSCs (Fig 4.4B). In all 8 E2-responsive cells in females, NASPM reduced the potentiated EPSC amplitude within 15-20 mins, from 77.8 $\pm$ 13% to

21.1 ± 8% above baseline (Fig 4.4C). Within cell ANOVA analysis revealed that in 4 out of 8 E2- responsive cells, NASPM completed reversed the potentiation to baseline whereas in the other 4, NASPM partially reversed the potentiation and the EPSCs in NASPM were still significantly higher than baseline. In males, there was low sensitivity to NASPM after E2 application and NASPM only changed the potentiated EPSC amplitude from 77.6 ± 10% to 56 ± 9% above baseline (Fig 4.4D). Within cell ANOVA demonstrated that in 6 out of 8 E2-responsive cells, NASPM had no significant effect on the potentiated EPSCs (ANOVA, $p>0.08$). In the other 2 E2-responsive cells in males, NASPM partially reversed the EPSC amplitude within 10-15 mins (ANOVA, $p<0.05$). In both females (n=3) and males (n=2), NASPM applied 10-15 mins after E2 application had no effect on the potentiated EPSCs and the EPSC amplitude stayed potentiated (82 ± 6% to 86 ± 6.4% above baseline, Fig 4.4E, F). Overall, these experiments show that cpAMPAR are required for stabilization of E2-potentiation in females. In males, cpAMPAR may contribute to E2-potentiation, but in most cells are not required. These results also corroborate with our previous finding and suggest that synaptic activation is required to activate cpAMPAR mediated signaling to express/stabilize E2-potentiation in females.


A
females
pre
NASPM
E2
E2
NASPM
EPSC amplitude (pA)
time (mins)
B
males
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EPSC amplitude (pA)
time (mins)
C
females
EPSC amp. (pA)
pre
E2
NASPM
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E
pre
E2
NASPM
E2
NASPM
EPSC amplitude (pA)
time (mins)
F
EPSC amplitude (pA)
pre
E2
NASPM

**Figure 4.4: Calcium permeable AMPARs are required for stabilization of E2-induced EPSC potentiation in females but not in males.**

**A)** Raw traces and EPSC time course of a representative cell in females, where NASPM (40μM) application reversed the E2-induced potentiated EPSC amplitude back to baseline EPSC amplitude. **B)** Raw traces and the EPSC time course of a representative cell in males, where NASPM (40μM) application failed to change the E2-induced potentiated EPSC amplitude. **C)** Group EPSC amplitude data of all E2-responsive spines in females, E2 condition shown in red and NASPM condition shown in grey. **D)** Group EPSC amplitude data of all E2-responsive spines in males, E2 condition shown in red and NASPM condition shown in white in 6 out 8 cells, and shown in grey in the other two. **E)** Raw traces and EPSC time course of a representative cell in females, where NASPM (40μM) application 15 min after E2 washout, , had no effect on the E2-induced potentiated EPSC amplitude. **F)** Summary of all NASPM experiments in males (n=2) and females (n=3) where NASPM application 15 mins after E2 washout had no effect on potentiated EPSCs Scale bar: 50 pA, 25 ms.

### 4.4.3 E2 increases two-photon evoked currents in a synapse specific manner in both sexes

A previous single channel study of different AMPARs has shown that cpAMPARs have inherently higher conductance than other AMPARs (Swanson et al., 1997). If cpAMPARs are incorporated at the synapse following E2-application, then they could also result in an overall increase in conductance at the synapse. To address this possibility, we focused on measuring the changes of single synapse currents before, during and after E2 application. To study the postsynaptic mechanisms underlying E2-potentiation, we performed glutamate uncaging experiments in acute hippocampal slices from adult gonadectomized males and females. To validate the uncaging experimental design, three different types of control experiments were performed in both males and females before doing the experiments with E2. The first control experiment tested whether two-photon evoked EPSCs (2pEPSCs) can be reliably recorded from dendritic spines for a period of 30-40 mins (Fig 4.5A). In these experiments, two aCSF containing solution lines were installed. For every recording, solution line 1 was switched to line 2 after 10-15 mins for 10 mins and then switched back to line 1. This mimicked switching solution during actual E2 experiments. Also, these control experiments were performed at room temp (19$^{o}$ C) in males and females. Additional control experiments in females were also performed at elevated temp (~33$^{o}$ C).

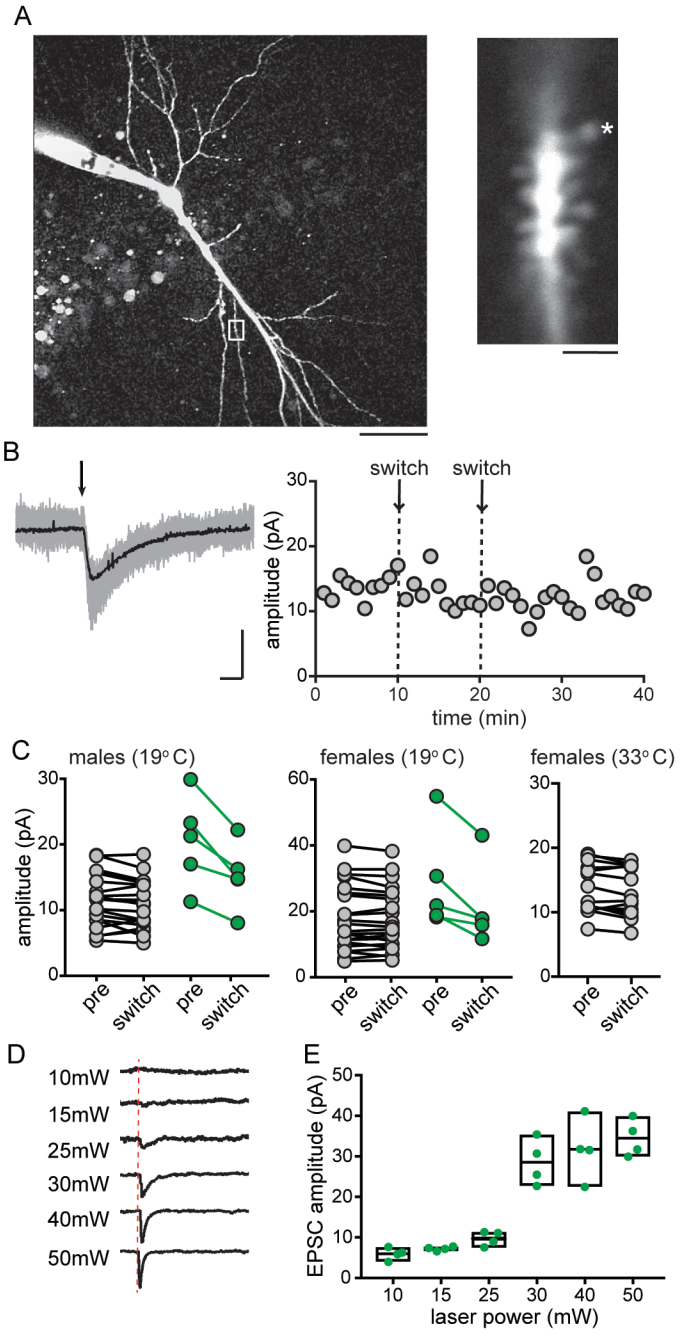


**Figure 4.5: Control two-photon glutamate uncaging experiments.**

A) Representative cell filled with Alexa 594 dye, magnified (9.52x) dendritic shaft from that cell. **B)** Raw traces (mean is shown in black) from one of the spines evoked by uncaging glutamate at 15 mW, 4 ms pulse width (arrow indicates uncaging pulse) and

time course of the representative spine, where glutamate uncaging was performed stably for 40 mins and switching aCSF line did not change 2pEPSC amplitude. **C)** Group EPSC amplitude data showing all control experiments done in males at 19 $^{o}$C (n=25; left), females at 19 $^{o}$C (n=27; middle) and females at 33 $^{o}$C (n=27; right). Spines shown in grey showed no change in the EPSC amplitude following switch. Spines shown in green showed a decrease in EPSC amplitude following switch. **D)** Representative traces from a dendritic spine with increasing laser power. **E)** 4 spines where increasing laser power showed a big jump from 25 to 30 mW laser power and 30 mW was used for the experiments. Scale bar for CA1 image is 50 μm, dendrite is 5 μm, raw traces 10 pA, 5 ms.

In both sexes, the majority of the spines showed no changes in the 2pEPSC amplitude throughout the recording (Fig 4.5B). In males, out of 25 spines recorded at room temperature from 7 cells, 20 showed no change in the 2pEPSC amplitude. 5 spines showed a reduction in a 2pEPSC amplitude after switching the aCSF solution (Fig 4.5C, left). Similarly, in females, out of 27 spines recorded at room temp. from 4 cells, 22 showed no change in the 2pEPSC amplitude. Similar to males, 5 spines showed a reduction in the 2pEPSC amplitude (Fig 4.5C, middle). We also performed similar experiments at elevated temperatures in females ($33^0$C) and found that none of the 14 spines recorded from 4 cells showed any change in EPSC amplitude (Fig 4.5C, right). As at 33 $^o$C temperature we did not find any reduction in EPSC amplitude, to maintain the consistency for our E2 experiments, we performed uncaging experiments at elevated temperature (33 $^o$C) in both males and females.

Second control experiments were performed to characterize the optimal laser power and uncaging focal points. Control laser power measurements were taken during the initial E2 experiments. To test the optimal uncaging laser power, the spine was given uncaging pulses of increasing laser power ranging from 10-50 mW (Fig 4.5D). The minimum laser power was chosen such that, all spines on a dendritic shaft show 30-50% of the maximal response (Fig 4.5E). The laser power used to uncage on dendritic spines were dependent on the depth of the cell in the hippocampal slice.

We next performed 2pEPSC experiments with E2, where we recorded 2pEPSCs from 3-5 spines per cell and found that, similar to our previous finding (Oberlander and Woolley, 2016), E2 potentiated 2pEPSC amplitude in a subset of spines in both females

and males. Within spine t-test showed that E2 increased 2pEPSC amplitude in 37 out of 78 spines from 18 cells in females by 83 ± 11% above baseline (Fig 4.6 A,B,C). The effect was similar in males where E2 increased 2pEPSC amplitude in 33 out of 54 spines from 12 cells by 91± 8% above baseline in males (Fig 4.6D). The magnitude of potentiation (unpaired t-test, $p=0.09$) and the frequency of responsiveness (Fisher's exact test, $p>0.5$) was not statistically different between sexes.

### 4.4.4 Sex difference in the single channel AMPAR properties that underlie E2-induced synaptic potentiation

Next, we investigated whether the E2-induced increase in 2pEPSC amplitude is due to an increase in synaptic AMPAR number or an increase in conductance of synaptic AMPARs. Single channel properties of AMPARs in slices have been previously estimated using nonstationary fluctuation analysis (NSFA) on 2pEPSCs (Matsuzaki et al., 2001; Momiyama et al., 2003). To estimate E2-induced changes in the number or conductance of AMPARs, we performed conventional NSFA on a subset of 2pEPSCs recordings. The recordings were selected such that there were at least 18 events in each condition (pre and E2). 29 spines from 9 cells met the criterion for analysis in females out of which 17 were responsive to E2 (overall 72 ± 7 % increase over baseline, Fig 4.6E). Similarly, 30 spines from 8 cells met the criterion for analysis in

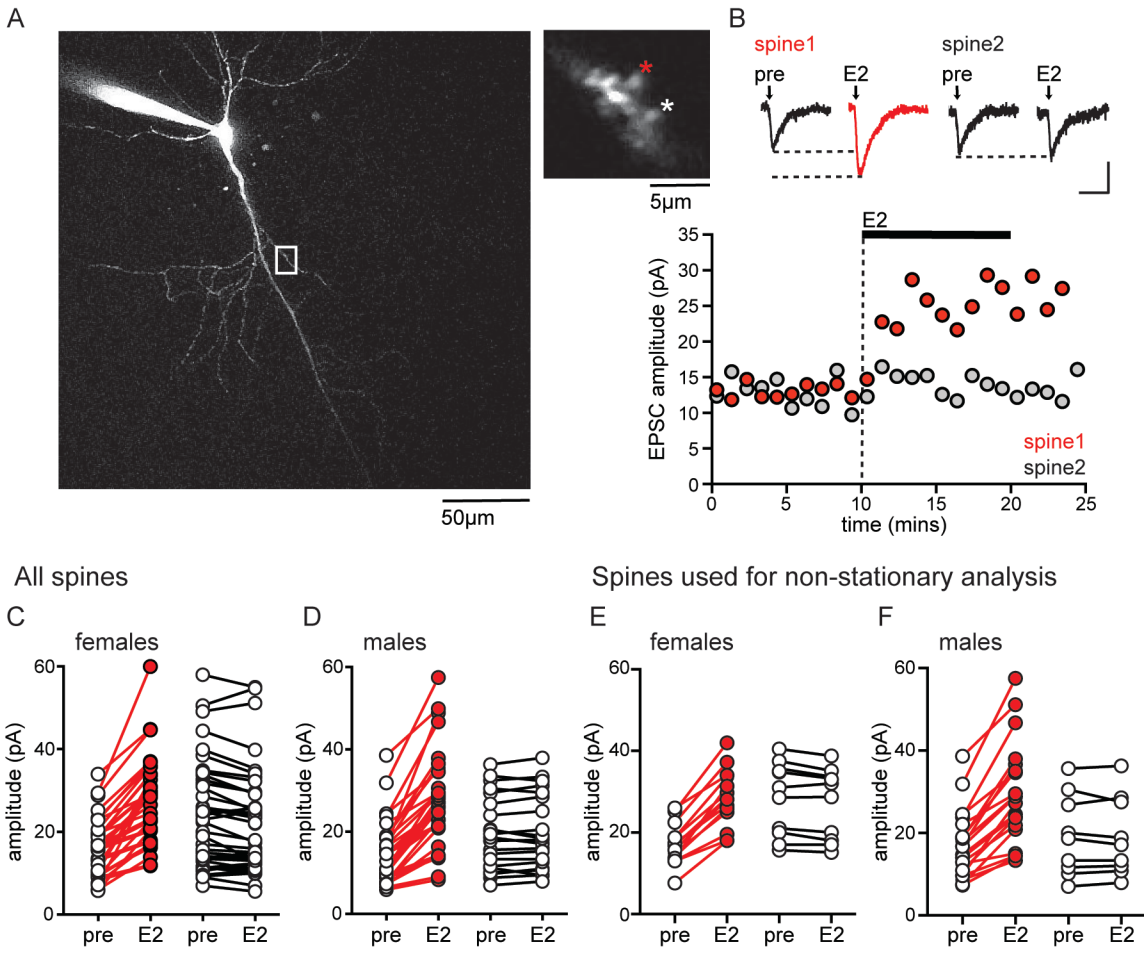


**Figure 4.6: E2-potentiates two-photon evoked EPSCs at individual spines similarly in both sexes.**

**A**) CA1 pyramidal cell filled with Alexa 594 dye during recording and white box showing the dendrite targeted for 2p-glutamate uncaging (left). Higher-magnification view of the dendrite (right) showing two spines that were targeted for uncaging (*). **B**) 2pEPSC raw traces during uncaging at the spines shown in **A** (top, arrow indicates uncaging pulse). Spine 1 showed an increase in 2pEPSC amplitude and is shown with red asterisk in **A** whereas spine 2 on the same dendrite showed no change in 2pEPSC amplitude and is shown white Asterix in **A**. Time course of 2pEPSC amplitude of spine 1 and spine 2

(bottom). Spine 1 showed a significant increase in 2pEPSC amplitude following treatment with E2, while spine 2 showed no E2-induced changes in 2pEPSC amplitude. In this recording, 3 uncaging pulses were given per minute and each dot represent the average of 3 2pEPSC amplitude. **C**) Group EPSC amplitude data of all 2pEPSC experiments performed in females. Red circles represent the spines in which E2 significantly increased the 2pEPSC amplitude whereas grey circles are the spines where E2 did not change the 2pEPSC amplitude (Also in **D**, **E**, **F**). **D**) Group EPSC amplitude data of all 2pEPSC experiments performed in males. **E**) Group EPSC amplitude data of the spines chosen from **C** to perform the non-stationary fluctuation analysis in females. **F**) Group EPSC amplitude data of the spines chosen from **D** to perform the non-stationary fluctuation analysis in males. Scale bar of the 2pEPSCs, 10pA, 10 ms.

males out of which 20 were responsive to E2 (overall 84±11 % increase over baseline, Fig 4.6F).

