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Sex-specific mechanisms underlie long-term potentiation at hippocampus-medium spiny neuron synapses in the medial shell of the nucleus accumbens

<https://doi.org/10.1523/JNEUROSCI.0100-24.2024>

Received: 15 January 2024

Revised: 16 May 2024

Accepted: 21 May 2024

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This Early Release article has been peer reviewed and accepted, but has not been through the composition and copyediting processes. The final version may differ slightly in style or formatting and will contain links to any extended data.

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1 Title: Sex-specific mechanisms underlie long-term potentiation at hippocampus-medium spiny
2 neuron synapses in the medial shell of the nucleus accumbens

3 Abbreviated title: Sex-specific mechanisms of LTP at Hipp-NAc synapses

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13
14 Number of pages: 36

15 Number of figures: 8

16 Number of words, abstract: 250

17 Number of words, introduction: 627

18 Number of words, discussion: 1500

19
20 Conflict of interest statement: The authors declare no competing financial interests.

21
22 Acknowledgments: We would like to thank Drs. Tracy Bale, Brian Mathur, and Scott Thompson
23 for their helpful suggestions on this project. Special thanks to Tyler Nguyen and Jennifer Pham
24 for their assistance in maintaining the mouse colony. This work was supported by startup funds
25 provided by UMBC and T32GM144876-02.

ABSTRACT

Sex differences have complicated our understanding of the neurobiological basis of many behaviors that are key for survival. As such, continued elucidation of the similarities and differences between sexes is necessary to gain insight into brain function and vulnerability. The connection between the hippocampus (Hipp) and nucleus accumbens (NAc) is a crucial site where modulation of neuronal activity mediates reward-related behavior. Our previous work demonstrated that long-term potentiation (LTP) of Hipp-NAc synapses is rewarding, and mice can establish learned associations between LTP of these synapses and the contextual environment in which LTP occurred. Here, we investigated sex differences in the mechanisms underlying Hipp-NAc LTP using whole-cell electrophysiology and pharmacology. We observed similarities in basal synaptic strength between males and females and found that LTP occurs postsynaptically with similar magnitudes in both sexes. However, key sex differences emerged as LTP in males required NMDA receptors (NMDAR) whereas LTP in females utilized an NMDAR-independent mechanism involving L-type voltage-gated Ca^{2+} channels (VGCC) and estrogen receptor α (ER α). We also uncovered sex-similar features as LTP in both sexes depended on CaMKII activity and occurred independently of dopamine-1 receptor (D1R) activation. Our results have elucidated sex-specific molecular mechanisms for LTP in an integral pathway that mediates reward-related behaviors, emphasizing the importance of considering sex as a variable in mechanistic studies. Continued characterization of sex-specific mechanisms underlying plasticity will offer novel insight into the neurophysiological basis of behavior, with significant implications for understanding how diverse processes mediate behavior and contribute to vulnerability to developing psychiatric disorders.

SIGNIFICANCE STATEMENT

Strengthening of hippocampus-nucleus accumbens (Hipp-NAc) synapses drives reward-related behaviors. Long-term potentiation (LTP) occurs with a similar magnitude in males and females,

and both sexes have a predicted postsynaptic locus of plasticity. Despite these similarities, here we illustrate that sex-specific molecular mechanisms underlie LTP at Hipp-NAc synapses. Given the bidirectional relationship between Hipp-NAc synaptic strength in mediating reward-related behaviors, the use of distinct molecular mechanisms may explain sex differences observed in stress susceptibility or response to rewarding stimuli. Uncovering these latent sex differences offers a deeper understanding of the sex-specific function of this behaviorally-relevant synapse with widespread implications for circuits that underlie learning and reward-related behavior.

INTRODUCTION

Sex differences in reward-related behaviors are prevalent across a variety of species. For instance, humans and rodents show clear sex differences in sensitivity to rewarding stimuli and reward value (Alarcón et al., 2017; Aubry et al., 2022; Becker, 2016; Cullity et al., 2021; Holly et al., 2012; Legget et al., 2018; Sinclair et al., 2017; Warthen et al., 2011; Westbrook et al., 2018; Yararbas et al., 2010). There are also well-documented sex differences in related disorders like major depressive disorder (Brody et al., 2018; Huang et al., 2019; Marcus et al., 2005) and depressive-like behaviors in rodents (Baratta et al., 2019; Burke et al., 2016; Dalla et al., 2008; Goodwill et al., 2019; L.-L. Liu et al., 2019; Pitzer et al., 2022; Song et al., 2018; Trainor et al., 2011; Williams et al., 2020), and males and females tend to respond differently to antidepressant treatment (reviewed in (LeGates et al., 2019)). This may be explained by clear sex differences in depression-related neuronal activity in humans and preclinical models (Bangasser & Cuarenta, 2021; X. Wang et al., 2023). However, the precise neuronal mechanisms underlying sex differences in behavior and circuit function remain unknown.

