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Estrous cycle-induced sex differences in medium spiny neuron excitatory synaptic transmission and intrinsic excitability in adult rat nucleus accumbens core

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Proaño SB, Morris HJ, Kunz LM, Dorris DM, Meitzen J. Estrous cycle-induced sex differences in medium spiny neuron excitatory synaptic transmission and intrinsic excitability in adult rat nucleus accumbens core. J Neurophysiol 120: 1356-1373, 2018. First published June 27, 2018; doi:10.1152/jn.00263.2018.-Naturally occurring hormone cycles in adult female humans and rodents create a dynamic neuroendocrine environment. These cycles include the menstrual cycle in humans and its counterpart in rodents, the estrous cycle. These hormone fluctuations induce sex differences in the phenotypes of many behaviors, including those related to motivation, and associated disorders such as depression and addiction. This suggests that the neural substrate instrumental for these behaviors, including the nucleus accumbens core (AcbC), likewise differs between estrous cycle phases. It is unknown whether the electrophysiological properties of AcbC output neurons, medium spiny neurons (MSNs), change between estrous cycle phases. This is a critical knowledge gap given that MSN electrophysiological properties are instrumental for determining AcbC output to efferent targets. Here we test whether the intrinsic electrophysiological properties of adult rat AcbC MSNs differ across female estrous cycle phases and from males. We recorded MSNs with whole cell patch-clamp technique in two experiments, the first using gonad-intact adult males and females in differing phases of the estrous cycle and the second using gonadectomized males and females in which the estrous cycle was eliminated. MSN intrinsic electrophysiological and excitatory synaptic input properties robustly changed between female estrous cycle phases and males. Sex differences in MSN electrophysiology disappeared when the estrous cycle was eliminated. These novel findings indicate that AcbC MSN electrophysiological properties change across the estrous cycle, providing a new framework for understanding how biological sex and hormone cyclicity regulate motivated behaviors and other AcbC functions and disorders.

NEW & NOTEWORTHY This research is the first demonstration that medium spiny neuron electrophysiological properties change across adult female hormone cycle phases in any striatal region. This influence of estrous cycle engenders sex differences in electrophysiological properties that are eliminated by gonadectomy. Broadly,

these findings indicate that adult female hormone cycles are an important factor for neurophysiology.

estrous cycle; excitability; medium spiny neurons; nucleus accumbens; sex steroid hormones

INTRODUCTION

Naturally occurring hormone cycles in adult female humans and rodents create a dynamic neuroendocrine environment that can potentially influence neuron structure and function (Breedlove and Arnold 1981; Cahill 2014; Gorski 1985; McCarthy 2008; Micevych et al. 2017; Woolley 1998; Woolley et al. 1990). These cycles include the menstrual cycle in humans and its counterpart in rodents, the estrous cycle (Hubscher et al. 2005). Similar to the menstrual cycle, the estrous cycle can be divided into phases that feature differing concentrations of gonadal hormones 17β -estradiol (estradiol) and progesterone. In the diestrus phase, circulating plasma levels of estradiol and progesterone are low. In the proestrus phase, first estradiol and then progesterone levels rapidly peak. Finally, in the estrus phase, estradiol and progesterone levels fall but hormone effects remain. It is during this phase that follicular maturation in the ovaries induces ovulation and heightened sexual receptivity. Although these cycles induce large changes in sex steroid concentrations in neural tissue, including in the striatum (Morissette et al. 1992), the effects of these cycles on neuron electrophysiology are unexplored outside of a few brain regions (Blume et al. 2017; Calizo and Flanagan-Cato 2000; Cooke and Woolley 2005; Hao et al. 2006; Okamoto et al. 2003; Woolley 1998; Woolley and McEwen 1993). This lack of knowledge reflects a general neglect of females in basic neuroscience research (Beery and Zucker 2011; Shansky and Woolley 2016; Will et al. 2017). This is unfortunate given that the robust behavioral changes in females in different estrous cycle phases must manifest in some manner in the neural substrate.