Due to low open probability of AMPARs, most of the data fit the initial linear part of the parabolic fitting of the NSFA equation, and thus only conductance can be estimated reliably using conventional NSFA. In females, we found that an increase in conductance largely accounts for the E2-induced 2pEPSC potentiation. In females, E2 increased conductance in 13 out of 17 responsive spines by 100 ± 24% above baseline (range pre: 7 - 28.5 pS; range E2: 13 - 44 pS), while 2 spines showed no change in conductance (Fig 4.7 A, C). In 2 responsive spines, we also found a decrease in the conductance (-51% above baseline, Fig 4.7C). In males, we found that E2-increased in conductance in 8 out of 20 responsive spines conductance by 111 ± 28% as compared to baseline (range pre: 10 - 16 pS; range E2: 13.5 - 46 pS, Fig 4.7B, D), while in the other 12 responsive spines, there was no E2-induced change in conductance (Fig 4.7D). This shows that in both females and males the increase in conductance partly explains the E2-induced 2pEPSC increase. E2-responsive spines that do not show any change in conductance, could get potentiated due to changes in other single channel parameters such as, an increase in the overall AMPAR number at the postsynaptic density.

To estimate other single channel parameters that could account for potentiation in the E2-responsive spines that don’t show changes in conductance, we next performed peak scaled NSFA (psNSFA, Traynelis et al., 1993). In psNSFA, variance at peak amplitude of 2pEPSCs is constrained to be minimum, which compensates for the low

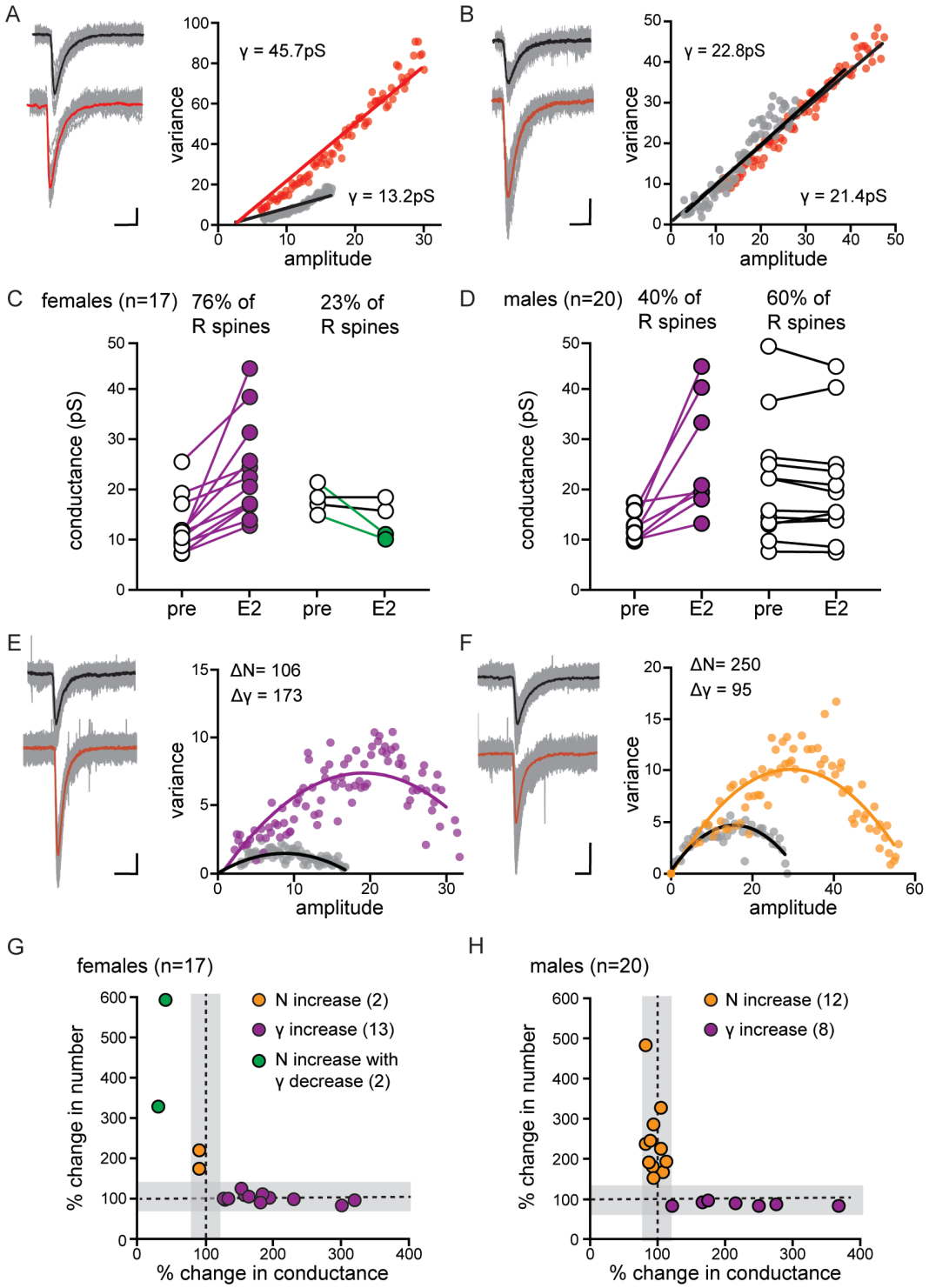
A
γ = 45.7pS
γ = 13.2pS
variance
amplitude
B
γ = 22.8pS
γ = 21.4pS
variance
amplitude
C
females (n=17)
76% of R spines
23% of R spines
conductance (pS)
pre
E2
pre
E2
D
males (n=20)
40% of R spines
60% of R spines
conductance (pS)
pre
E2
pre
E2
E
ΔN= 106
Δγ = 173
variance
amplitude
F
ΔN= 250
Δγ = 95
variance
amplitude
G
females (n=17)
N increase (2)
γ increase (13)
N increase with γ decrease (2)
% change in number
% change in conductance
H
males (n=20)
N increase (12)
γ increase (8)
% change in number
% change in conductance

**Figure 4.7: Sex difference in AMPAR properties that underlie E2-induced 2pEPSC potentiation.**

**A)** Raw traces of baseline (mean is shown in black) and E2 (mean is shown in red) and a representative mean-variance plot of the decay phase of 2pEPSCs showing an increase in conductance in E2 spines (23 events, 45.7 pS) as compared to pre (18 events, 13.2 pS). **B)** Raw traces of baseline (mean is shown in black) and E2 (mean is shown in red) and a representative mean-variance plot of the decay phase of 2pEPSCs showing no change in conductance in E2 spines (28 events, 22.8 pS) as compared to pre (21 events, 21.4 pS). **C)** Group conductance measurements obtained by performing conventional non-stationary fluctuation analysis in females. Purple circles (n=13) represent spines that show >20% increase in conductance as compared to baseline, white circles (n=2) represent spines that show <20 % change in conductance and green circles (n=2) represent spines that show >20% reduction in conductance. **D)** Group conductance measurements obtained by performing conventional non-stationary fluctuation analysis in males. Purple circles (n=8) represent spines that show >20% increase in conductance as compared to baseline and white circles (n=12) represent spines that show <20 % change in conductance. **E)** Raw traces of baseline (mean is shown in black) and E2 (mean is shown in red) and a representative mean-variance plot of the decay phase of peak-scaled 2pEPSCs showing an increase in conductance in E2 spines (173% above baseline) but no change in number (106% above baseline). **F)** Raw traces of baseline (mean is shown in black) and E2 (mean is shown in red) and a representative mean-variance plot of the decay phase of peak-scaled 2pEPSCs

showing an increase in the number in E2 spines (250% above baseline) but no change mean open probability of AMPAR. Although the absolute values of AMPARs number or conductance cannot be in conductance (95% above baseline). **G)** Normalized change in conductance versus number plots of psNSFA in females, where grey shaded region shows the 20% change in either properties. In females, 13 spines showed a >20% increase in conductance (purple circles), 2 spines showed no change in conductance but >20% increase in number (orange circles) and 2 spines showed >20% reduction in conductance and 20% increase in number (green circles). **H)** Normalized change in conductance versus number plots of psNSFA in males, where grey shaded region regions shows the 20% change in either properties. In males, 8 spines showed a >20% increase in conductance (purple circles), 12 spines showed no change in conductance but >20% increase in number (orange circles). Scale bar for raw traces 10pA, 25 ms.

estimated with this analysis, it can still be used to measure the relative changes. In females, psNSFA confirmed the increase in conductance in same 13 E2-responsive spines out of 17 (as estimated by conventional NSFA), by 84±15% with no changes in number as compared to baseline. In the other 4 spines, we found an increase in receptor number (112± 4% above baseline, Fig 4.7E, G) with no change in conductance. Out of these 4, the spines that showed a decrease in conductance in the conventional NSFA, also showed reduced conductance with psNSFA and showed a relatively higher increase in the receptor number (Fig 4.7G, green). This analysis shows that in females, E2-induced increase in 2pEPSCs is due to an increase in the conductance in 76% of spines whereas in the other 23 % percent there is an E2-induced increase in the number. Similarly, in males, psNSFA confirmed that the same 8 out of 20 spines showed an increase in conductance, that previously showed conductance change in conventional NSFA. Furthermore, in these spines there was no change in receptor number (conductance: 107±18% above baseline, Fig 4.7H). In the other 12 spines that showed no change in conductance in conventional NSFA, showed an E2-induced increase in number as compared to baseline (117±16% above baseline, Fig 4.7F, H). Thus, in males 40% of the E2-responsive spines show an increase in conductance whereas 60 % of the E2-responsive spines show an increase in number. None of the spines in females and males showed an increase in both number and conductance in the same E2-responsive spine. Fisher's exact test revealed a sex difference in the single channel properties that underlie E2-induced potentiation of 2pEPSCs, where 13 out of 17 spines in females and 8 out of 20 in males showed an

increase in conductance (p=0.03). Moreover, in both females and males, conventional and psNSFA showed that there were no E2-induced changes in conductance or number in any of the non-responsive spine in males (1±3.7% above baseline, Fig 4.8A, B).

Further analysis was performed to determine if we can correlate the E2-induced changes in the single channel properties with properties of 2pEPSCs. We measure rise time, decay, half width of 2pEPSCs in pre and E2 conditions. We found that in both females and males, a subset of E2-responsive spines showed a significant increase in the decay constant tau of potentiated 2pEPSCs as compared to baseline 2pEPSCs. Interestingly, among E2-responsive spines, the increase in decay tau correlated with the increase in AMPAR number (Pearson correlation, $r=0.58$, $p<0.001$, Fig 4.8C). We found no change in the rise times of 2pEPSCs in E2-responsive spines of both sexes (Fig 4.8D). Moreover, in both females and males, we found no correlation of changes in either increase in number (Pearson correlation, $r<0.001$, $p>0.1$, Fig 8E) or conductance (Pearson correlation, $r<0.001$, $p>0.1$, Fig 4.8F) with the magnitude of E2 potentiation. Overall, NSFA showed a sex difference in the postsynaptic mechanisms of E2-potentiation and moreover, corroborated our cpAMPAR result. In females, the majority of the responsive spines showed an increase in conductance, possibly due to replacement with cpAMPARs at these synapses and consistent with a high sensitivity to NASPM. However, in males, there increase in number or conductance could occur in E2 responsive spines, and thus there are fewer synapses with cpAMPARs incorporation and consistent with a much lower sensitivity to NASPM.

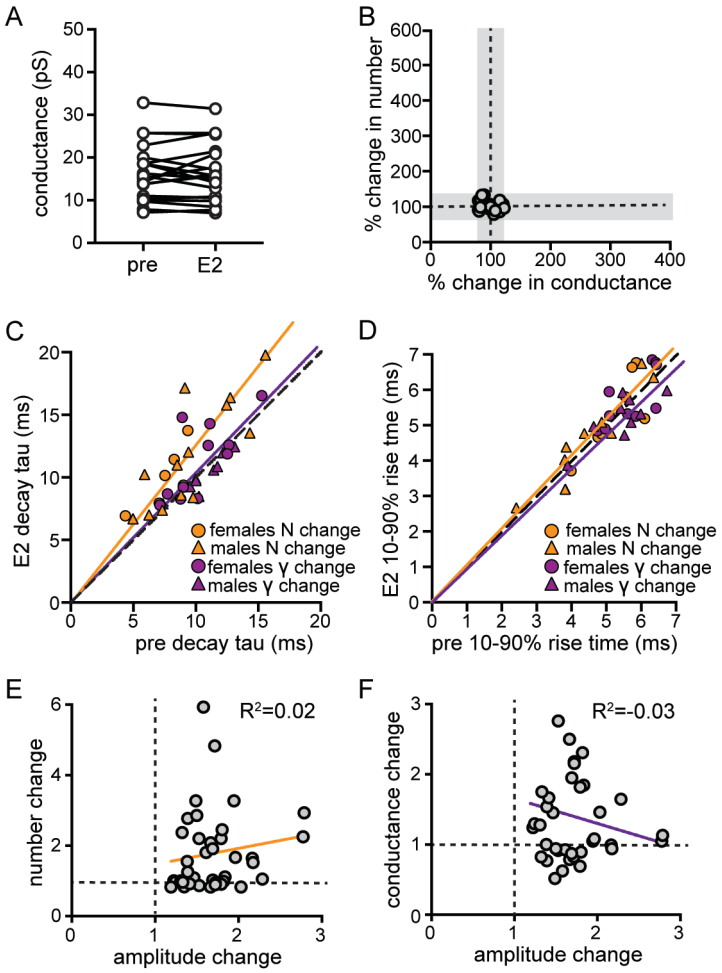


**Figure 4.8: Non-stationary fluctuation analysis of E2-non-responsive spines and correlations of 2pEPSC kinetics with conductance, number and magnitude of E2-potentiation.**

**A)** Group conductance measurements obtained by performing conventional NSFA all non-responsive spines (females and males) showing no change in conductance. **B)** Normalized change in conductance versus number plots of psNSFA of all non-responsive spine showing no change in conductance and number in any of the non-

responsive spine. **C)** Tau of decay phase in pre vs E2 of responsive spines. The spines are categorized as female that showed a change in number (orange circles), males that showed a change in number (orange triangles), females that showed a change in conductance ( purple circles) and males that showed a change in conductance (purple triangles) (Also in **D**). This shows that the spines that showed an increase in Tau in E2 also showed an increase in number. **D)** Rise time of 2pEPSCs in pre vs E2 of responsive spines. **E)** Correlation plots of change in number with the 2pEPSC amplitude change (all female and male responsive spines combined). Pearson correlation analysis showed no correlation of number change with amplitude. **E)** Correlation plots of change in conductance with the 2pEPSC amplitude change (all female and male responsive spines combined). Pearson correlation analysis showed no correlation of conductance change with amplitude.