The nucleus accumbens (NAc) is a key node of the reward pathway that responds to rewarding stimuli (Richter et al., 2020), integrates information from various sources to mediate goal-directed behavior (Francis & Lobo, 2017; Gruber et al., 2009), and is altered in preclinical depression models (Drysdale et al., 2017; Wacker et al., 2009). The hippocampus (Hipp) provides

79 crucial excitatory input to the NAc, which influences NAc activity and conveys spatial and
80 contextual information to guide reward-related behavior (Bagot et al., 2015; Belujon & Grace,
81 2008; Britt et al., 2012; Floresco et al., 2001; Gauthier & Tank, 2018; Gill & Grace, 2013; Ito et
82 al., 2008; LeGates et al., 2018; Lind et al., 2023; O'Donnell et al., 1999; Okuyama et al., 2016;
83 Oliva et al., 2016; Sjulson et al., 2018; Trouche et al., 2019; Williams et al., 2020; Y. Zhou et al.,
84 2019). Our previous work revealed that LTP of Hipp-NAc synapses drives reward-related
85 behaviors while exposure to chronic stress reduced Hipp-NAc excitatory synaptic strength,
86 abolished LTP, and produced a concomitant aberration in reward-related behaviors (LeGates et
87 al., 2018). This is supported by data from human subjects showing that functional connectivity of
88 Hipp-striatal pathways is correlated to fluctuations in positive affect due to experiential diversity
89 (Heller et al., 2020). These findings demonstrate a key bidirectional relationship between the
90 strength of Hipp-NAc synapses and reward-related behaviors, highlighting the Hipp-NAc pathway
91 as a crucial component of reward circuitry.

92 Given numerous examples of sex differences in reward behaviors that may be impacted
93 by the Hipp-NAc pathway, we were interested in characterizing the molecular mechanisms
94 underlying Hipp-NAc LTP in male and female mice. We previously found that LTP at Hipp-NAc
95 medium spiny neuron (MSN) synapses in the medial shell of male mice requires NMDARs,
96 postsynaptic Ca^{2+} influx, and CaMKII activity, but occurs independently of D1R activation
97 (LeGates et al., 2018). Here, we performed whole-cell electrophysiology and pharmacology to
98 characterize the mechanisms underlying Hipp-NAc MSN plasticity in females, comparing them to
99 mechanisms used in males. We found that high frequency stimulation (HFS) of hippocampal
100 axons induced LTP of similar magnitude in males and females. While LTP was supported by
101 postsynaptic mechanisms in both sexes, we observed several key sex differences: LTP in males
102 was NMDAR-dependent while LTP in females occurred through an NMDAR-independent
103 mechanism involving L-type VGCCs and $\text{ER}\alpha$ activity. LTP at Hipp-NAc synapses in both sexes
104 required CaMKII activation and occurred independent of D1R activity suggesting important sex

similarities exist as well. Taken together, these data reveal latent sex differences produce similar LTP at Hipp-NAc synapses, which may be a key factor contributing to sex differences in behavior and disorder.

MATERIALS & METHODS

Animals

Adult (8–10-week-old) male and female D1dra-tdTomato or C57BL/6J mice were bred in-house or purchased directly from Jackson Laboratories. Use of D1dra-tdTomato mice allowed us to identify dopamine-1-receptor and putative dopamine-2-receptor-expressing MSNs (D1-MSN and pD2-MSN): D1-MSNs expressed tdTomato while pD2-MSNs were unlabeled. D1dra-tdTomato mice were used for the experiments described in Figures 1 and 6 while C57BL/6J mice were used for the remaining experiments. Mice were housed with same-sex cage mates in a temperature- and humidity-controlled environment under a 12:12 light cycle (lights on at 07:00). We did not track estrous cycle in females. All experiments were performed in accordance with the regulations set forth by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore County.

Mouse Brain Slice Preparation

Acute parasagittal slices (Lateral 0.36-0.72) containing the fornix and nucleus accumbens were prepared for whole-cell patch-clamp electrophysiology. Animals were deeply anesthetized with isoflurane, decapitated, and brains were quickly dissected and submerged in ice-cold, bubbled (carbogen: 95% O₂/5% CO₂) N-methyl-D-glucamine (NMDG) recovery solution containing the following (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 11 glucose, 25 NaHCO₃, 1.2 MgCl₂, and 2.4 CaCl₂, pH=7.3-7.4, osmolarity=300-310 mOsm. Using a vibratome (VT1000S, Leica Microsystems), parasagittal slices (400 µm) were cut in cold, oxygenated NMDG. Slices were transferred to 32-34°C NMDG for 7-12 minutes to recover and were then transferred to room-temperature artificial cerebrospinal fluid (aCSF) containing the following (in mM): 120 NaCl,

3 KCl, 1.0 NaH₂PO₄, 20 glucose, 25 NaHCO₃, 1.5 MgCl₂·7H₂O, and 2.5 CaCl₂, pH=7.3-7.4. Slices were allowed to recover for 1-hour at room-temperature before beginning electrophysiological recordings.

Whole-Cell Recordings

We performed whole-cell patch-clamp recordings using an Axopatch 200B amplifier (Axon Instruments, Molecular Devices) and a Digidata 1550B digitizer (Axon Instruments). Slices were placed in a submersion-type recording chamber and superfused with room-temperature aCSF (flow rate 0.5-1mL/min). Patch pipettes (4-8MΩ) were made from borosilicate glass (World Precision Instruments) using a Sutter Instruments P-97 model puller. Cells were visualized using a 60x water immersion objective (Nikon Eclipse FN-1). D1R-MSNs were identified by the expression of tdTomato while putative D2R-MSNs were cells with a similar morphology that lacked expression of tdTomato.