## 4.5 Discussion

In this study, we described mechanisms that underlie the postsynaptic component of E2-potentiation and found latent sex differences in these synaptic mechanisms. Using electrical stimulation experiments, we found that calcium permeable AMPARs (cpAMPARs) are required for E2-induced EPSC potentiation in females but not in males and synaptic activity is required, possibly to activate the cpAMPAR mediated downstream signaling, in females but not in males. Further, non-stationary fluctuation analysis on 2pEPSCs showed that both increase in number or conductance could underlie E2-potentiation. However, we found a difference in which mechanism(s) predominate in each sex. While in females, the majority of the spines show an increase in the conductance in postsynaptic E2-potentiation, in males almost equal proportion of spines show an increase in either conductance or number. Overall, our study shows that E2 can activate distinct mechanisms to increase postsynaptic glutamate sensitivity for example either an increase in conductance or number and the relative contributions of these mechanisms vary between sexes.

### 4.5.1 Interpretation of E2-induced increase in AMPAR conductance or number

Performing non-stationary fluctuation analysis on 2pEPSCs is more interpretable than doing the analysis on miniature EPSCs because miniature EPSCs arise from different locations in a cell, while 2pEPSCs are synaptic events that are recorded from one location on the dendrite. Thus, there is less variability between events and noise in the

decay occurs mainly due to the stochastic closing of AMPAR channels. Theoretically non-stationary fluctuation analysis of the EPSCs estimates 3 main properties of AMPAR - conductance, number and mean open probability. It has been previously reported that the mean open probability of AMPAR is around 0.2-0.3 (Matsuzaki et al., 2001) and our conventional non-stationary fluctuation analysis corroborate this low open probability as our data mainly falls within the linear part of the parabola. Conventional analysis estimates values of conductance that are closer to the absolute values at the dendritic spines of CA1 cells in adult hippocampus. However, as there are multiple conductance states of AMPARs (Rosenmund et al.,1998), what we estimate here is the weighted mean average conductance of AMPARs at these synapses. Peak-scaled non-stationary fluctuation analysis (psNSFA) accounts for the low mean open probability of AMPARs. Using psNSFA we conclude that E2-responsive spines that did not show an increase in conductance, showed an increase in number. However, during peak scaling we artificially increase the mean open probability at 1, and consequently, the spines that showed an increase in number could also have an E2-induced change in mean open probability which is masked in psNSFA. Thus, the apparent increase in number could be due to an actual increase in mean open probability. Our correlation analysis showed an increase in AMPAR number with an increase in decay time. A previous study that showed an increase in AMPAR number also showed an overall increase in decay time of the EPSCs (Andrasfalvy and Magee, 2004) and suggests that changes in AMPAR numbers in our experiments are more likely.

The challenge in performing NSFA on 2pEPSCs is the number of events in each condition because it is difficult to maintain a whole cell configuration with a constant series resistance throughout recording. So far, two other labs have performed NSFA on 2pEPSCs but both these studies were performed in neonatal hippocampal and cerebellar slices (Matsuzaki et al., 2001; Momiyama et al., 2003). Our study provides a useful information of conductance measurements in adult CA1 dendritic spines with intact synaptic transmission.

### 4.5.2 Role of calcium permeable AMPARs in E2-induced potentiation

Previous studies have extensively investigated the postsynaptic mechanisms that underlie different forms of synaptic plasticity at CA3-CA1 synapses in the hippocampus. For example, we now know that there are many different forms of LTP, some that require NMDAR and PKA and some that don't. Recent studies have shown that LTP that requires PKA also requires insertion of cpAMPAR during induction (Park et al., 2016). Interestingly, we have found in previous studies that PKA is required for induction of E2-potentiation and LTP in females but not in males (Chapter 3). Studies with single channel recordings in the outside out patches have shown that cpAMPAR have inherently a higher basal conductance than calcium impermeable AMPARs. Moreover, calcium influx via cpAMPAR activates downstream signaling such as Rac/PAK/LIM to regulate actin cytoskeleton and increase spine volume following LTP stimulus (Fortin et al., 2010). Actin polymerization has shown to be required for E2-potentiation at CA3-CA1 synapses in males (Kramar et al., 2009). Therefore, we

hypothesize that in females cpAMPAR are inserted into the synapses following E2 application. Synaptic activity is then required to initiate cpAMPAR mediated signaling to express and stabilize E2-potentiation. We know from our previous experiments that CaMKII is also required for expression and maintenance of E2-potentiation. Thus, one possibility is that calcium influx via cpAMPAR activates CaMKII. Moreover, E2-induced modification of AMPARs pool at the synapse with more cpAMPARs, results in an overall increase in conductance (Benke and Traynelis, 2019). In males, in addition to the above mechanism where cpAMPAR insertion in different E2-responsive spines, there could be either an increase in conductance or an overall increase in the AMPAR number with to cause EPSC potentiation. Thus, in males, there could be insertion of cpAMPAR following E2-application similar to females, but this not required. Potentiation is expressed even in the absence of cpAMPAR induced signaling. Thus, signaling via cpAMPARs may only contribute to synaptic potentiation in males. Alternatively, in males the increase in conductance could occur via a different mechanism like phosphorylation of S841 subunit by CaMKII (Oh and Derkach, 2005). The increase in either number or conductance in males adds to our previous experiments with kinases and calcium sources, where we found parallel signaling cascades underlie E2-induced potentiation in males.

The role of cpAMPARs in expression of LTP has been debated. While some labs show a clear involvement of cpAMPARs in LTP (Plant et al., 2006), other labs using the same experimental design could not replicate these findings (Adesnik and Nicoll, 2007). Neither of the studies mentioned the sex of animals used in their studies. We found that

the requirement of cpAMPARs in E2-potentiation is different between females and males and this could possibly explain the discrepancy found in the literature. AMPAR modulation occurs in several different forms of plasticity and finding sex differences in AMPAR modulation emphasizes in the inclusion and separate examination of both sexes in future mechanistic studies.

# 5. General Discussion

## 5.1 S*ummary: Latent sex differences in the mechanisms that underlie acute E2-induced potentiation in the hippocampus*

17β-Estradiol (E2) can be locally and acutely synthesized in the hippocampus and we investigated the role of E2 as a neuromodulator. Through different experimental approaches, we characterized the acute effects of E2 on CA3-CA1 synaptic transmission in the hippocampal slices of gonadectomized adult females and male rats. We found that E2 acutely potentiates excitatory synaptic transmission similarly in both males and females and this potentiation occurs via largely independent pre or postsynaptic mechanisms. Although the overall magnitude of E2-potentiation is similar between sexes, while investigating the molecular mechanisms underlying this potentiation, we found multiple sex differences.

We investigated the role of estrogen receptors (ERs) and found that different ERs initiate pre and postsynaptic component of E2-potentiation in males versus females. While ERβ activation in females induces the presynaptic component of E2-potentiation, in males it induces the postsynaptic component of E2-potentiation. GPER-1 activation in females induces the postsynaptic E2-potentiation and ERα activation induces the presynaptic E2-potentiation in males. Thus, mechanisms to induce E2-potentiation start to differ between females and males at the level of ERs.

Downstream of the ERs, we also found differences in the molecular signaling in males versus females. Specifically, we found that calcium sources and PKA are differentially required in males versus females. While PKA is required in the initiation of

E2-potentiation in females, it is not required in males. Additionally, we found that L-type calcium channels and calcium release from internal stores are each required for E2-potentiation in females, while in males one can compensate the other to initiate E2-potentiation. However, not all signaling was different. In contrast to PKA, other kinases like SRC, ROCK and MAPK were found to be required in both females and males.

Studying these mechanisms in both sexes, revealed novel distinctions in the roles of popularly studied kinases that can be generalized to other forms of plasticity. In an attempt to draw parallels between mechanisms of E2-potentiation and mechanisms of more commonly studied forms of plasticity, we investigated the role of PKA in long term potentiation (LTP). Interestingly, we found that the sex difference in the requirement of PKA is generalizable to different forms of LTP. In the most commonly used LTP protocols- 1x high frequency stimulation (HFS) and 3x HFS, we found that while in males PKA was not required for LTP induction, in females PKA was required in 1xHFS and contributed to 3x HFS LTP induction. Moreover, in pairing induced protocol, we found that while PKA contributed to the LTP induction in males, it was required for LTP induction in females. This demonstrates that the sex difference in the requirement of PKA is generalizable and thus, is likely independent of differential requirement of ERs in males versus females.

To further understand where these distinct mechanisms underlying E2-potentiation in males and females converge to produce E2-potentiation of same magnitude, we focused on the mechanisms underlying the postsynaptic component of potentiation. We found that these distinct signaling pathways do not converge at the postsynaptic

terminal and even modulate AMPARs differently to potentiate AMPAR currents. While in females, E2 largely caused an increase in AMPARs conductance to induce potentiation, in males, E2 increased either the number or conductance to induce potentiation. Moreover, we found that the increase in the conductance is due to transient replacement of AMPARs with calcium permeable AMPARs (cpAMPARs). Matching our non-stationary results, in our experiments with NASPM we found that cpAMPARs are required in females but not in males. Overall, we have shown how E2 can act as a neuromodulator to potentiate excitatory synapses. Moreover, we found that comparing mechanisms underlying neuromodulation between males and females is important because although the overall E2-neuromodulation effect appears to be similar, the underlying molecular signaling differs between the sexes.

### 5.2 *Neurosteroid E2 as a neuromodulator*

E2 follows some basic principles of an intrinsic neuromodulation. An intrinsic neuromodulator is released within the brain region and modulates the strength of few synapses via specific receptors. E2 can be locally synthesized in the hippocampus (Sato and Woolley, 2016) and can acutely modulate both excitatory (Smejkalova and Woolley, 2010) and inhibitory synaptic transmission (Huang and Woolley, 2012). Moreover, we found that the effect of E2 on excitatory synaptic transmission at CA3-CA1 synapses is synapse specific in both sexes. Recently, work from our lab also found acute E2 effects on the inhibitory synaptic transmission. We have shown that E2 suppresses somatic inhibitory synaptic transmission in females but not males

(Tabatadze et al., 2015), and it suppresses dendritic inhibition much more commonly in males than in females (unpublished, Huang and Woolley). These changes at excitatory or inhibitory synapses occur within 5-7 minutes of bath E2 application. The time required for E2 to induce/express the synaptic changes in our experiments and previous studies is similar to the effects of other neuromodulators at these synapses like dopamine (Otmakhova and Lisman, 1999) that requires GPCR signaling, but is slower than neurosteroids that directly bind to GABAR and modulate their currents within seconds (Carver and Reddy, 2016).

We found that E2-induced potentiation occurred in a subset of CA1 neurons in electrical stimulation experiment or in a subset of spines in two-photon experiments. A subset of synapses or cells being non-responsive to E2, could have two possible explanations. 1) ERs are not expressed in every spine of presynaptic axonal terminal. Evidence from different immunohistochemical studies suggests heterogeneous expression of ERα, ERβ and GPER throughout the CA1 neurons and also at the CA3 presynaptic axonal terminals of the hippocampus (Milner et al., 2001; Milner et al., 2005; Waters et al., 2015). Thus, spines that do not express ERs might be unable to initiate downstream signaling and fail to potentiate synapse. 2) The non-responsiveness of a subset of synapses or cells could be a homeostatic mechanism that gets activated. When a few spines get potentiated, they actively suppress the potentiation of other synapses to maintain electrotonic stability within the cell.

It is likely that most actions of E2 occur via secondary messenger signaling pathways like calcium, IP3 or cAMP. We found that calcium release/influx from different

calcium sources are required in both sexes to initiate E2-potentiation. Moreover, some of these secondary messengers can also be activated by increased association with other metabotropic receptors. For example, our lab found that at somatic inhibitory synapses, E2 promotes association with ERα and mGluA1 to increase IP3 levels. IP3 then binds to IP3R at the internal stores to increases cytosolic calcium. This increase in calcium activates anandamide synthesis, which then retrogradely binds to presynaptic CB1 receptors and activates Gi secondary messenger signaling to suppress GABA release (Tabatadze et al., 2015). Overall, these results demonstrate how neurosteroid E2 acts as an intrinsic neuromodulator in the hippocampus. However, further studies are required to understand what physiological or pathological conditions activate E2 synthesis in the hippocampus, by what mechanisms and how local or widespread is the E2 release?

### 5.*3 Proposed mechanisms to describe how E2-potentiates excitatory synapses in males and females*

One of the advantages of studying the acute effects of E2 at the CA1 synapses is that there is an extensive literature that describes the mechanisms underlying various synaptic plasticity forms at these synapses. Using our results and the evidence provided by other groups, we propose some mechanisms of how E2 acutely potentiates excitatory synaptic transmission. The pre and postsynaptic mechanisms that the underlie initiation, expression and maintenance of E2-potentiation are outlined in Figure 5.1.

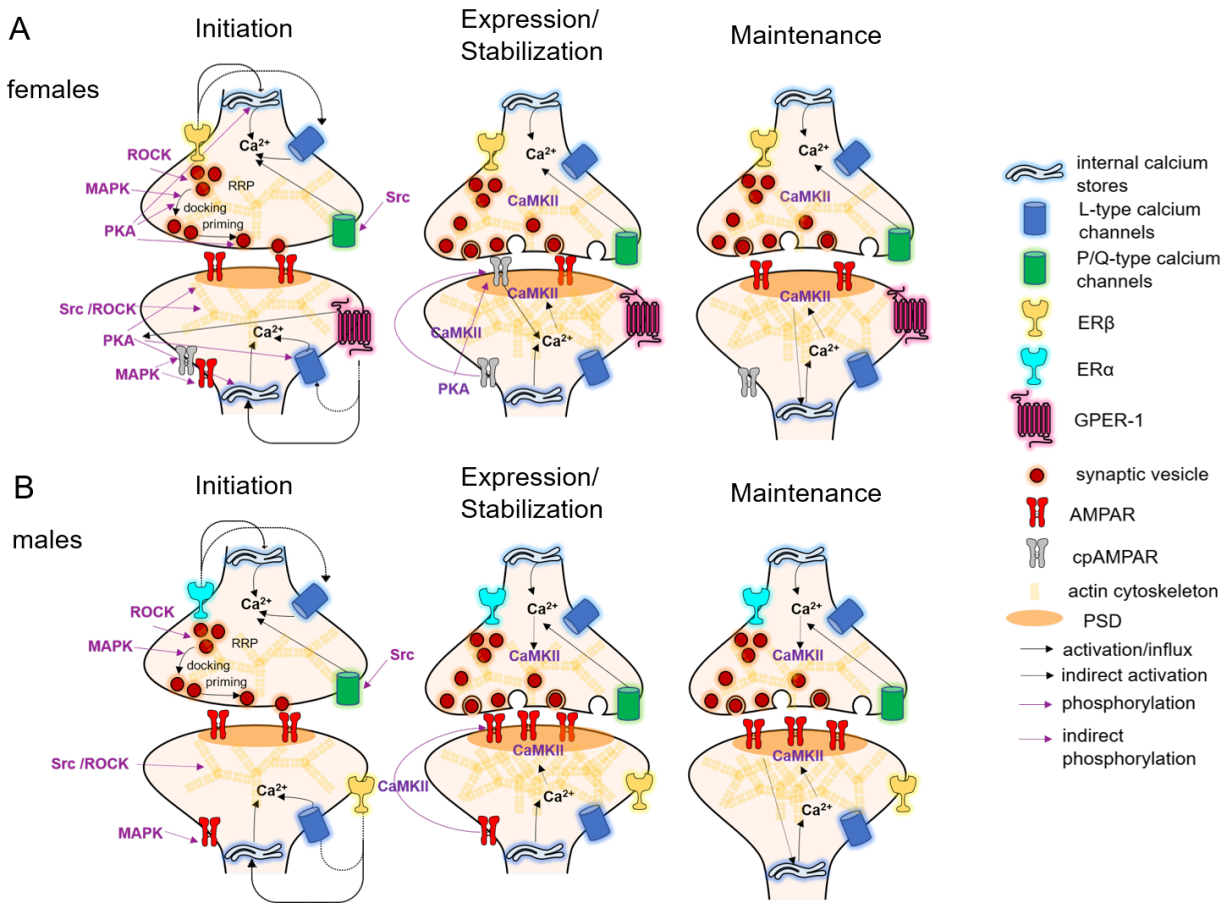


**Figure 5.1: Proposed model for pre and postsynaptic mechanisms that underlie initiation, expression and maintenance of E2-induced potentiation in both sexes.**

**A)** Initiation, expression and maintenance mechanisms in females. Presynaptically, ERβ plays a role in initiating downstream signaling. ERβ indirectly activates both calcium sources (internal stores and L-type calcium channels) and this leads to an increase in intracellular calcium. Calcium acts as a secondary messenger and regulates downstream kinases. PKA and MAPK can regulate docking of synaptic vesicles via phosphorylation of synapsin-1. PKA can additionally regulate priming and direct vesicular exocytosis by interacting with RIM1a and munc-13. Src can increase calcium

influx via P/Q type calcium channels. ROCK can maintain the readily releasable pool (RRP). Src/ROCK can also regulate actin cytoskeleton via presynaptic machinery. The functions of Src, ROCK and MAPK at the presynaptic terminal are also similar in males (shown in **B**). Postsynaptically, GPER-1 activates downstream signaling in females. GPER-1 can activate both calcium channels indirectly via PKA or other secondary messengers to increase intracellular calcium. Src/ ROCK indirectly promote actin polymerization via inhibiting cofilin. Actin polymerization can increase the spine size to allow insertion of AMPAR receptors. MAPK indirectly plays a role in AMPAR trafficking to the perisynaptic sites. In females in higher proportions of synapses, CaMKII translocates calcium permeable AMPARs (grey) to the PSD. Similar to the presynaptic terminal, the roles of Src, ROCK and MAPK functions are similar in males (shown in **B**). For expression and maintenance, we only know that CaMKII is required for the presynaptic mechanisms. Postsynaptically, in females, MAPK specifically traffics cpAMPARs with the aid of PKA to the synapse. This insertion of cpAMPARs is transient and occurs only during stabilization of E2-potentiation. There is no overall change in AMPAR number. One function of cpAMPAR insertion might be to facilitate CaMKII activity. Postsynaptically in females CaMKII plays a role. CaMKII gets translocated following E2 application and remains in PSD by being bound to NR2B subunit of NMDAR. CaMKII at PSD can increase conductance in females by phosphorylating AMPARs. It can also immobilize AMPARs at the synapse (also in **B**). **B)** Initiation, expression and maintenance mechanisms in males. Presynaptically, ERα initiates downstream signaling. There is no PKA and the function of Src, ROCK and MAPK

proposed are similar to A. ERα can activate downstream signaling by activating only one of the calcium sources. This is in contrast to females where both calcium sources are required for E2-potentiation. Postsynaptically, ERβ initiates downstream signaling again via recruiting either of the calcium source. The function of the Src, ROCK and MAPK proposed are similar to A. For expression of E2-potentiation in males, there can be two mechanisms. One is similar to females where there is transient increase in cpAMPARs. In the second mechanism, CAMKII translocate more AMPARs (but calcium impermeable subunits) to the synapse and results in an overall increase in AMPAR number. Both these mechanisms can exist in equal proportion of cells. Postsynaptic mechanisms of E2-potentiation in males are similar to females as described in A.

We have covered the postsynaptic part of this model in great detail in the discussion of chapter 3 and 4. Here, we focus on theories to explain the latent sex differences in the requirement of ERs, PKA, calcium sources and AMPAR modulation that underlie E2-potentiation. Physiologically, it would all start with E2 synthesis in the hippocampus. Aromatase activation and E2 release mechanisms are purely speculative and will be discussed in greater detail later, but essentially, we suspect that the E2 release is local and probably affects a few synapses. Upon E2 release it could activate ERs at the pre and/or postsynaptic terminals in autocrine or paracrine manner, similar to other neuromodulators. We have demonstrated that different ERs participate in the pre or postsynaptic component of E2-potentiation in males versus females.

### 5.3.1 *Membrane associated ERs initiate non-genomic signaling*

We described in the introduction that ERs can be membrane associated with the help of caveolins or can get transiently localized to the membrane (Dominguez and Micevych, 2010; Tabatadze et al., 2013). Once ERs are membrane associated, they can initiate biochemical signaling at pre or postsynaptic sites. In our experiments we found that distinct ERs activate the pre or postsynaptic components of potentiation in males versus females. Based on the biochemical evidence, we speculate two possibilities of how sex differences in requirement of ERs occur. 1) One explanation could be the heterogenous expression of ERs at the membrane at pre or postsynaptic terminal in males versus females to activate specific signaling. For example, ERβ is expressed at the presynaptic terminals more abundantly in females than in males. This

heterogenous expression could be stochastic or due to some differences in trafficking mechanisms involving different caveolins or lipid rafts to specifically target each ER to a particular synaptic location. 2) Another explanation for the distinct function of ERs could be that, although the expression patterns of different ERs are similar throughout the cell, the downstream signaling components that are associated with them are different. Thus, it is the specific signaling that drives specific function of ERs in E2-potentiation. GPER-1 in contrast to the classical ERs is a G-protein coupled receptor and is shown to be expressed at both pre and postsynaptic terminals in the hippocampus (Waters et al., 2015). The mechanisms to regulate GPCR at the membrane will be different than other ERs. Future experiments should determine how do these distinct functions of ERs arise during the development of the hippocampal physiology.

Membrane associated ERs and their downstream signaling components could also potentially explain a curious result in our whole cell recordings. In our experiments, we sometimes observe E2-induced potentiation for more than 60-70 mins after the whole cell configuration has been achieved. Whole cell recording is known to dialyze the intracellular components and studies have shown the lack of LTP induction 15-20 mins after whole cell access due to dialysis of cellular signaling components (Malinow and Tsien, 1990). Even in our whole cell LTP experiments, having a longer baseline (>15 mins) failed to induce LTP. Thus, one potential explanations of late E2-effects in our whole cell recordings could be that signaling components are membrane compartmentalized and don't get dialyzed easily. Alternatively, if the signaling components are not all membrane associated, dialysis could be a reason why we see

some cells/spines to be non-responsive to E2. If signaling components are cytoplasmic, dialysis could wash out these signaling components and E2 will fail to potentiate excitatory synaptic transmission at those synapses.

### 5.3.2 *ERβ mediated signaling strongly influences E2-induced excitatory synaptic potentiation in both sexes*

In both sexes, E2 potentiates excitatory synaptic transmission via different ERs at the pre or postsynaptic terminals. With miniature EPSC experiments we found that while in females, ERβ agonist (WAY-20070) mimicked the presynaptic effects of E2-potentiation by increasing mEPSC frequency, it mimicked the postsynaptic effects of E2 in males by increasing mEPSC amplitude. Three pieces of evidence suggest a trend where ERβ plays a stronger role in inducing E2-potentiation in both males and females. 1) Our lab has shown using electrical stimulation experiments that in females DPN, an ERβ agonist, is sufficient to potentiate synapses and occludes the effect of E2. However, the GPER agonist G-1 induced potentiation in only 2 out of 15 cells (Smejkalova and Woolley, 2010). 2) In our miniature EPSC experiments, although not significant, we found that a higher percentage of E2-response (pre or postsynaptic component) is mediated by ERβ in both males and females. Among the responsive cells, relatively more cells showed E2-induced increase in mEPSC frequency in females (27%) as compared to males (16%), whereas relatively more cells showed E2-induced increase in mEPSC amplitude in males (25%) as compared to females (17%). 3) In our 2pEPSC experiments, where we investigated the postsynaptic component of E2-

potentiation, we found a trend where higher proportion of cells in males showed response to E2 as compared to females (ERβ mediates postsynaptic component in males). Furthermore, in these experiments a similar trend was seen in magnitude of E2-induced increase in EPSCs; higher in males. Overall based on these observations we hypothesize that downstream of E2, ERβ probably plays a more dominant role to acutely potentiation excitatory synaptic transmission. ERα and GPER-1 activation also contribute to E2-potentiation but not as strongly as ERβ. It is curious how activation of different ERs in males and females leads to similar magnitude of potentiation. We speculate that the sex difference arises from the dominant pre versus post synaptic ERβ activation. The other ERs compensate for the sex specific ERβ signaling by activating additional signaling that leads to the similar appearing potentiation in both sexes. It will be interesting to study whether the sex difference in the requirement of ERs causes downstream sex differences in the requirement of PKA and calcium sources or these sex differences are independent of each other. Following sections have been divided into two parts- one component that is different between sexes and one that is similar between sexes.

### 5.3.3 *Sex difference in the activation and function of PKA in E2-induced excitatory synaptic potentiation*

Although we have not investigated the specific functions of kinases, based on the roles of kinases described in other forms of plasticity like LTP, we speculate that each kinase performs a distinct function to potentiate excitatory synapses. Results from our

experiments showed that while in females signaling components that we tested are each required for E2-induced potentiation, in males the molecular signaling is parallel and one pathway can compensate the other. The sex difference in the requirement of PKA is most likely independent of E2 or differential ER activation because we found a similar PKA sex difference in LTP. In this section I describe how the sex difference we observe in PKA, calcium sources and cpAMPARs could be linked mechanistically to induce E2-potentiation.

In electrical stimulation experiments, PKA inhibitors blocked the E2-induced synaptic potentiation in all of the responsive cells in females, but not in males (Chapter 3). This suggests that in females, a PKA-dependent signaling gets activated at both pre and postsynaptic terminals. In contrast in males, in addition to a PKA dependent signaling, other PKA-independent signaling pathways also get activated. PKA is not completely dispensable for E2-induced EPSC potentiation in males, because we found that applying both CAMKII and PKA inhibitor together blocked the initiation of E2-potentiation. However, in males, whether PKA plays a role at both pre and postsynaptic terminals is not yet clear.

PKA can be activated pre and post-synaptically by different calcium sources that get recruited by E2. There is evidence of both internal calcium stores and L-type calcium channels to be expressed at pre and postsynaptic terminals. Calcium influx can activate calcium sensitive adenylyl cyclase to activate PKA signaling. Specifically, adenylyl cyclase 8 has been reported to be calcium sensitive in the brain (Cali et al., 1994). Alternatively, in females GPER-1 can activate PKA independent of calcium, for example

activation via canonical GPCR signaling (Prossnitz and Maggiolini, 2009). Furthermore, PKA can also modulate calcium sources to activate other kinases that are required for E2-potentiation. For example, it has been shown that PKA can phosphorylate IP3R on internal stores to increase their mean open probability (Banke et al., 2000). Calcium dependence of the pre and postsynaptic components of E2-potentiation should be further explored in both sexes and also determined what calcium sources are required for each component of potentiation by either blocking one calcium source or another.

Pre-synaptically, we observed that E2-increases glutamate release probability in both sexes. This increase in release probability could occur by two mechanisms. 1) Direct modulation of presynaptic calcium channels or activating internal stores to increase calcium influx. 2) Modulation of proteins that participate in the synaptic vesicular machinery. We hypothesize that the role of PKA is more dominant in females than in males. In females, PKA-dependent signaling can increase the glutamate release probability by phosphorylating proteins that are involved in synaptic vesicular priming and exocytosis. Preliminary biochemical results from our lab shows that E2 increases the phosphorylation of synapsin1 at Ser 9, which is a PKA substrate, in females but not in males (unpublished). PKA could also increase vesicular priming by regulating other proteins such as RIM1α as shown previously (Lonart et al., 2003).

Post-synaptically, PKA has been shown to perform multiple functions. During E2-potentiation PKA can regulate the activities of other kinases. Specifically, PKA promotes CAMKII activity by indirectly inhibiting PP1 phosphatase as is described previously (Blitzer et al., 1998). PKA mediated signaling downstream can also transiently

incorporate calcium permeable AMPARs during expression of E2-induced synaptic potentiation. This hypothesis is based on previous literature where PKA phosphorylation at Ser-845 was required to stabilize cpAMPARs (He et al., 2009). Additionally, recent data have shown that cpAMPARs are required for PKA dependent LTP (Park et al., 2016). Thus, difference in the requirement of PKA could, at the postsynaptic site, result in the sex difference in insertion of cpAMPARs. This suggests that the sex difference we observe in the requirement of cpAMPARs is possibly just a consequence of the sex difference we observe in the requirement of PKA in E2-potentiation. Future experiments are required to better understand the mechanistic details of these sex differences and to address how sex difference occur in the requirement of all these different signaling components.

### 5.3.4 *Mechanisms of Src, ROCK, MAPK and CAMKII that underlie E2-potentiation*

The pre and postsynaptic functions of Src, ROCK, MAPK and CAMKII have been described in other forms of plasticity. In contrast to the role of PKA, given their similar requirement in E2 potentiation in both males and females, we hypothesize that the functions of these kinases are also similar between both sexes.

Presynaptically, Src, ROCK, MAPK and CAMKII can each modulate different parts of synapse cycle to increase glutamate release probability. Preliminary biochemical data from our lab shows that E2- increases synapsin-1 phosphorylation at Ser 603, which is a MAPK substrate in both sexes (unpublished). There is evidence that Src kinase regulates the increase in presynaptic calcium influx possibly by regulating calcium

channels (Wang, 2003). Additionally, ROCK can maintain the readily releasable pool of glutamate vesicles (Gonzalez-Forero et al., 2012).

Even at the postsynaptic site, each kinase is shown to play a distinct role to initiate potentiation. Some of the functions have been discussed in chapter 3. E2 activates Src and ROCK signaling, which could promote actin polymerization that would increase the spine volume. Requirement of actin polymerization in E2-potentiation has been shown previously (Kramar et al., 2009). Moreover, studies have also shown acute E2-induced increase in spine volume (Soma et al., 2018). With an increase in spine volume and possibly the post synaptic density (PSD), it gives opportunity for more AMPAR insertion. Based on previous studies, MAPK pathway can regulate trafficking of both GluA1 and GluA2 containing AMPAR subunits (Gu and Stornetta, 2007). The activation of the Ras-MEK-ERK pathway stimulates phosphorylation of GluA2 at S841 and of GluA1 at S845. S841 phosphorylation of GluA2 is sufficient to drive GluA2 containing AMPARs into synapses, while S845 phosphorylation of GluA1 is required for the exocytosis of GluA1-containing AMPARs (Qin et al., 2005). Based on our non-stationary analysis, we found that in males half of the responsive spines show an increase in AMPAR number, whereas the other half show an increase in conductance. Thus, in males in half of the responsive spines, there could be more GluA2 containing AMPARs trafficked to the synapses, which result in an overall increase in the number of AMPARs. In the other half and in females, GluA1 containing AMPAR get trafficked to the synapses, but the overall AMPAR number does not change. Here, the calcium impermeable AMPARs (GluA2) get replaced by cpAMPAR pool (GluA1). As cpAMPAR have inherently higher

conductance (Swanson et al., 1997), in these spines we observe an overall change in conductance.