All recordings were performed in voltage-clamp conditions from MSNs in the NAc medial shell. A bipolar stimulating electrode (FHC) was placed in the fornix to electrically stimulate hippocampal axons and record evoked excitatory postsynaptic currents (EPSC). For local stimulation experiments (Fig 6c,d), a bipolar stimulating electrode (FHC) was placed in the NAc to non-specifically stimulate all inputs to NAc MSNs. For LTP experiments, patch pipettes were filled with a solution containing 130 mM K-gluconate, 5 mM KCl, 2 mM MgCl₆-H₂O, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na₂-GTP, 10 mM Na₂-phosphocreatine, and 1 mM EGTA; pH=7.3-7.4; osmolarity=285-295mOsm. EPSCs were recorded from paired pulses (100ms apart) performed every 10s. A 5-minute baseline EPSC recording was obtained, then high-frequency stimulation (HFS: four trains of 100Hz stimulation for 1s with 15s between trains while holding the cell at -40mV) was used to induce LTP, followed by a 30-minute recording of EPSCs. For experiments determining I-V relationship, the patch pipette solution was composed of 135 mM CsCl, 2 mM MgCl₆-H₂O, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na₂-GTP, 10 mM Na₂-phosphocreatine, 1 mM EGTA, 5 mM QX-314, and 100 μM spermine; pH=7.3-7.4;

osmolarity=285-295mOsm. EPSCs were collected from holding potentials ranging from -80mV to +40mV to create an I-V curve. For all pharmacological experiments, drugs (APV (Tocris, 50uM), NASPM (Tocris, 20uM), nimodipine (Tocris, 3uM), KN-62 (Tocris, 3uM), SCH23390 (Tocris, 3uM), MPP dihydrochloride (Tocris, 3uM)) were superfused over the slice for at least 15 minutes prior to recording. We used the following exclusion criteria to eliminate unhealthy cells and unreliable recordings: 1) We only proceeded with experiments on cells with series resistances $<10\text{M}\Omega$, 2) Cells were excluded if their series resistance changed by $>20\%$ (comparing the resistance at the beginning and end of the experiment), 3) Cells in poor health or poor recording status were excluded (i.e. cell partially or fully sealed up, a decrease in holding current $>100\text{pA}$ that is consistent with the cell dying, an increase in jitter post HFS, and/or an increase in response failure rate to $>50\%$).

Quantification, statistical analysis, and reproducibility

A total of 223 cells were recorded from 194 mice for these experiments. Males and females were kept separate in our analyses. We found no statistically significant difference between D1R- and pD2R-MSNs, so they are plotted together unless otherwise indicated. Both the number of cells and number of mice is reported for each experiment. The sample size (n) per condition represents the number of cells unless otherwise indicated in the figure caption. For LTP experiments, the five-minute baseline and last five minutes of recording were used for statistical comparisons. Two-tailed, paired Wilcoxon tests were used to determine whether a group of cells had a significant increase above baseline, indicating LTP. Pairwise comparisons using the Mann Whitney u-test were used to assess experimental condition differences due to non-normal distributions of data. Significant pairwise comparisons were reported. A p -value of <0.05 was considered statistically significant, where exact p -values can be found in the figure captions. For statistical tests considering both sex and cell-type, a two-way ANOVA was used. All statistical analyses were performed using Graphpad Prism 9/10 software. For box plots, the line in the

middle of the box is plotted at the median. The box extends from the 25th to 75th percentiles. Whiskers represent minimum and maximum. Figures were created with BioRender.com.

RESULTS

HFS induces Hipp-NAc MSN LTP of similar magnitude in males and females

We performed whole-cell patch-clamp electrophysiology while electrically stimulating the fornix to record Hipp-evoked excitatory postsynaptic currents (EPSC) in MSNs in the medial shell of the NAc (Fig. 1a). Slices were taken from D1dra-tdTomato mice, allowing us to distinguish between dopamine-1- and putative dopamine-2-receptor-expressing MSNs (D1-MSN and pD2-MSN) based on expression of tdTomato. In response to high frequency stimulation (HFS), we observed LTP of similar magnitude in male and female mice, with no difference between D1- and pD2-MSNs (Fig. 1). Comparison of paired-pulse ratio (PPR) baseline and post-HFS 25-30min values suggests that LTP involves postsynaptic mechanisms in both male and female mice (Fig. 1c,e). These results indicate that LTP occurs at Hipp-NAc MSN synapses similarly in males and females, with no difference in baseline EPSC amplitude (Fig. 1b), LTP magnitude (Fig. 1c-f), or predicted locus of plasticity mechanisms between the sexes (Fig. 1c,e). Since there was no difference between D1- and pD2-MSNs, cells were pooled for the remainder of the data shown.

NMDAR activity is required for male, but not female, Hipp-NAc MSN LTP

The prediction that both sexes use postsynaptic mechanisms for LTP suggests a rise in postsynaptic Ca^{2+} levels. At Hipp-NAc synapses in male mice, the proximate means of this Ca^{2+} is NMDARs (LeGates et al., 2018). We reproduced this result by repeating the experiment described above in the presence of 50uM 2-Aminophosphonovaleric acid (APV), an NMDAR antagonist (Fig. 2a,b). However, in slices taken from female mice, we found that APV was unable to block LTP (Fig. 2c,d), suggesting that while NMDARs are necessary for LTP at Hipp-NAc synapses in males, they not required for LTP females. We measured AMPA:NMDA ratio at Hipp-

NAC synapses in male and female mice and found no sex difference in this ratio (Fig. 2e), suggesting that there is no difference in the relative strength of these synapses. The ability to collect NMDAR-mediated currents also shows that females have NMDARs present at Hipp-NAC synapses, suggesting that the lack of requirement for NMDARs in female LTP is not explained by a lack of NMDARs in the synapse. These experiments reveal the surprising sex-specific use of an NMDAR-independent pathway for LTP at Hipp-NAC synapses in females.

L-type VGCC is required for Hipp-NAC MSN LTP in females

There are various NMDAR-independent mechanisms that have been shown to underlie postsynaptically-expressed LTP which involve Ca^{2+} -permeable AMPA receptors (CP-AMPA), mobilization of intracellular Ca^{2+} stores, or VGCCs (Alkadhi, 2021; Nanou & Catterall, 2018; Padamsey et al., 2019; Park et al., 2018). To begin to understand which mechanisms may underlie LTP at Hipp-NAC synapses, we modified our LTP induction protocol to deliver HFS while holding the cell at -70mV rather than the depolarized (-40mV) potential we typically use. This modification effectively prevents the activation of any voltage-dependent processes during LTP induction. We found that delivering HFS in the absence of simultaneous depolarization prevented LTP induction (Fig. 3a-c), implicating the involvement of a voltage-dependent means of external Ca^{2+} in LTP at female Hipp-NAC synapses.

From here, we wanted to determine which type of voltage-gated channel was necessary for LTP at female Hipp-NAC synapses. L-type VGCCs have been implicated in postsynaptic forms of LTP in the amygdala and CA1 region of the hippocampus (Huber et al., 1995; Weisskopf et al., 1999) and are expressed postsynaptically within the NAC, allowing for voltage-dependent influx of Ca^{2+} into MSNs. Therefore, we hypothesized that L-type VGCCs mediate LTP at Hipp-NAC synapses in females. We tested this idea by pretreating slices with the L-type VGCC antagonist nimodipine (10uM). Bath application of nimodipine was sufficient to block LTP in female mice (Fig. 3d,e), suggesting that L-type VGCCs are required for LTP at Hipp-NAC synapses in females. In

contrast, inhibition of L-type VGCCs was not sufficient to block LTP in males, although LTP magnitude was reduced in the presence of nimodipine (Fig. 3f,g). Together, with our recordings in the presence of APV, this demonstrates that males and females utilize distinct sources of postsynaptic Ca^{2+} to mediate LTP at Hipp-NAc synapses, where males rely primarily on NMDARs with some contribution from L-type VGCCs while females utilize an NMDAR-independent mechanism that requires L-type VGCCs.

CP-AMPA are not involved in Hipp-NAc MSN LTP

Given the role of CP-AMPA receptors in sex-specific mechanisms of synaptic potentiation in hippocampal CA1 neurons (Jain & Woolley, 2023), their contributions to multiple forms of NAc plasticity and behaviors that occur in response to drug exposure (Carr, 2020; Guire et al., 2008; Mameli et al., 2009; McCutcheon et al., 2011; Park et al., 2021; Terrier et al., 2016; Wolf & Tseng, 2012), and presence at ventral subiculum-NAc synapses (Boxer et al., 2023), we sought to investigate their potential role in LTP at Hipp-NAc synapses in females. CP-AMPA receptors have unique electrophysiological properties and are inwardly rectifying, whereas Ca^{2+} -impermeable AMPARs display a linear current-voltage relationship (Cull-Candy et al., 2006; S. J. Liu & Zukin, 2007). This allows us to electrophysiologically determine the predominant population of AMPARs present at a particular synapse. We found that basal current voltage relationships at Hipp-NAc MSN synapses were linear, demonstrating the absence of CP-AMPA receptors prior to HFS and precluding their involvement in LTP induction (Fig. 4a). Since CP-AMPA receptors can also be involved in LTP through preferential insertion following HFS (Whitehead et al., 2013), we aimed to determine whether they might instead play a role in LTP at Hipp-NAc synapses through this mechanism. We washed in 1-naphthylacetyl spermine (NASPM; 20 μM), a CP-AMPA receptor antagonist, 10-minutes after HFS, but found that NASPM wash-in had no effect on EPSC amplitude (Fig. 4b,c), suggesting that insertion of CP-AMPA receptors does not contribute to LTP at Hipp-NAc MSN synapses in female mice. Altogether, these results rule out the involvement of CP-AMPA receptors in LTP at Hipp-

NAC MSN synapses in females which aligns with our previous observations in males (LeGates et al., 2018).

CaMKII is required for LTP in female mice

Despite males and females differing in their proximate means of postsynaptic Ca^{2+} , we hypothesized that similar, Ca^{2+} -dependent molecular players may be recruited downstream of this Ca^{2+} to mediate LTP. In male mice, the postsynaptic rise in Ca^{2+} initiates activation of CaMKII to cause LTP (LeGates et al., 2018). To determine whether this is consistent for LTP at Hipp-NAC synapses in females, we applied a CaMKII inhibitor (KN-62, 3 μM) before recording from MSNs. We found that blocking CaMKII prevented LTP in female mice (Fig. 5), suggesting that CaMKII activity is required for LTP in both sexes.

Female Hipp-NAC MSN LTP occurs independently of dopamine

Dopamine is a well-known modulator of reward-related behaviors and plays a crucial role in regulating excitatory synapses within the NAC (Speranza et al., 2021). Many characterized forms of LTP at excitatory synapses within the NAC require D1R activity (Du Hoffmann & Nicola, 2014; Floresco et al., 2001; Goto & Grace, 2005; Hernandez et al., 2005; Madadi Asl et al., 2018; Mameli & Lüscher, 2011; Pignatelli & Bonci, 2015; Yu et al., 2022), but at Hipp-NAC synapses, HFS-induced LTP in males is unaffected by dopamine receptor blockade, demonstrating that LTP at these synapses in males occurs independent of dopamine receptor signaling (LeGates et al., 2018). To test whether this was also true at Hipp-NAC synapses in females, we blocked D1R activity with SCH 23390 (3 μM) and found that pretreatment of slices with SCH 23390 had no impact on Hipp-NAC MSN LTP in female mice (Fig. 6a-c). In separate slices, we used local stimulation to elicit EPSCs that were not pathway specific and found that HFS induces LTP that was blocked by pretreatment with SCH 23390 (Fig. 6d-e). Together, these results support previous findings on the importance of D1Rs in excitatory synaptic plasticity broadly in the NAC

and highlight a key distinction at Hipp-NAc synapses where LTP occurs independent of dopamine receptor signaling in male and female mice.