MAPK signaling does not traffic AMPARs directly to the synapse but to the perisynaptic site. CAMKII mediated phosphorylation of auxiliary subunits like stargazin translocates AMPARs to the PSD (Opazo et al., 2010). Additionally, the function of CAMKII at the PSD can be facilitated by the calcium influx through cpAMPARs. The maintenance of E2-potentiation is speculative. I hypothesize that the autophosphorylation property of CAMKII, which keeps it active even in the absence of active signal is responsible for maintenance of E2-potentiation. Active CAMKII has been shown to immobilize AMPAR at the PSD and can potentially maintain increased synaptic strength (Opazo et al., 2010). It will be interesting to address how different ERs are associated with each of these kinases and activate them similarly in both females and males. Future experiments will confirm some of these hypotheses and moreover investigate how CAMKII maintains presynaptic component of E2-potentiation.

### 5.4 *How do sex differences that underlie synaptic plasticity occur?*

It is now generally believed that the basis of sex difference originates from the presence of different sex chromosomes- XX in females and XY in males. The presence or absence of Y chromosome leads to different gonad development and results in different hormone production. One way, sex differences are established in the brain is during development by the actions of gonadal steroids. Another way is that the sex chromosomes can directly influence the brain development and physiology differently

between sexes (Arnold, 2004). However, the most robust brain sex differences studied so far are those that are related to reproduction. Sex differences in non-reproductive processes like cognition, stress and anxiety and locomotion have also been reported but we know very little about the neural substrates and mechanistic basis of these sex differences. In our studies, we observed sex differences in the mechanisms that underlie acute effects of E2 in adult hippocampus, specifically in the requirements of estrogen receptors, calcium sources, PKA and in AMPAR modulation. What mechanisms result in these mechanisms? Do they exist from birth due to some organization effect of the hormones or are expressed only in adults? In the following section I provide evidence for some sex specific gene expression or environmental factor like stress that could underlie sex difference in the requirement of PKA.

In contrast to the sex chromosomes being different between males and females, the autosomal genetic content is the same between sexes. However, the regulatory genome that controls the expression of these genes is sexually dimorphic and could introduce sex-bias in the expression of certain genes, which potentially influences brain physiology differently between sexes (Nishida et al., 2005; Yang et al., 2006; Reinius et al., 2008). An evidence from human studies observed genetic polymorphisms in the human adenylate cyclase gene, adenylate cyclase 8 (ADCY8), that correlate with glioma risk in patients with type I neurofibromatosis in a sex-specific manner, elevating risk in females while reducing risk in males (Warrington et al., 2015). Interestingly adenylyl cyclase VIII is the subtype of adenylate cyclase, which is calcium sensitive and increases cAMP levels upon activation. There is evidence from animal studies that

calcium release from internal stores is one source of calcium that can activate PKA signaling via adenylyl cyclase VIII (Wong et al., 1999). Based on this evidence, it is possible that ADCY8 levels are higher in female CA1 neurons and thus more readily activate PKA signaling to induce E2-potentiation.

Alternative to sex difference in the activation of cAMP signaling, there could be sex difference in the removal of cAMP. Studies show that different levels phosphodiesterase 4B (PDE4B) control the amount of PKA in the system. In cardiac myocytes, different levels of PDE4B caused different cAMP levels and this underlies the sex differences observed in the role of PKA in cardiac calcium spark production (Parks et al., 2014). This study also showed that sex difference in the PKA levels caused a sex difference in the magnitude of calcium sparks generated by internal stores. Similarly, using transgenic mice when PDE4B was disrupted, sex difference was shown in the hippocampal LTP induction (Campbell et al., 2017). Thus, it is also possible that different levels of PDE4B expression or activity in males versus females in CA1 neurons could also influence the differential requirement of PKA.

Alternative to the sex-biased gene expression leading to different levels of the protein as potential explanation for sex difference, there could be some factors in the environment, like certain chemicals or stressors, that in combination with the varying hormone levels, differentially modulate the neuronal cellular processes in adult males versus females. Sex differences in the mechanisms underlying chronic and acute stress have been described in the brain. For example, corticotrophin releasing factor (CRF) is released following a stressful event and is known to modulate neurons. CRF binds to

CRF-1 receptors and activates secondary messenger signaling and sex differences in some components have been observed. CRF-1 receptors are found to be more highly coupled to $G_{as}$ type G-protein in females than males (Valentino et al., 2013). Accordingly, overexpression of CRF induces greater cAMP-PKA signaling in female mice, while in males, activation of the B-arrestin pathway downstream of CRF-1 receptors is more common (Bangasser et al., 2010). Similar sex difference in CRF-1 receptor signaling has also been observed in phosphor-proteomic analysis in mouse cerebral cortex (Bangasser et al., 2017). Effects of CRF-1 have also been shown in the hippocampus and it is likely that these effects could prime PKA signaling differently in the hippocampus of females versus males. Overall, comparing the mechanisms that underlie E2- potentiation in both sexes unmasked sex differences in the roles of important kinases and calcium sources in one of the most popularly studied synapses in the field of neuroscience.

### 5.5 *Limitation of using pharmacological tools*

One of the limitations to conclusively interpret the requirement of ERs, kinases and calcium sources is the use of pharmacological agents. In most of my experiments we either used specific agonists to activate different ERs or used antagonists to block specific kinases or calcium sources. Classical drugs can have off-target effects at high concentrations. For example, studies have shown that H89 at 10μM and higher concentration could block kinases like PKC with equal efficiency (Lochner and Moolman, 2006). Previous biochemical and electrophysiology literature was thoroughly

studied before choosing the drug and in most experiments the lower end from the range of concentration previous studies was used. We investigated and observed the effects of mPKI and tatCN21 at 0.5 μM and 1 μM respectively, which is lower than the average concentration used in the literature – ~0.5-3μM for mPKI and ~1-20 μM for tatCN21 (Vest et al., 2010). Other factors such as specificity, mechanism of action, cell permeability, solubility, concentration, sensitivity to light, whether it can be washed out, and so on, were also considered before choosing a drug.

Apart from the potential off-target effect problem, another limitation in designing the experimental protocols is not having a positive control for the antagonists/inhibitors. In all my experiments except thapsigargin, inhibitors had no effect on the basal synaptic transmission. Thus, the lack of an effect could either mean that the protein is not involved or that the drug is not effective. All of the drugs we chose in our experiments have been extensively studied and tested in different experimental approaches before. Also, to make our experiments more interpretable 3 additional control steps were taken. 1) For determining the role of PKA, experiments were performed in male and female hippocampal slices on the same day, where mPKI solution blocked the effect of E2 in females, the same solution failed to block the effect in males (n = 2 responsive cells in each sex). 2) We also confirmed the lack of effect of a drug by testing the same drug at a higher concentration. For example, we used two different concentrations of H89 (1 μM and 5 μM) and found that, at both concentrations H89 failed to block the effect of E2 in males. 3) Lastly, to conclusively determine whether the protein is actually involved in initiation/expression or maintenance of E2-potentiation, we used two drugs that block

the protein's activity by different mechanisms. For example, to block PKA we used H89 and mPKI, where H89 competes with ATP to bind to the ATP binding site of the catalytic subunit, mPKI is a peptide inhibitor that competitively binds to the enzymatic subunit of PKA and blocks its activity. Similarly, we used two different drugs to block CAMKII-KN93 and tatCN21 that block CaMKII activation and its activity, respectively. Additionally, tatCN21 also blocks the autophosphorylation of CAMKII activity that allows it to remain active in the absence of active signal (Calcium/calmodulin complex). In spite of all above described controls, we interpret all the results with caution and future experiments with genetic manipulation of each signaling components should be performed to confirm our proposed molecular mechanisms that underlie E2-induced potentiation.

5.6 *Studying mechanisms in both sexes will potentially improve scientific reproducibility*

Mechanisms underlying LTP and other forms of synaptic plasticity have been extensively studied at CA3-CA1 synapses using many different types of techniques. Some parts of the LTP mechanisms have been established and reproduced by several labs. For example, CAMKII has been found to be involved in induction of LTP at CA3-CA1 synapses by many groups. However, there are some mechanisms for which the data are contradicting between different labs and thus their roles have been debated for years. In our study, we found sex differences in the mechanisms underlying E2-synaptic plasticity which suggests that ignoring sex as a biological variable could have accounted for some of the contradicting evidence across labs. Here we describe the two

mechanisms whose involvement in LTP has been debated, and we found that these two mechanisms are different between females and males.

One big argument has been in the requirement of PKA in initiation of early LTP. Majority of the initial studies show that PKA is not required for the early component of LTP, but it is required for the late component (Frey et al., 1993). However, later studies demonstrated that PKA is required for LTP and is dependent on the type of stimulus used to induce LTP (Otmakhova et al., 2000). In most of these studies, either males were used, or the sex was not mentioned. In males, we found that the requirement of PKA is similar to what was previously described by the earlier studies. We found that PKA inhibition does not affect/block LTP induction. However, in contrast we found that PKA inhibition completely blocks LTP induction in females. Thus, comparing the requirement of PKA in both sexes could account for some differences observed across different labs.

Another argument in the LTP mechanisms has been in the requirement of calcium permeable AMPARs (cpAMPARs). A group showed that cpAMPARs are transiently inserted following LTP induction, and cpAMPAR signaling is important for expression or maintenance of LTP (Plant et al., 2006). Other groups failed to replicate this study and suggested that cpAMPAR are not required for LTP expression (Adesnik and Nicoll, 2007; Gray et al., 2007). Once again in all these studies the sex of the animals used was not mentioned. We found that while cpAMPAR are required for expression of E2-potentiation in females, they only partially contribute to E2-potentiation in males. Thus, to improve the reproducibility across labs, our results strongly emphasize on the

inclusion of both sexes even while studying mechanisms that underlie non-reproductive physiology.

Synaptic plasticity and neuromodulation are fundamental phenomena that occur in almost all brain regions. My thesis contributed towards understanding how E2 could act as an intrinsic neuromodulator in the hippocampus. More importantly, comparing the mechanisms that underlie E2-neuromodulation in both sexes, shows sex differences. How adult females recruits some mechanisms differently than males in synaptic plasticity, adds to the computational complexity of the brain, via which information can be processed and stored. Clinically, sex-specific mechanisms can also provide an understanding of how some neuropsychiatric disorders differ between sexes. For example, there is a sex difference in the incidence of Major Depressive Disorder (MDD), but the molecular mechanisms that underlie this sex difference are poorly understood. A recent study investigating molecular mechanisms that underlie MDD showed sex differences in the genes that get altered or activated following MDD in both humans and mice (Labonte et al., 2017). Understanding molecular mechanisms that differ between sexes provides a wider range of therapeutic strategies to target signaling cascades that get altered in these disorders.

**References**

Abdelgadir SE, Resko JA, Ojeda SR, Lephart ED, McPhaul MJ, Roselli CE (1994) Androgens regulate aromatase cytochrome P450 messenger ribonucleic acid in rat brain. Endocrinology 135:395-401.

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R (1997) Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell 88:615-626.

Adashi EY, Hsueh AJ (1982) Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. J Biol Chem 257:6077-6083.

Adesnik H, Nicoll RA (2007) Conservation of glutamate receptor 2-containing AMPA receptors during long-term potentiation. J Neurosci 27:4598-4602.

Akama KT, Thompson LI, Milner TA, McEwen BS (2013) Post-synaptic density-95 (PSD-95) binding capacity of G-protein-coupled receptor 30 (GPR30), an estrogen receptor that can be identified in hippocampal dendritic spines. J Biol Chem 288:6438-6450.

Alberini CM (2009) Transcription factors in long-term memory and synaptic plasticity. Physiol Rev 89:121-145.

Allen PB, Hvalby O, Jensen V, Errington ML, Ramsay M, Chaudhry FA, Bliss TV, Storm-Mathisen J, Morris RG, Andersen P, Greengard P (2000) Protein

phosphatase-1 regulation in the induction of long-term potentiation: heterogeneous molecular mechanisms. J Neurosci 20:3537-3543.

Alves SE, McEwen BS, Hayashi S, Korach KS, Pfaff DW, Ogawa S (2000) Estrogen-regulated progestin receptors are found in the midbrain raphe but not hippocampus of estrogen receptor alpha (ER alpha) gene-disrupted mice. J Comp Neurol 427:185-195.

Andrasfalvy BK, Magee JC (2004) Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones. J Physiol 559:543-554.

Arnold AP (2004) Sex chromosomes and brain gender. Nat Rev Neurosci 5:701-708.

Arnold AP (2017) A general theory of sexual differentiation. J Neurosci Res 95:291-300.

Babayan AH, Kramar EA (2013) Rapid effects of oestrogen on synaptic plasticity: interactions with actin and its signalling proteins. J Neuroendocrinol 25:1163-1172.

Balthazart J, Baillien M, Ball GF (2006) Rapid control of brain aromatase activity by glutamatergic inputs. Endocrinology 147:359-366.

Balthazart J, Baillien M, Charlier TD, Cornil CA, Ball GF (2003) Multiple mechanisms control brain aromatase activity at the genomic and non-genomic level. J Steroid Biochem Mol Biol 86:367-379.

Bangasser DA, Curtis A, Reyes BA, Bethea TT, Parastatidis I, Ischiropoulos H, Van Bockstaele EJ, Valentino RJ (2010) Sex differences in corticotropin-releasing factor receptor signaling and trafficking: potential role in female vulnerability to stress-related psychopathology. Mol Psychiatry 15:877, 896-904.

Bangasser DA, Dong H, Carroll J, Plona Z, Ding H, Rodriguez L, McKennan C, Csernansky JG, Seeholzer SH, Valentino RJ (2017) Corticotropin-releasing factor overexpression gives rise to sex differences in Alzheimer's disease-related signaling. Mol Psychiatry 22:1126-1133.

Banke TG, Bowie D, Lee H, Huganir RL, Schousboe A, Traynelis SF (2000) Control of GluR1 AMPA receptor function by cAMP-dependent protein kinase. J Neurosci 20:89-102.

Barcomb K, Hell JW, Benke TA, Bayer KU (2016) The CaMKII/GluN2B Protein Interaction Maintains Synaptic Strength. J Biol Chem 291:16082-16089.

Baulieu EE (1991) Neurosteroids: a new function in the brain. Biol Cell 71:3-10.

Bayer J, Rune G, Schultz H, Tobia MJ, Mebes I, Katzler O, Sommer T (2015) The effect of estrogen synthesis inhibition on hippocampal memory. Psychoneuroendocrinology 56:213-225.

Bean BP (1989) Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. Nature 340:153-156.

Beery AK, Zucker I (2011) Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev 35:565-572.

Ben-Ari Y, Aniksztejn L, Bregestovski P (1992) Protein kinase C modulation of NMDA currents: an important link for LTP induction. Trends Neurosci 15:333-339.