Estrogen receptor activity is required for female Hipp-NAc MSN LTP

Estrogen can alter excitatory synapse function and plasticity (described in detail by Frick et al., 2015; Jain & Woolley, 2023; Oberlander & Woolley, 2017) and can regulate Ca^{2+} influx via L-type VGCCs in the striatum (Mermelstein et al., 1996; Sarkar et al., 2008). Additionally, while the mechanism underlying LTP at hippocampal CA1 is similar in male and females, females have an additional requirement of membrane-localized estrogen receptor- α ($\text{ER}\alpha$) activation (Gall et al., 2023; X. Wang et al., 2018). Within the NAc, $\text{ER}\alpha$ is expressed primarily at the membrane in adult mice (Almey et al., 2022) and has been shown to interact with GPCRs to promote other forms of plasticity (Krentzel & Meitzen, 2018; Tonn Eisinger et al., 2018). Since $\text{ER}\alpha$ is moderately expressed in the NAc (Mitra et al., 2003) and has the potential to alter postsynaptic Ca^{2+} influx, we postulated that $\text{ER}\alpha$ is required for LTP at Hipp-NAc synapses in females. We used bath application of the $\text{ER}\alpha$ antagonist, MPP dihydrochloride (3 μM), to test the involvement of $\text{ER}\alpha$ in LTP at Hipp-NAc synapses. We found that pretreating slices with MPP prevented LTP in female mice but not male mice (Fig. 7), demonstrating the sex-specific requirement of $\text{ER}\alpha$ activation for LTP.

DISCUSSION

Our data reveal key sex-specific and -similar molecular mechanisms underlying LTP at Hipp- NAc synapses (Fig. 8). Males and females displayed LTP of similar magnitude that relies on common mechanisms like postsynaptic Ca^{2+} influx and CaMKII activity. However, key differences emerged when we investigated the proximate means of postsynaptic Ca^{2+} ; NMDARs are required for LTP at Hipp-NAc synapses in males, while L-type VGCCs are required in females. Furthermore, we identified a requirement for $\text{ER}\alpha$ in females that was not observed in males.

Together, our results highlight the discovery of latent sex differences in the molecular mechanisms underlying LTP at Hipp-NAc synapses. Given the important role for these synapses in mediating reward-related behaviors, the identified sex differences have major implications for uncovering the neurobiological basis of sex variation in motivated behaviors and related psychiatric disorders.

Similarities in synaptic strength and LTP magnitude across sex and cell subtype

A wealth of evidence has established clear sex differences in excitatory circuitry throughout the brain (Bangasser & Cuarenta, 2021; Cao et al., 2018; Duarte-Guterman et al., 2015; Johnson et al., 2023; McEwen, 2010; McLaughlin et al., 2009). This includes the NAc core where it is related to sex differences in cocaine-induced behaviors and synaptic plasticity (Catalfio et al., 2023; Forlano & Woolley, 2010; Knouse et al., 2023; Lewitus & Blackwell, 2023; Wissman et al., 2011, 2012). Our investigation of Hipp-NAc shell synapses revealed no differences in AMPA:NMDA ratio, AMPA subunit composition (CP-AMPA versus AMPA), or LTP magnitude across sex or MSN subtype demonstrating similarities between sexes and subtypes in basal synaptic strength and activity-dependent plasticity. We also noted that a small subset of cells across our study displayed HFS-induced long-term depression (LTD). Given its infrequency, we did not investigate this further, but HFS-induced LTD has been observed within the nucleus accumbens (Chergui, 2011; Kombian & Malenka, 1994) and at Hipp-NAc synapses in a rodent model of schizophrenia (Belujon et al., 2014) making it an interesting avenue of future research.

Sex differences in excitatory synaptic plasticity mechanisms

Despite similarities in LTP magnitude and the locus of plasticity, our experiments demonstrate latent sex differences underlie LTP at Hipp-NAc synapses. Hipp-NAc LTP in males requires NMDARs while L-type VGCCs facilitate typical LTP magnitude (Fig. 2&3). In contrast, LTP in females occurs independent of NMDARs and instead relies on L-type VGCC (Fig. 3). This key difference in the type of calcium channel involved is a particularly unique finding as numerous studies have shown that other excitatory synapses onto MSNs primarily use NMDAR-dependent

forms of plasticity (Floresco et al., 2001; Popescu et al., 2007; Thomas & Malenka, 2003; Vega-Villar et al., 2019), and to our knowledge, this is the first description of latent sex differences comprised of NMDAR-dependent and NMDAR-independent mechanisms.

L-type VGCCs and NMDARs, which are both critical in long-lasting synaptic plasticity, are both voltage-dependent channels that facilitate Ca^{2+} influx to bind calmodulin, leading to Ca^{2+} -dependent activation of CaMKII that is required for Hipp-NAc LTP in both sexes (Ataman et al., 2007; Berger & Bartsch, 2014; LeGates et al., 2018; Fig. 5). Despite these similarities, NMDARs and L-type VGCCs are differentially regulated, and their dysregulation is implicated in different behaviors and diseases (Laryushkin et al., 2021; Mielnik et al., 2021; Myers et al., 2019; Ortner & Striessnig, 2015; Sanderson et al., 2022; Q. Zhou & Sheng, 2013). For example, deletion of L-type VGCCs impairs learning and memory in females (Klomp et al., 2022; Zanos et al., 2015). Moreover, *Cacna1c*, which encodes the $\text{Ca}_v1.2$ subunit of the L-type VGCC, is associated with genetic risk for multiple mood disorders (Bigos et al., 2010; Dedic et al., 2018; Jiang et al., 2023; Moon et al., 2018; Sklar et al., 2008) and shows sex-specific interactions influencing depression (Dao et al., 2010). Since Hipp-NAc communication is a key mediator of reward-related behaviors, elucidation of sex-specific mechanisms underlying LTP may offer essential insight into sex differences in behavior and pathophysiology.

Hormones regulate Hipp-NAc synapses

Estrogen and testosterone are important regulators of synaptic transmission and plasticity in both sexes (Barth et al., 2015; Chen et al., 2022; Lu et al., 2019; W. Wang et al., 2016; Williams et al., 2020). We did not track estrous cycle in our experiments but observed variability in LTP magnitude in females, which may suggest a role for the estrous cycle in modulating plasticity at Hipp-NAc synapses. Additionally, the bimodal distribution of our data obtained in the presence of NMDAR antagonism may indicate estrous cycle-dependent differences in LTP mechanisms. To our knowledge, there are no descriptions of shifts in LTP mechanisms across the estrous cycle (eg: NMDA-dependent to -independent), though there is evidence indicating estrous cycle-

dependent changes in hippocampal LTP magnitude as well as expression and posttranslational modifications of key synaptic proteins including NMDARs (Bi et al., 2001; Diao et al., 2007; Good et al., 1999; Iqbal et al., 2020; Smith et al., 2009; Tada et al., 2015; Warren et al., 1995; Waters et al., 2009). Given the behavioral role for Hipp-NAc synapses in mediating learning and motivation, determining how the estrogen impacts plasticity of these synapses will provide key insight into the synaptic basis for sex different and estrous cycle-dependent alterations in behavior.

A key distinction we observed was in the sex-specific requirement of ER α in LTP in females (Fig. 7). This is congruent with observations in the hippocampus demonstrating sex differences in the requirement of estrogen receptors for LTP in CA1 (Vierk et al., 2012; W. Wang et al., 2018) and may stem from differences in expression or function. Membrane-localized ER α (mER α) is prevalent in the hippocampus and NAc where nuclear ER α is less abundant (Almey et al., 2022; Grove-Strawser et al., 2010; Krentzel et al., 2020; Krentzel & Meitzen, 2018; Mitra et al., 2003; Schultz et al., 2009; Stanić et al., 2014; Vasudevan & Pfaff, 2007), and females express higher levels of synapse-localized ER α (W. Wang et al., 2018). mER α influences dendritic structure and synaptic function (Mazid et al., 2023; P. Micevych & Christensen, 2012; W. Wang et al., 2018), and in the NAc, where dendritic spine density is modulated by estradiol, mER α can rapidly modulate mEPSCs (Beeson & Meitzen, 2023; Krentzel et al., 2019; Miller et al., 2023; Peterson et al., 2015; Proaño et al., 2018, 2020; Staffend et al., 2011). mER α can functionally couple with mGluRs (Grove-Strawser et al., 2010; P. E. Micevych & Mermelstein, 2008; Tonn Eisinger et al., 2018) to influence L-type VGCCs (Subbamanda & Bhargava, 2022), supporting the idea that estradiol can have rapid, transcriptionally-independent effects on excitatory synapses. Alternatively, our observed sex-specific effect of ER α antagonism could be due to sex-specific functions of ER α activation, which has been reported in brain regions with no sex differences in ER α expression (Krentzel & Meitzen, 2018; Oberlander & Woolley, 2017). Further

studies are necessary to identify the source of the sex-specific requirement for ER α in LTP at Hipp-NAc synapses.

D1R is not required for LTP at Hipp-NAc synapses

Dopamine is a critical regulator of the reward system and typically an important factor in LTP within the NAc (Floresco et al., 2001; Goto & Grace, 2005; Jay et al., 2004; Madadi Asl et al., 2018; Mameli & Lüscher, 2011; Pignatelli & Bonci, 2015; Speranza et al., 2021; Yu et al., 2022). While D1R is required for LTP at many excitatory synapses in the NAc, our results (Fig. 6) and previous work (LeGates et al., 2018) show that D1R activation is not required for LTP at Hipp-NAc synapses; a finding observed elsewhere in the striatum and in hedonic reward learning behaviors (Berridge & Robinson, 1998; Cannon & Palmiter, 2003; Pennartz et al., 1993). Our findings do not preclude the possibility that dopamine can influence plasticity in this pathway. In fact, others have shown dopamine-dependent modulation of LTP magnitude even when not required for induction (FitzGerald et al., 2015; Otani et al., 2003; Palacios-Filardo & Mellor, 2019). Further examination is required to fully understand dopamine-dependent effects on Hipp-NAc synaptic plasticity.