Benke T, Traynelis SF (2019) AMPA-Type Glutamate Receptor Conductance Changes and Plasticity: Still a Lot of Noise. Neurochem Res 44:539-548.

Benke TA, Luthi A, Isaac JT, Collingridge GL (1998) Modulation of AMPA receptor unitary conductance by synaptic activity. Nature 393:793-797.

Berlucchi G, Buchtel HA (2009) Neuronal plasticity: historical roots and evolution of meaning. Exp Brain Res 192:307-319.

Bi GQ, Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. J Neurosci 18:10464-10472.

Bi R, Broutman G, Foy MR, Thompson RF, Baudry M (2000) The tyrosine kinase and mitogen-activated protein kinase pathways mediate multiple effects of estrogen in hippocampus. Proc Natl Acad Sci U S A 97:3602-3607.

Bliss TV, Lomo T (1973) Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J Physiol 232:331-356.

Blitzer RD, Connor JH, Brown GP, Wong T, Shenolikar S, Iyengar R, Landau EM (1998) Gating of CaMKII by cAMP-regulated protein phosphatase activity during LTP. Science 280:1940-1942.

Boulware MI, Kordasiewicz H, Mermelstein PG (2007) Caveolin proteins are essential for distinct effects of membrane estrogen receptors in neurons. J Neurosci 27:9941-9950.

Briz V, Zhu G, Wang Y, Liu Y, Avetisyan M, Bi X, Baudry M (2015) Activity-dependent rapid local RhoA synthesis is required for hippocampal synaptic plasticity. J Neurosci 35:2269-2282.

Busquets-Garcia A, Bains J, Marsicano G (2018) CB1 Receptor Signaling in the Brain: Extracting Specificity from Ubiquity. Neuropsychopharmacology 43:4-20.

Cali JJ, Zwaagstra JC, Mons N, Cooper DM, Krupinski J (1994) Type VIII adenylyl cyclase. A Ca2+/calmodulin-stimulated enzyme expressed in discrete regions of rat brain. J Biol Chem 269:12190-12195.

Campbell SL, van Groen T, Kadish I, Smoot LHM, Bolger GB (2017) Altered phosphorylation, electrophysiology, and behavior on attenuation of PDE4B action in hippocampus. BMC Neurosci 18:77.

Carver CM, Reddy DS (2016) Neurosteroid Structure-Activity Relationships for Functional Activation of Extrasynaptic deltaGABA(A) Receptors. J Pharmacol Exp Ther 357:188-204.

Charlier TD, Cornil CA, Balthazart J (2013) Rapid modulation of aromatase activity in the vertebrate brain. J Exp Neurosci 7:31-37.

Chen L, Chetkovich DM, Petralia RS, Sweeney NT, Kawasaki Y, Wenthold RJ, Bredt DS, Nicoll RA (2000) Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408:936-943.

Choi YB, Li HL, Kassabov SR, Jin I, Puthanveettil SV, Karl KA, Lu Y, Kim JH, Bailey CH, Kandel ER (2011) Neurexin-neuroligin transsynaptic interaction mediates learning-related synaptic remodeling and long-term facilitation in aplysia. Neuron 70:468-481.

Clements JD, Lester RA, Tong G, Jahr CE, Westbrook GL (1992) The time course of glutamate in the synaptic cleft. Science 258:1498-1501.

Colgan LA, Yasuda R (2014) Plasticity of dendritic spines: subcompartmentalization of signaling. Annu Rev Physiol 76:365-385.

Collingridge GL, Kehl SJ, McLennan H (1983) The antagonism of amino acid-induced excitations of rat hippocampal CA1 neurones in vitro. J Physiol 334:19-31.

Colmers WF, Lukowiak K, Pittman QJ (1987) Presynaptic action of neuropeptide Y in area CA1 of the rat hippocampal slice. J Physiol 383:285-299.

Cunha RA (2001) Adenosine as a neuromodulator and as a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int 38:107-125.

De Vries GJ (2004) Minireview: Sex differences in adult and developing brains: compensation, compensation, compensation. Endocrinology 145:1063-1068.

Derkach V, Barria A, Soderling TR (1999) Ca2+/calmodulin-kinase II enhances channel conductance of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. Proc Natl Acad Sci U S A 96:3269-3274.

Dominguez R, Micevych P (2010) Estradiol rapidly regulates membrane estrogen receptor alpha levels in hypothalamic neurons. J Neurosci 30:12589-12596.

Dragunow M, Abraham WC, Goulding M, Mason SE, Robertson HA, Faull RL (1989) Long-term potentiation and the induction of c-fos mRNA and proteins in the dentate gyrus of unanesthetized rats. Neurosci Lett 101:274-280.

Dudel J (1965) Facilitatory Effects of 5-Hydroxy-Tryptamine on the Crayfish Neuromuscular Junction. Naunyn Schmiedebergs Arch Exp Pathol Pharmakol 249:515-528.

Dunwiddie T, Lynch G (1978) Long-term potentiation and depression of synaptic responses in the rat hippocampus: localization and frequency dependency. J Physiol 276:353-367.

English JD, Sweatt JD (1997) A requirement for the mitogen-activated protein kinase cascade in hippocampal long term potentiation. J Biol Chem 272:19103-19106.

Fatt P, Katz B (1952) Spontaneous subthreshold activity at motor nerve endings. J Physiol 117:109-128.

Fiala JC, Feinberg M, Popov V, Harris KM (1998) Synaptogenesis via dendritic filopodia in developing hippocampal area CA1. J Neurosci 18:8900-8911.

Filardo E, Quinn J, Pang Y, Graeber C, Shaw S, Dong J, Thomas P (2007) Activation of the novel estrogen receptor G protein-coupled receptor 30 (GPR30) at the plasma membrane. Endocrinology 148:3236-3245.

Fitzpatrick SL, Richards JS (1991) Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. Endocrinology 129:1452-1462.

Fitzpatrick SL, Carlone DL, Robker RL, Richards JS (1997) Expression of aromatase in the ovary: down-regulation of mRNA by the ovulatory luteinizing hormone surge. Steroids 62:197-206.

Fortin DA, Davare MA, Srivastava T, Brady JD, Nygaard S, Derkach VA, Soderling TR (2010) Long-term potentiation-dependent spine enlargement requires synaptic Ca2+-permeable AMPA receptors recruited by CaM-kinase I. J Neurosci 30:11565-11575.

Frey U, Huang YY, Kandel ER (1993) Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. Science 260:1661-1664.

Fujii S, Matsumoto M, Igarashi K, Kato H, Mikoshiba K (2000) Synaptic plasticity in hippocampal CA1 neurons of mice lacking type 1 inositol-1,4,5-trisphosphate receptors. Learn Mem 7:312-320.

Futatsugi A, Kato K, Ogura H, Li ST, Nagata E, Kuwajima G, Tanaka K, Itohara S, Mikoshiba K (1999) Facilitation of NMDAR-independent LTP and spatial learning in mutant mice lacking ryanodine receptor type 3. Neuron 24:701-713.

Gonzalez-Forero D, Montero F, Garcia-Morales V, Dominguez G, Gomez-Perez L, Garcia-Verdugo JM, Moreno-Lopez B (2012) Endogenous Rho-kinase signaling maintains synaptic strength by stabilizing the size of the readily releasable pool of synaptic vesicles. J Neurosci 32:68-84.

Gorski RA, Gordon JH, Shryne JE, Southam AM (1978) Evidence for a morphological sex difference within the medial preoptic area of the rat brain. Brain Res 148:333-346.

Gray EE, Fink AE, Sarinana J, Vissel B, O'Dell TJ (2007) Long-term potentiation in the hippocampal CA1 region does not require insertion and activation of GluR2-lacking AMPA receptors. J Neurophysiol 98:2488-2492.

Grover LM, Kim E, Cooke JD, Holmes WR (2009) LTP in hippocampal area CA1 is induced by burst stimulation over a broad frequency range centered around delta. Learn Mem 16:69-81.

Gu Q, Moss RL (1996) 17 beta-Estradiol potentiates kainate-induced currents via activation of the cAMP cascade. J Neurosci 16:3620-3629.

Gu Q, Moss RL (1998) Novel mechanism for non-genomic action of 17 beta-oestradiol on kainate-induced currents in isolated rat CA1 hippocampal neurones. J Physiol 506 ( Pt 3):745-754.

Gu Y, Stornetta RL (2007) Synaptic plasticity, AMPA-R trafficking, and Ras-MAPK signaling. Acta Pharmacol Sin 28:928-936.

Gulledge AT, Kawaguchi Y (2007) Phasic cholinergic signaling in the hippocampus: functional homology with the neocortex? Hippocampus 17:327-332.

Haage D, Johansson S (1999) Neurosteroid modulation of synaptic and GABA-evoked currents in neurons from the rat medial preoptic nucleus. J Neurophysiol 82:143-151.

Haage D, Druzin M, Johansson S (2002) Allopregnanolone modulates spontaneous GABA release via presynaptic Cl- permeability in rat preoptic nerve terminals. Brain Res 958:405-413.

Hamid J, Nelson D, Spaetgens R, Dubel SJ, Snutch TP, Zamponi GW (1999) Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. J Biol Chem 274:6195-6202.

Hansel C (2019) Deregulation of synaptic plasticity in autism. Neurosci Lett 688:58-61.

Hardingham N, Dachtler J, Fox K (2013) The role of nitric oxide in pre-synaptic plasticity and homeostasis. Front Cell Neurosci 7:190.

Hartveit E, Veruki ML (2007) Studying properties of neurotransmitter receptors by non-stationary noise analysis of spontaneous postsynaptic currents and agonist-evoked responses in outside-out patches. Nat Protoc 2:434-448.

Harvey J, Collingridge GL (1992) Thapsigargin blocks the induction of long-term potentiation in rat hippocampal slices. Neurosci Lett 139:197-200.

Hasegawa Y, Hojo Y, Kojima H, Ikeda M, Hotta K, Sato R, Ooishi Y, Yoshiya M, Chung BC, Yamazaki T, Kawato S (2015) Estradiol rapidly modulates synaptic plasticity of hippocampal neurons: Involvement of kinase networks. Brain Res 1621:147-161.

Hata Y, Davletov B, Petrenko AG, Jahn R, Sudhof TC (1993) Interaction of synaptotagmin with the cytoplasmic domains of neurexins. Neuron 10:307-315.

He K, Song L, Cummings LW, Goldman J, Huganir RL, Lee HK (2009) Stabilization of Ca2+-permeable AMPA receptors at perisynaptic sites by GluR1-S845 phosphorylation. Proc Natl Acad Sci U S A 106:20033-20038.

Herlitze S, Garcia DE, Mackie K, Hille B, Scheuer T, Catterall WA (1996) Modulation of Ca2+ channels by G-protein beta gamma subunits. Nature 380:258-262.

Hille B (1994) Modulation of ion-channel function by G-protein-coupled receptors. Trends Neurosci 17:531-536.

Hojo Y, Hattori TA, Enami T, Furukawa A, Suzuki K, Ishii HT, Mukai H, Morrison JH, Janssen WG, Kominami S, Harada N, Kimoto T, Kawato S (2004) Adult male rat hippocampus synthesizes estradiol from pregnenolone by cytochromes

P45017alpha and P450 aromatase localized in neurons. Proc Natl Acad Sci U S A 101:865-870.

Huang GZ, Woolley CS (2012) Estradiol acutely suppresses inhibition in the hippocampus through a sex-specific endocannabinoid and mGluR-dependent mechanism. Neuron 74:801-808.

Huang YY, Kandel ER (1994) Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. Learn Mem 1:74-82.

Irie M, Hata Y, Takeuchi M, Ichtchenko K, Toyoda A, Hirao K, Takai Y, Rosahl TW, Sudhof TC (1997) Binding of neuroligins to PSD-95. Science 277:1511-1515.

Isaac JT, Nicoll RA, Malenka RC (1995) Evidence for silent synapses: implications for the expression of LTP. Neuron 15:427-434.

Itoh Y, Arnold AP (2015) Are females more variable than males in gene expression? Meta-analysis of microarray datasets. Biol Sex Differ 6:18.

Jain A, Huang GZ, Woolley CS (2019) Latent Sex Differences in Molecular Signaling That Underlies Excitatory Synaptic Potentiation in the Hippocampus. J Neurosci 39:1552-1565.

Jiang M, Spicher K, Boulay G, Wang Y, Birnbaumer L (2001) Most central nervous system D2 dopamine receptors are coupled to their effectors by Go. Proc Natl Acad Sci U S A 98:3577-3582.

Kato HK, Kassai H, Watabe AM, Aiba A, Manabe T (2012) Functional coupling of the metabotropic glutamate receptor, InsP3 receptor and L-type Ca2+ channel in mouse CA1 pyramidal cells. J Physiol 590:3019-3034.

Kato J, Yamada-Mouri N, Hirata S (1997) Structure of aromatase mRNA in the rat brain. J Steroid Biochem Mol Biol 61:381-385.

Koleske AJ (2013) Molecular mechanisms of dendrite stability. Nat Rev Neurosci 14:536-550.

Kopec CD, Li B, Wei W, Boehm J, Malinow R (2006) Glutamate receptor exocytosis and spine enlargement during chemically induced long-term potentiation. J Neurosci 26:2000-2009.

Kosaka T, Kosaka K, Tateishi K, Hamaoka Y, Yanaihara N, Wu JY, Hama K (1985) GABAergic neurons containing CCK-8-like and/or VIP-like immunoreactivities in the rat hippocampus and dentate gyrus. J Comp Neurol 239:420-430.

Kramar EA, Chen LY, Brandon NJ, Rex CS, Liu F, Gall CM, Lynch G (2009) Cytoskeletal changes underlie estrogen's acute effects on synaptic transmission and plasticity. J Neurosci 29:12982-12993.

Kretz O, Fester L, Wehrenberg U, Zhou L, Brauckmann S, Zhao S, Prange-Kiel J, Naumann T, Jarry H, Frotscher M, Rune GM (2004) Hippocampal synapses depend on hippocampal estrogen synthesis. J Neurosci 24:5913-5921.

Krezel W, Dupont S, Krust A, Chambon P, Chapman PF (2001) Increased anxiety and synaptic plasticity in estrogen receptor beta -deficient mice. Proc Natl Acad Sci U S A 98:12278-12282.

Kristensen AS, Jenkins MA, Banke TG, Schousboe A, Makino Y, Johnson RC, Huganir R, Traynelis SF (2011) Mechanism of Ca2+/calmodulin-dependent kinase II regulation of AMPA receptor gating. Nat Neurosci 14:727-735.

Kullmann DM, Perkel DJ, Manabe T, Nicoll RA (1992) Ca2+ entry via postsynaptic voltage-sensitive Ca2+ channels can transiently potentiate excitatory synaptic transmission in the hippocampus. Neuron 9:1175-1183.

Kumar A, Foster TC (2002) 17beta-estradiol benzoate decreases the AHP amplitude in CA1 pyramidal neurons. J Neurophysiol 88:621-626.

Kumar A, Bean LA, Rani A, Jackson T, Foster TC (2015) Contribution of estrogen receptor subtypes, ERalpha, ERbeta, and GPER1 in rapid estradiol-mediated enhancement of hippocampal synaptic transmission in mice. Hippocampus 25:1556-1566.

Kuno M (1964) Quantal Components of Excitatory Synaptic Potentials in Spinal Motoneurones. J Physiol 175:81-99.

Labonte B et al. (2017) Sex-specific transcriptional signatures in human depression. Nat Med 23:1102-1111.