Implications of latent sex differences in LTP at Hipp-NAc synapses

The modulation of Hipp-NAc synaptic transmission is a critical contributor to reward-related behaviors, and these synapses are altered in response to stress and cocaine (LeGates et al., 2018; Sjulson et al., 2018; Williams et al., 2020). As such, the sex differences in LTP mechanisms at Hipp-NAc synapses holds significant implications for stress- and reward-related behavior and physiology. Recent studies support the idea of sex-specific LTP mechanisms underlying sex differences in spatial learning and memory (Gall et al., 2023; Monfort et al., 2015; Safari et al., 2021; Sneider et al., 2015), so the use of distinct mechanisms for Hipp-NAc LTP may explain some sex-dependent behavioral changes that occur in response to stress and mood disorders (Brancato et al., 2017; Hodes et al., 2015; Huang et al., 2019; Salk et al., 2017; Seney & Sibille, 2014; Wei et al., 2014; Williams et al., 2020). Together, our findings highlight sex-specific

415 mechanisms underlying plasticity in a reward pathway that redefines our knowledge about LTP
416 and offers potential molecular targets for therapeutics to treat conditions linked to aberrant reward
417 processing and stress.

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FIGURES

Figure 1. Both sexes display a similar magnitude of LTP and a predicted postsynaptic locus of plasticity. a) Recording strategy with stimulating electrode placed in the fornix and

recording electrode in the NAc medial shell to record Hipp-evoked EPSCs from MSNs. The shown
 parasagittal section represents a slice from Lateral +0.48. b) Comparison of baseline, non-
 normalized EPSC amplitudes in male and female D1- and pD2-MSNs reveals no difference
 among sex or cell subtype (M D1-MSN $n=7$ cells from 6 mice, M pD2-MSN $n=7$ cells from 7 mice,
 F D1-MSN $n=8$ cells from 7 mice, D2-MSN $n=8$ cells from 8 mice; two-way ANOVA, ns $p=0.5191$).
 c) Hippocampal-evoked EPSC amplitudes and PPR from D1- and pD2-MSNs in males. Data
 represent 1-minute bins (means of all cells in each condition $\pm$ SEM). (Comparison of baseline
 PPR to 25-30min PPR: M D1 $n=7$ cells from 6 mice, $p=0.0781$, two-tailed paired Wilcoxon test; M
 pD2 $n=7$ cells from 7 mice, $p>0.9999$, two-tailed paired Wilcoxon test). d) Summary EPSC data
 from 25-30 min post-HFS revealing similar magnitudes of LTP (M D1-MSN $n=7$ cells from 6 mice,
 $\#p=0.0469$, two-tailed paired Wilcoxon test; M pD2-MSN $n=7$ cells from 7 mice, $\#p=0.0312$, two-
 tailed paired Wilcoxon test; $p=0.3829$, Mann-Whitney U-test). Representative traces with scale
 bars = 40pA/10ms. e) Hippocampal-evoked EPSC amplitudes and PPR from D1- and pD2-MSNs
 in females. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM).
 (Comparison of baseline PPR to 25-30min PPR: F D1 $n=7$ cells from 6 mice, $p=0.5781$, two-tailed
 paired Wilcoxon test; M pD2 $n=8$ cells from 8 mice, $p=0.3125$, two-tailed paired Wilcoxon test). f)
 Summary EPSC data from 25-30 min post-HFS revealing similar magnitudes of LTP (F D1-MSN
 $n=8$ cells from 7 mice, $\#p=0.0078$, two-tailed paired Wilcoxon test; F pD2-MSN $n=8$ cells from 8
 mice, $\#p=0.0156$, two-tailed paired Wilcoxon test; $p=0.9591$, Mann-Whitney U-test). Comparison
 of LTP magnitude between male and female D1- and pD2-MSNs reveal no significant difference
 (two-way ANOVA, ns $p=0.6067$). Representative traces with scale bars = 40pA/10ms.
 *Differences between treatment and control by two-tailed Mann-Whitney U-test. $\#$ Significant
 increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 2. LTP is NMDAR-independent at Hipp-NAc synapses in females. a) Comparison of
 LTP in the presence and absence of NMDAR antagonist, APV, in males. Data represent 1-minute

bins (means of all cells in each condition $\pm$ SEM). b) Summary EPSC data from 25-30 min post-HFS showing abolishment of LTP in APV condition (Ctrl M $n=10$ cells from 9 mice, $^{\#}p=0.0248$, two-tailed paired Wilcoxon test; APV M $n=9$ cells from 8 mice, $p=0.0750$, two-tailed paired Wilcoxon test; $*p=0.0172$, Mann-Whitney U test). Statistical difference is not driven by the control cell with large magnitude LTP (exclusion of this cell results in $*p=0.0315$, Mann-Whitney U-test). Representative trace scale bars = 20pA/10ms. c) Comparison of LTP in the presence and absence of NMDAR antagonist, APV, in female mice. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). d) Summary EPSC data from 25-30 min post-HFS showing similar LTP magnitude in control and APV conditions (Ctrl F $n=6$ cells from 6 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; APV F $n=7$ cells from 5 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; $p=0.4248$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. e) AMPA:NMDA ratio comparison in male and female mice reveals no sex differences in basal Hipp-NAC synaptic properties (Male $n=6$ cells from 4 mice; Female $n=6$ cells from 3 mice; $p=0.7338$, Mann-Whitney U test). Representative trace scale bars = 20pA/50ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 3. L-type VGCCs are required for LTP at Hipp-NAC synapses in females. a) Control (depolarizing cell to -40mV) and experimental (-70mV) HFS protocols. b) Comparison of LTP with control HFS and HFS @ -70mV protocols. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). c) Summary EPSC data from 25-30 min post-HFS showing that HFS while holding the cell at -70mV prevents LTP (Control $n=10$ cells from 9 mice, $^{\#}p=0.0006$, two-tailed paired Wilcoxon test; HFS @ -70mV $n=5$ cells from 5 mice, $p=0.5998$, two-tailed paired Wilcoxon test; $*p=0.0047$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. d) Comparison of LTP in presence and absence of L-type VGCC antagonist, nimodipine (Nim), in female mice. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). e)

Summary EPSC data from 25-30 min post-HFS reveals that Nim prevents LTP (Control $n=7$ cells from 6 mice, $^{\#}p=0.0223$, two-tailed paired Wilcoxon test; Nim $n=6$ cells from 5 mice, $p=0.9624$, two-tailed paired Wilcoxon test; $^*p=0.0221$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. f) Comparison of LTP in presence and absence of L-type VGCC antagonist, nimodipine (Nim), in male mice. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). g) Summary EPSC data from 25-30 min post-HFS reveals that Nim causes a decrease in the magnitude of LTP in males (Control $n=8$ cells from 7 mice, $^{\#}p=0.0078$, two-tailed paired Wilcoxon test; Nim $n=9$ cells from 8 mice, $^{\#}p=0.0195$, two-tailed paired Wilcoxon test; $^*p=0.0206$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 4. CP-AMPA receptors are not present at Hipp-NAC synapses and insertion of CP-AMPA receptors is not required for LTP in females. a) Linear I-V relationship demonstrates AMPARs at Hipp-NAC synapses are Ca^{2+} -impermeable (Male $n=7$ cells from 4 mice; Female $n=9$ cells from 3 mice). b) CP-AMPA receptor antagonist, NASPM, wash-on 10-minutes after HFS. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). c) Summary EPSC data from 25-30 min post-HFS showing that NASPM wash has no effect on LTP (Control $n=6$ cells from 6 mice, $^{\#}p=0.0156$, two-tailed paired Wilcoxon test; NASPM wash $n=6$ cells from 6 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; $p=0.3939$, Mann-Whitney U test). Representative trace scale bars = 40pA/10ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 5. Downstream of Ca^{2+} influx, CAMKII activity is required for LTP in females. a) Comparison of LTP in the presence and absence of CAMKII antagonist, KN-62. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). b) Summary EPSC data from 25-30

min post-HFS showing that KN-62 prevents LTP (Control $n=6$ cells from 6 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; KN-62 $n=6$ cells from 6 mice, $p=0.1562$, two-tailed paired Wilcoxon test; $^*p=0.0411$, Mann-Whitney U test). Representative trace scale bars = 40pA/10ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 6. Dopamine receptor activity is not required for Hipp-NAc LTP in females. a) Schematic of D1R downstream signaling that can contribute to LTP. b) Comparison of LTP in presence and absence of D1R antagonist, SCH23390 (SCH). Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). c) Summary EPSC data from 25-30 min post-HFS showing that SCH does not prevent LTP (Control $n=10$ cells from 10 mice, $^{\#}p=0.0020$, two-tailed paired Wilcoxon test; SCH $n=11$ cells from 9 mice, $^{\#}p=0.0020$, two-tailed paired Wilcoxon test; $p=0.3867$, Mann-Whitney U test). Representative trace scale bars = 40pA/10ms. d) Comparison of LTP induced by local stimulation of NAc in presence and absence of SCH. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). e) Summary EPSC data from 25-30 min post-HFS showing that LTP induced by local stimulation is prevented by application of SCH (Control $n=6$ cells from 5 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; SCH $n=5$ cells from 4 mice, $p=0.8125$, two-tailed paired Wilcoxon test; $^*p=0.0173$, Mann-Whitney U test). Representative trace scale bars = 40pA/10ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 7. Sex-specific requirement for ER α activity for Hipp-NAc LTP. a) Comparison of LTP in the presence and absence of an ER α antagonist, MPP dihydrochloride (MPP) in female mice. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). b) Summary EPSC

data from 25-30 min post-HFS showing that ER α inhibition prevents LTP in female mice (Ctrl F $n=6$ cells from 6 mice, $^{\#}p=0.0312$, two-tailed paired Wilcoxon test; MPP F $n=5$ cells from 5 mice, $p=0.8125$, two-tailed paired Wilcoxon test; $^{**}p=0.0087$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. c) Comparison of LTP in the presence and absence of an ER α antagonist, MPP dihydrochloride (MPP) in male mice. Data represent 1-minute bins (means of all cells in each condition $\pm$ SEM). d) Summary EPSC data from 25-30 min post-HFS showing that ER α inhibition has no effect on LTP in male mice (Ctrl M $n=7$ cells from 7 mice, $^{\#}p=0.0156$, two-tailed paired Wilcoxon test; MPP M $n=8$ cells from 8 mice, $^{\#}p=0.0391$, two-tailed paired Wilcoxon test; $p=0.6126$, Mann-Whitney U test). Representative trace scale bars = 20pA/10ms. *Differences between treatment and control by two-tailed Mann-Whitney U-test. $^{\#}$ Significant increase in EPSC amplitude above baseline revealed by two-tailed paired Wilcoxon test.

Figure 8. Comparison of sex-specific mechanisms involved in Hipp-NAc LTP. a) LTP at Hipp-NAc synapses in males requires NMDAR-mediated Ca $^{2+}$ influx and CAMKII activation but does not require ER α or D1R activity. b) In females, LTP at Hipp-NAc synapses occurs with a mechanism involving L-type VGCCs instead of NMDARs for Ca $^{2+}$ influx, CAMKII, and ER α activity but does not require D1R activity.