Lebesgue D, Chevaleyre V, Zukin RS, Etgen AM (2009) Estradiol rescues neurons from global ischemia-induced cell death: multiple cellular pathways of neuroprotection. Steroids 74:555-561.

Lebesgue D, Traub M, De Butte-Smith M, Chen C, Zukin RS, Kelly MJ, Etgen AM (2010) Acute administration of non-classical estrogen receptor agonists

attenuates ischemia-induced hippocampal neuron loss in middle-aged female rats. PLoS One 5:e8642.

Lee SJ, Escobedo-Lozoya Y, Szatmari EM, Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. Nature 458:299-304.

Lee SJ, Campomanes CR, Sikat PT, Greenfield AT, Allen PB, McEwen BS (2004) Estrogen induces phosphorylation of cyclic AMP response element binding (pCREB) in primary hippocampal cells in a time-dependent manner. Neuroscience 124:549-560.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 96:3239-3244.

Liao GY, Wagner DA, Hsu MH, Leonard JP (2001) Evidence for direct protein kinase-C mediated modulation of N-methyl-D-aspartate receptor current. Mol Pharmacol 59:960-964.

Lisman J, Yasuda R, Raghavachari S (2012) Mechanisms of CaMKII action in long-term potentiation. Nat Rev Neurosci 13:169-182.

Lochner A, Moolman JA (2006) The many faces of H89: a review. Cardiovasc Drug Rev 24:261-274.

Lonart G, Schoch S, Kaeser PS, Larkin CJ, Sudhof TC, Linden DJ (2003) Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. Cell 115:49-60.

Lu Q, Pallas DC, Surks HK, Baur WE, Mendelsohn ME, Karas RH (2004) Striatin assembles a membrane signaling complex necessary for rapid, nongenomic activation of endothelial NO synthase by estrogen receptor alpha. Proc Natl Acad Sci U S A 101:17126-17131.

Lu Y, Christian K, Lu B (2008) BDNF: a key regulator for protein synthesis-dependent LTP and long-term memory? Neurobiol Learn Mem 89:312-323.

Lu Y, Allen M, Halt AR, Weisenhaus M, Dallapiazza RF, Hall DD, Usachev YM, McKnight GS, Hell JW (2007) Age-dependent requirement of AKAP150-anchored PKA and GluR2-lacking AMPA receptors in LTP. EMBO J 26:4879-4890.

Lu YM, Roder JC, Davidow J, Salter MW (1998) Src activation in the induction of long-term potentiation in CA1 hippocampal neurons. Science 279:1363-1367.

Luscher C, Malenka RC (2012) NMDA receptor-dependent long-term potentiation and long-term depression (LTP/LTD). Cold Spring Harb Perspect Biol 4.

Lynch G, Larson J, Kelso S, Barrionuevo G, Schottler F (1983) Intracellular injections of EGTA block induction of hippocampal long-term potentiation. Nature 305:719-721.

MacLusky NJ, Walters MJ, Clark AS, Toran-Allerand CD (1994) Aromatase in the cerebral cortex, hippocampus, and mid-brain: ontogeny and developmental implications. Mol Cell Neurosci 5:691-698.

Magee JC, Avery RB, Christie BR, Johnston D (1996) Dihydropyridine-sensitive, voltage-gated Ca2+ channels contribute to the resting intracellular Ca2+

concentration of hippocampal CA1 pyramidal neurons. J Neurophysiol 76:3460-3470.

Malamas MS, Manas ES, McDevitt RE, Gunawan I, Xu ZB, Collini MD, Miller CP, Dinh T, Henderson RA, Keith JC, Jr., Harris HA (2004) Design and synthesis of aryl diphenolic azoles as potent and selective estrogen receptor-beta ligands. J Med Chem 47:5021-5040.

Malenka RC (1994) Synaptic plasticity in the hippocampus: LTP and LTD. Cell 78:535-538.

Malinow R, Tsien RW (1990) Presynaptic enhancement shown by whole-cell recordings of long-term potentiation in hippocampal slices. Nature 346:177-180.

Malinow R, Schulman H, Tsien RW (1989) Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science 245:862-866.

Mannaioni G, Marino MJ, Valenti O, Traynelis SF, Conn PJ (2001) Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J Neurosci 21:5925-5934.

Marder E (2012) Neuromodulation of neuronal circuits: back to the future. Neuron 76:1-11.

Marinissen MJ, Gutkind JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 22:368-376.

Markram H, Lubke J, Frotscher M, Sakmann B (1997) Regulation of synaptic efficacy by coincidence of postsynaptic APs and EPSPs. Science 275:213-215.

Marsicano G, Lutz B (2006) Neuromodulatory functions of the endocannabinoid system. J Endocrinol Invest 29:27-46.

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H (2004) Structural basis of long-term potentiation in single dendritic spines. Nature 429:761-766.

Matsuzaki M, Ellis-Davies GC, Nemoto T, Miyashita Y, Iino M, Kasai H (2001) Dendritic spine geometry is critical for AMPA receptor expression in hippocampal CA1 pyramidal neurons. Nat Neurosci 4:1086-1092.

Mayer ML, Westbrook GL (1984) Mixed-agonist action of excitatory amino acids on mouse spinal cord neurones under voltage clamp. J Physiol 354:29-53.

McCarthy MM (2009) The two faces of estradiol: effects on the developing brain. Neuroscientist 15:599-610.

McCarthy MM, Arnold AP, Ball GF, Blaustein JD, De Vries GJ (2012) Sex differences in the brain: the not so inconvenient truth. J Neurosci 32:2241-2247.

McEwen BS, Akama KT, Spencer-Segal JL, Milner TA, Waters EM (2012) Estrogen effects on the brain: actions beyond the hypothalamus via novel mechanisms. Behav Neurosci 126:4-16.

Megias M, Emri Z, Freund TF, Gulyas AI (2001) Total number and distribution of inhibitory and excitatory synapses on hippocampal CA1 pyramidal cells. Neuroscience 102:527-540.

Mendoza-Garces L, Mendoza-Rodriguez CA, Jimenez-Trejo F, Picazo O, Rodriguez MC, Cerbon M (2011) Differential expression of estrogen receptors in two

hippocampal regions during the estrous cycle of the rat. Anat Rec (Hoboken) 294:1913-1919.

Menegon A, Bonanomi D, Albertinazzi C, Lotti F, Ferrari G, Kao HT, Benfenati F, Baldelli P, Valtorta F (2006) Protein kinase A-mediated synapsin I phosphorylation is a central modulator of Ca2+-dependent synaptic activity. J Neurosci 26:11670-11681.

Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA (2001) Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J Med Chem 44:4230-4251.

Milner TA, McEwen BS, Hayashi S, Li CJ, Reagan LP, Alves SE (2001) Ultrastructural evidence that hippocampal alpha estrogen receptors are located at extranuclear sites. J Comp Neurol 429:355-371.

Milner TA, Ayoola K, Drake CT, Herrick SP, Tabori NE, McEwen BS, Warrier S, Alves SE (2005) Ultrastructural localization of estrogen receptor beta immunoreactivity in the rat hippocampal formation. J Comp Neurol 491:81-95.

Mitterling KL, Spencer JL, Dziedzic N, Shenoy S, McCarthy K, Waters EM, McEwen BS, Milner TA (2010) Cellular and subcellular localization of estrogen and progestin receptor immunoreactivities in the mouse hippocampus. J Comp Neurol 518:2729-2743.

Mizuno K, Antunes-Martins A, Ris L, Peters M, Godaux E, Giese KP (2007) Calcium/calmodulin kinase kinase beta has a male-specific role in memory formation. Neuroscience 145:393-402.

Momiyama A, Silver RA, Hausser M, Notomi T, Wu Y, Shigemoto R, Cull-Candy SG (2003) The density of AMPA receptors activated by a transmitter quantum at the climbing fibre-Purkinje cell synapse in immature rats. J Physiol 549:75-92.

Morgan SL, Teyler TJ (2001) Electrical stimuli patterned after the theta-rhythm induce multiple forms of LTP. J Neurophysiol 86:1289-1296.

Morris RG (1999) D.O. Hebb: The Organization of Behavior, Wiley: New York; 1949. Brain Res Bull 50:437.

Moser EI, Krobert KA, Moser MB, Morris RG (1998) Impaired spatial learning after saturation of long-term potentiation. Science 281:2038-2042.

Murakoshi H, Wang H, Yasuda R (2011) Local, persistent activation of Rho GTPases during plasticity of single dendritic spines. Nature 472:100-104.

Murphy DD, Segal M (1997) Morphological plasticity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. Proc Natl Acad Sci U S A 94:1482-1487.

Nair D, Hosy E, Petersen JD, Constals A, Giannone G, Choquet D, Sibarita JB (2013) Super-resolution imaging reveals that AMPA receptors inside synapses are dynamically organized in nanodomains regulated by PSD95. J Neurosci 33:13204-13224.

Nakahata Y, Yasuda R (2018) Plasticity of Spine Structure: Local Signaling, Translation and Cytoskeletal Reorganization. Front Synaptic Neurosci 10:29.

Nanou E, Catterall WA (2018) Calcium Channels, Synaptic Plasticity, and Neuropsychiatric Disease. Neuron 98:466-481.

Nishida Y, Yoshioka M, St-Amand J (2005) Sexually dimorphic gene expression in the hypothalamus, pituitary gland, and cortex. Genomics 85:679-687.

Niswender CM, Conn PJ (2010) Metabotropic glutamate receptors: physiology, pharmacology, and disease. Annu Rev Pharmacol Toxicol 50:295-322.

Nottebohm F, Arnold AP (1976) Sexual dimorphism in vocal control areas of the songbird brain. Science 194:211-213.

Oberlander JG, Woolley CS (2016) 17beta-Estradiol Acutely Potentiates Glutamatergic Synaptic Transmission in the Hippocampus through Distinct Mechanisms in Males and Females. J Neurosci 36:2677-2690.

Oh MC, Derkach VA (2005) Dominant role of the GluR2 subunit in regulation of AMPA receptors by CaMKII. Nat Neurosci 8:853-854.

Opazo P, Watabe AM, Grant SG, O'Dell TJ (2003) Phosphatidylinositol 3-kinase regulates the induction of long-term potentiation through extracellular signal-related kinase-independent mechanisms. J Neurosci 23:3679-3688.

Opazo P, Labrecque S, Tigaret CM, Frouin A, Wiseman PW, De Koninck P, Choquet D (2010) CaMKII triggers the diffusional trapping of surface AMPARs through phosphorylation of stargazin. Neuron 67:239-252.

Otmakhov N, Griffith LC, Lisman JE (1997) Postsynaptic inhibitors of calcium/calmodulin-dependent protein kinase type II block induction but not maintenance of pairing-induced long-term potentiation. J Neurosci 17:5357-5365.

Otmakhova NA, Lisman JE (1999) Dopamine selectively inhibits the direct cortical pathway to the CA1 hippocampal region. J Neurosci 19:1437-1445.

Otmakhova NA, Otmakhov N, Mortenson LH, Lisman JE (2000) Inhibition of the cAMP pathway decreases early long-term potentiation at CA1 hippocampal synapses. J Neurosci 20:4446-4451.

Oyola MG, Portillo W, Reyna A, Foradori CD, Kudwa A, Hinds L, Handa RJ, Mani SK (2012) Anxiolytic effects and neuroanatomical targets of estrogen receptor-beta (ERbeta) activation by a selective ERbeta agonist in female mice. Endocrinology 153:837-846.

Park P, Volianskis A, Sanderson TM, Bortolotto ZA, Jane DE, Zhuo M, Kaang BK, Collingridge GL (2014) NMDA receptor-dependent long-term potentiation comprises a family of temporally overlapping forms of synaptic plasticity that are induced by different patterns of stimulation. Philos Trans R Soc Lond B Biol Sci 369:20130131.

Park P, Sanderson TM, Amici M, Choi SL, Bortolotto ZA, Zhuo M, Kaang BK, Collingridge GL (2016) Calcium-Permeable AMPA Receptors Mediate the Induction of the Protein Kinase A-Dependent Component of Long-Term Potentiation in the Hippocampus. J Neurosci 36:622-631.

Park-Chung M, Malayev A, Purdy RH, Gibbs TT, Farb DH (1999) Sulfated and unsulfated steroids modulate gamma-aminobutyric acidA receptor function through distinct sites. Brain Res 830:72-87.

Park-Chung M, Wu FS, Purdy RH, Malayev AA, Gibbs TT, Farb DH (1997) Distinct sites for inverse modulation of N-methyl-D-aspartate receptors by sulfated steroids. Mol Pharmacol 52:1113-1123.

Parks RJ, Ray G, Bienvenu LA, Rose RA, Howlett SE (2014) Sex differences in SR Ca(2+) release in murine ventricular myocytes are regulated by the cAMP/PKA pathway. J Mol Cell Cardiol 75:162-173.

Passani MB, Pugliese AM, Azzurrini M, Corradetti R (1994) Effects of DAU 6215, a novel 5-hydroxytryptamine3 (5-HT3) antagonist on electrophysiological properties of the rat hippocampus. Br J Pharmacol 112:695-703.

Patterson MA, Szatmari EM, Yasuda R (2010) AMPA receptors are exocytosed in stimulated spines and adjacent dendrites in a Ras-ERK-dependent manner during long-term potentiation. Proc Natl Acad Sci U S A 107:15951-15956.

Pavlov PI (2010) Conditioned reflexes: An investigation of the physiological activity of the cerebral cortex. Ann Neurosci 17:136-141.

Perrot-Sinal TS, Kostenuik MA, Ossenkopp KP, Kavaliers M (1996) Sex differences in performance in the Morris water maze and the effects of initial nonstationary hidden platform training. Behav Neurosci 110:1309-1320.

Phoenix CH, Goy RW, Gerall AA, Young WC (1959) Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig. Endocrinology 65:369-382.

Picconi B, Piccoli G, Calabresi P (2012) Synaptic dysfunction in Parkinson's disease. Adv Exp Med Biol 970:553-572.

Pigott BM, Garthwaite J (2016) Nitric Oxide Is Required for L-Type Ca(2+) Channel-Dependent Long-Term Potentiation in the Hippocampus. Front Synaptic Neurosci 8:17.

Plant K, Pelkey KA, Bortolotto ZA, Morita D, Terashima A, McBain CJ, Collingridge GL, Isaac JT (2006) Transient incorporation of native GluR2-lacking AMPA receptors during hippocampal long-term potentiation. Nat Neurosci 9:602-604.

Poncer JC, Esteban JA, Malinow R (2002) Multiple mechanisms for the potentiation of AMPA receptor-mediated transmission by alpha-Ca2+/calmodulin-dependent protein kinase II. J Neurosci 22:4406-4411.

Prange-Kiel J, Wehrenberg U, Jarry H, Rune GM (2003) Para/autocrine regulation of estrogen receptors in hippocampal neurons. Hippocampus 13:226-234.

Prossnitz ER, Maggiolini M (2009) Mechanisms of estrogen signaling and gene expression via GPR30. Mol Cell Endocrinol 308:32-38.

Qi X, Zhang K, Xu T, Yamaki VN, Wei Z, Huang M, Rose GM, Cai X (2016) Sex Differences in Long-Term Potentiation at Temporoammonic-CA1 Synapses: Potential Implications for Memory Consolidation. PLoS One 11:e0165891.

Qin Y, Zhu Y, Baumgart JP, Stornetta RL, Seidenman K, Mack V, van Aelst L, Zhu JJ (2005) State-dependent Ras signaling and AMPA receptor trafficking. Genes Dev 19:2000-2015.

Radzicki D, Yau HJ, Pollema-Mays SL, Mlsna L, Cho K, Koh S, Martina M (2013) Temperature-sensitive Cav1.2 calcium channels support intrinsic firing of pyramidal neurons and provide a target for the treatment of febrile seizures. J Neurosci 33:9920-9931.

Ratner MH, Kumaresan V, Farb DH (2019) Neurosteroid Actions in Memory and Neurologic/Neuropsychiatric Disorders. Front Endocrinol (Lausanne) 10:169.

Raymond LA (2017) Striatal synaptic dysfunction and altered calcium regulation in Huntington disease. Biochem Biophys Res Commun 483:1051-1062.

Razandi M, Pedram A, Greene GL, Levin ER (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. Mol Endocrinol 13:307-319.

Reddy DS (2003) Is there a physiological role for the neurosteroid THDOC in stress-sensitive conditions? Trends Pharmacol Sci 24:103-106.

Reddy DS (2008) Mass spectrometric assay and physiological-pharmacological activity of androgenic neurosteroids. Neurochem Int 52:541-553.

Reddy DS, Jian K (2010) The testosterone-derived neurosteroid androstanediol is a positive allosteric modulator of GABAA receptors. J Pharmacol Exp Ther 334:1031-1041.

Reinius B, Saetre P, Leonard JA, Blekhman R, Merino-Martinez R, Gilad Y, Jazin E (2008) An evolutionarily conserved sexual signature in the primate brain. PLoS Genet 4:e1000100.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19:4337-4348.

Roselli CE, Horton LE, Resko JA (1985) Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. Endocrinology 117:2471-2477.

Rosenfield RL, Perovic N, Devine N (2000) Optimizing estrogen replacement in adolescents with Turner syndrome. Ann N Y Acad Sci 900:213-214.

Rui M, Qian J, Liu L, Cai Y, Lv H, Han J, Jia Z, Xie W (2017) The neuronal protein Neurexin directly interacts with the Scribble-Pix complex to stimulate F-actin assembly for synaptic vesicle clustering. J Biol Chem 292:14334-14348.

Sacktor TC, Hell JW (2017) The genetics of PKMzeta and memory maintenance. Sci Signal 10.

Sandstrom NJ, Williams CL (2001) Memory retention is modulated by acute estradiol and progesterone replacement. Behav Neurosci 115:384-393.

Saneyoshi T, Fortin DA, Soderling TR (2010) Regulation of spine and synapse formation by activity-dependent intracellular signaling pathways. Curr Opin Neurobiol 20:108-115.

Sanhueza M, Fernandez-Villalobos G, Stein IS, Kasumova G, Zhang P, Bayer KU, Otmakhov N, Hell JW, Lisman J (2011) Role of the CaMKII/NMDA receptor complex in the maintenance of synaptic strength. J Neurosci 31:9170-9178.

Sato SM, Woolley CS (2016) Acute inhibition of neurosteroid estrogen synthesis suppresses status epilepticus in an animal model. Elife 5.

Sattar Y, Wilson J, Khan AM, Adnan M, Azzopardi Larios D, Shrestha S, Rahman Q, Mansuri Z, Hassan A, Patel NB, Tariq N, Latchana S, Lopez Pantoja SC, Vargas S, Shaikh NA, Syed F, Mittal D, Rumesa F (2018) A Review of the Mechanism of Antagonism of N-methyl-D-aspartate Receptor by Ketamine in Treatment-resistant Depression. Cureus 10:e2652.

Scoville WB, Milner B (1957) Loss of recent memory after bilateral hippocampal lesions. J Neurol Neurosurg Psychiatry 20:11-21.

Segal M, Korkotian E (2014) Endoplasmic reticulum calcium stores in dendritic spines. Front Neuroanat 8:64.

Selkoe DJ (2002) Alzheimer's disease is a synaptic failure. Science 298:789-791.

Sharp AH, McPherson PS, Dawson TM, Aoki C, Campbell KP, Snyder SH (1993) Differential immunohistochemical localization of inositol 1,4,5-trisphosphate- and ryanodine-sensitive Ca2+ release channels in rat brain. J Neurosci 13:3051-3063.

Sheldahl LC, Shapiro RA, Bryant DN, Koerner IP, Dorsa DM (2008) Estrogen induces rapid translocation of estrogen receptor beta, but not estrogen receptor alpha, to the neuronal plasma membrane. Neuroscience 153:751-761.

Shors TJ, Chua C, Falduto J (2001) Sex differences and opposite effects of stress on dendritic spine density in the male versus female hippocampus. J Neurosci 21:6292-6297.

Sibley DR, Monsma FJ, Jr. (1992) Molecular biology of dopamine receptors. Trends Pharmacol Sci 13:61-69.

Simerly RB (2002) Wired for reproduction: organization and development of sexually dimorphic circuits in the mammalian forebrain. Annu Rev Neurosci 25:507-536.

Smejkalova T, Woolley CS (2010) Estradiol acutely potentiates hippocampal excitatory synaptic transmission through a presynaptic mechanism. J Neurosci 30:16137-16148.

Sohrabji F, Miranda RC, Toran-Allerand CD (1995) Identification of a putative estrogen response element in the gene encoding brain-derived neurotrophic factor. Proc Natl Acad Sci U S A 92:11110-11114.

Soma M, Kim J, Kato A, Kawato S (2018) Src Kinase Dependent Rapid Non-genomic Modulation of Hippocampal Spinogenesis Induced by Androgen and Estrogen. Front Neurosci 12:282.

Spacek J, Harris KM (1997) Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J Neurosci 17:190-203.

Srivastava DP, Woolfrey KM, Penzes P (2013) Insights into rapid modulation of neuroplasticity by brain estrogens. Pharmacol Rev 65:1318-1350.

Srivastava DP, Woolfrey KM, Jones KA, Shum CY, Lash LL, Swanson GT, Penzes P (2008) Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity. Proc Natl Acad Sci U S A 105:14650-14655.

Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA (2000) Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. J Med Chem 43:4934-4947.

Stocco C (2008) Aromatase expression in the ovary: hormonal and molecular regulation. Steroids 73:473-487.

Stricker C, Cowan AI, Field AC, Redman SJ (1999) Analysis of NMDA-independent long-term potentiation induced at CA3-CA1 synapses in rat hippocampus in vitro. J Physiol 520 Pt 2:513-525.

Sudhof TC (2017) Synaptic Neurexin Complexes: A Molecular Code for the Logic of Neural Circuits. Cell 171:745-769.

Swanson GT, Kamboj SK, Cull-Candy SG (1997) Single-channel properties of recombinant AMPA receptors depend on RNA editing, splice variation, and subunit composition. J Neurosci 17:58-69.

Szego CM, Davis JS (1967) Adenosine 3',5'-monophosphate in rat uterus: acute elevation by estrogen. Proc Natl Acad Sci U S A 58:1711-1718.

Tabatadze N, Smejkalova T, Woolley CS (2013) Distribution and posttranslational modification of synaptic ERalpha in the adult female rat hippocampus. Endocrinology 154:819-830.

Tabatadze N, Sato SM, Woolley CS (2014) Quantitative analysis of long-form aromatase mRNA in the male and female rat brain. PLoS One 9:e100628.

Tabatadze N, Huang G, May RM, Jain A, Woolley CS (2015) Sex Differences in Molecular Signaling at Inhibitory Synapses in the Hippocampus. J Neurosci 35:11252-11265.

Tardin C, Cognet L, Bats C, Lounis B, Choquet D (2003) Direct imaging of lateral movements of AMPA receptors inside synapses. EMBO J 22:4656-4665.

Terasawa E, Timiras PS (1968) Electrical activity during the estrous cycle of the rat: cyclic changes in limbic structures. Endocrinology 83:207-216.

Teyler TJ, Vardaris RM, Lewis D, Rawitch AB (1980) Gonadal steroids: effects on excitability of hippocampal pyramidal cells. Science 209:1017-1018.

Titus DJ, Wilson NM, Freund JE, Carballosa MM, Sikah KE, Furones C, Dietrich WD, Gurney ME, Atkins CM (2016) Chronic Cognitive Dysfunction after Traumatic Brain Injury Is Improved with a Phosphodiesterase 4B Inhibitor. J Neurosci 36:7095-7108.

Tomita S, Adesnik H, Sekiguchi M, Zhang W, Wada K, Howe JR, Nicoll RA, Bredt DS (2005) Stargazin modulates AMPA receptor gating and trafficking by distinct domains. Nature 435:1052-1058.

Traynelis SF, Silver RA, Cull-Candy SG (1993) Estimated conductance of glutamate receptor channels activated during EPSCs at the cerebellar mossy fiber-granule cell synapse. Neuron 11:279-289.

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62:405-496.

Tuscher JJ, Szinte JS, Starrett JR, Krentzel AA, Fortress AM, Remage-Healey L, Frick KM (2016) Inhibition of local estrogen synthesis in the hippocampus impairs hippocampal memory consolidation in ovariectomized female mice. Horm Behav 83:60-67.

Valentino RJ, Van Bockstaele E, Bangasser D (2013) Sex-specific cell signaling: the corticotropin-releasing factor receptor model. Trends Pharmacol Sci 34:437-444.

Vega-Vela NE, Osorio D, Avila-Rodriguez M, Gonzalez J, Garcia-Segura LM, Echeverria V, Barreto GE (2017) L-Type Calcium Channels Modulation by Estradiol. Mol Neurobiol 54:4996-5007.

Vest RS, O'Leary H, Coultrap SJ, Kindy MS, Bayer KU (2010) Effective post-insult neuroprotection by a novel Ca(2+)/ calmodulin-dependent protein kinase II (CaMKII) inhibitor. J Biol Chem 285:20675-20682.

Wagner LE, 2nd, Joseph SK, Yule DI (2008) Regulation of single inositol 1,4,5-trisphosphate receptor channel activity by protein kinase A phosphorylation. J Physiol 586:3577-3596.

Walker VR, Korach KS (2004) Estrogen receptor knockout mice as a model for endocrine research. ILAR J 45:455-461.

Waltereit R, Dammermann B, Wulff P, Scafidi J, Staubli U, Kauselmann G, Bundman M, Kuhl D (2001) Arg3.1/Arc mRNA induction by Ca2+ and cAMP requires protein

kinase A and mitogen-activated protein kinase/extracellular regulated kinase activation. J Neurosci 21:5484-5493.

Wang H, Eriksson H, Sahlin L (2000) Estrogen receptors alpha and beta in the female reproductive tract of the rat during the estrous cycle. Biol Reprod 63:1331-1340.

Wang JH, Kelly PT (1997) Postsynaptic calcineurin activity downregulates synaptic transmission by weakening intracellular Ca2+ signaling mechanisms in hippocampal CA1 neurons. J Neurosci 17:4600-4611.

Wang SJ (2003) A role for Src kinase in the regulation of glutamate release from rat cerebrocortical nerve terminals. Neuroreport 14:1519-1522.

Wang W, Le AA, Hou B, Lauterborn JC, Cox CD, Levin ER, Lynch G, Gall CM (2018) Memory-Related Synaptic Plasticity Is Sexually Dimorphic in Rodent Hippocampus. J Neurosci 38:7935-7951.

Warren SG, Humphreys AG, Juraska JM, Greenough WT (1995) LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. Brain Res 703:26-30.

Warrington NM et al. (2015) The cyclic AMP pathway is a sex-specific modifier of glioma risk in type I neurofibromatosis patients. Cancer Res 75:16-21.

Waters EM, Thompson LI, Patel P, Gonzales AD, Ye HZ, Filardo EJ, Clegg DJ, Gorecka J, Akama KT, McEwen BS, Milner TA (2015) G-protein-coupled estrogen receptor 1 is anatomically positioned to modulate synaptic plasticity in the mouse hippocampus. J Neurosci 35:2384-2397.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF (2006) Learning induces long-term potentiation in the hippocampus. Science 313:1093-1097.

Wong M, Moss RL (1992) Long-term and short-term electrophysiological effects of estrogen on the synaptic properties of hippocampal CA1 neurons. J Neurosci 12:3217-3225.

Wong ST, Athos J, Figueroa XA, Pineda VV, Schaefer ML, Chavkin CC, Muglia LJ, Storm DR (1999) Calcium-stimulated adenylyl cyclase activity is critical for hippocampus-dependent long-term memory and late phase LTP. Neuron 23:787-798.

Woo NH, Abel T, Nguyen PV (2002) Genetic and pharmacological demonstration of a role for cyclic AMP-dependent protein kinase-mediated suppression of protein phosphatases in gating the expression of late LTP. Eur J Neurosci 16:1871-1876.

Wood GE, Shors TJ (1998) Stress facilitates classical conditioning in males, but impairs classical conditioning in females through activational effects of ovarian hormones. Proc Natl Acad Sci U S A 95:4066-4071.

Woolley CS (2007) Acute effects of estrogen on neuronal physiology. Annu Rev Pharmacol Toxicol 47:657-680.

Woolley CS, McEwen BS (1992) Estradiol mediates fluctuation in hippocampal synapse density during the estrous cycle in the adult rat. J Neurosci 12:2549-2554.

Woolley CS, Gould E, McEwen BS (1990) Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res 531:225-231.

Wu J, Rowan MJ, Anwyl R (2006) Long-term potentiation is mediated by multiple kinase cascades involving CaMKII or either PKA or p42/44 MAPK in the adult rat dentate gyrus in vitro. J Neurophysiol 95:3519-3527.

Wu TW, Wang JM, Chen S, Brinton RD (2005) 17Beta-estradiol induced Ca2+ influx via L-type calcium channels activates the Src/ERK/cyclic-AMP response element binding protein signal pathway and BCL-2 expression in rat hippocampal neurons: a potential initiation mechanism for estrogen-induced neuroprotection. Neuroscience 135:59-72.

Yang X, Schadt EE, Wang S, Wang H, Arnold AP, Ingram-Drake L, Drake TA, Lusis AJ (2006) Tissue-specific expression and regulation of sexually dimorphic genes in mice. Genome Res 16:995-1004.

Yasuda H, Barth AL, Stellwagen D, Malenka RC (2003) A developmental switch in the signaling cascades for LTP induction. Nat Neurosci 6:15-16.

Zadran S, Qin Q, Bi X, Zadran H, Kim Y, Foy MR, Thompson R, Baudry M (2009) 17-Beta-estradiol increases neuronal excitability through MAP kinase-induced calpain activation. Proc Natl Acad Sci U S A 106:21936-21941.

Zamponi GW, Snutch TP (2002) Modulating modulation: crosstalk between regulatory pathways of presynaptic calcium channels. Mol Interv 2:476-478.

Zhao L, Brinton RD (2007) Estrogen receptor alpha and beta differentially regulate intracellular Ca(2+) dynamics leading to ERK phosphorylation and estrogen neuroprotection in hippocampal neurons. Brain Res 1172:48-59.

Zhao L, Woody SK, Chhibber A (2015) Estrogen receptor beta in Alzheimer's disease: From mechanisms to therapeutics. Ageing Res Rev 24:178-190.

Zhou Y, Takahashi E, Li W, Halt A, Wiltgen B, Ehninger D, Li GD, Hell JW, Kennedy MB, Silva AJ (2007) Interactions between the NR2B receptor and CaMKII modulate synaptic plasticity and spatial learning. J Neurosci 27:13843-13853.