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One class of behaviors sensitive to differing estrous cycle phases include those related to reward, motivation, and associated disorders such as depression and addiction (Baran et al. 2009, 2010; Becker 1999; Becker et al. 2001, 2012; Becker and Hu 2008; Jackson et al. 2006, Lebron-Milad and Milad 2012; Milad et al. 2009; Walf and Frye 2006). Investigations into the neural substrate instrumental for these behaviors and disorders have targeted striatal brain regions, including the nucleus accumbens core (AcbC). The AcbC is a crucial nexus region that links the limbic and premotor systems (Salgado and Kaplitt 2015) and, among other tasks, helps regulate reproductive reward and sexual motivation (Tonn Eisinger et al. 2018). Robust sex differences and hormone sensitivity in AcbC function have been documented, primarily in dopaminergic transmission and related behaviors and disorders (Becker 1999; Becker and Hu 2008; Czoty et al. 2009; Lebron-Milad and Milad 2012; Salgado and Kaplitt 2015; Yoest et al. 2014). The predominant neuron type in the AcbC is the medium spiny neuron (MSN), also called the spiny projection neuron, the region's major output neuron. AcbC MSNs integrate glutamatergic, dopaminergic, and other inputs to directly regulate motivated behaviors and AcbC-related disorders. In addition to dopaminergic action, more recently sex differences and hormone sensitivity have also been discovered in excitatory glutamatergic synapse number, markers, and activity onto MSNs (Bonansco et al. 2018; Cao et al. 2016; Forlano and Woolley 2010; Peterson et al. 2015; Staffend et al. 2011; Willett et al. 2016; Wissman et al. 2011, 2012). In females, excitatory synapse function as measured via miniature excitatory postsynaptic current (mEPSC) analysis is increased compared with males (Cao et al. 2016; Wissman et al. 2011). However, it is unknown whether the electrophysiological properties of AcbC MSNs change between estrous cycle phases. This is a critical knowledge gap given that MSN electrophysiological properties are critical for determining what information is communicated to AcbC efferent targets.

Here we address this question by testing the hypothesis that the excitatory synaptic input and intrinsic electrophysiological properties of adult rat AcbC MSNs differ across female estrous cycle phases and from males. To accomplish this, we employed whole cell patch-clamp technique to record MSNs in acute brain slices of the AcbC in two different experiments. In the first experiment, we recorded MSNs from gonad-intact males and females in the diestrus, proestrus, and estrus phases of the estrous cycle. We discovered robust differences in MSN electrophysiological properties across the estrous cycle and between females and males. MSN excitatory synaptic input as measured via mEPSC properties was generally elevated in females in proestrus and estrus phases compared with females in diestrus phase and males. In contrast, intrinsic neuronal excitability was decreased in females in proestrus and estrus phases compared with females in diestrus phase and males. In the second experiment, we tested whether the presence of the estrous cycle was necessary for differences in MSN electrical properties between female and males. Thus we recorded MSNs from gonadectomized males and females in which the estrous cycle was eliminated. Sex differences in MSN electrophysiology disappeared when the estrous cycle was eliminated. These findings indicate that the primary output neurons of the AcbC, the MSNs, are sensitive to natural hormone cycles in adult females, providing a new framework for understanding how

changes in cellular electrophysiology regulate hormone cycle influences on motivated behaviors and other AcbC functions and disorders.

METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committees at North Carolina State University and Charles River Laboratories. For experiments assessing estrous cycle, female and male Sprague-Dawley CD IGS rats were born from timed-pregnant females purchased from Charles River Laboratories. Rats were housed with their littermates and dam until weaning. After weaning at postnatal day (P)21, males (n = 7) were group housed and females were group housed until P60 to facilitate assessment of the following phases of the estrous cycle: diestrus (n = 11), proestrus (n = 8), and estrus (n = 7). Age at recording ranged from P70 to P85 and was matched between groups (mean \pm SE: diestrus: P77 \pm 1; proestrus: P79 \pm 1; estrus: P78 \pm 2; male: P78 \pm 2). All animals were housed in a temperature- and light-controlled room (23°C, 40% humidity, 12:12-h light-dark cycle with lights turned on and off at 7 AM and 7 PM, respectively) at the Biological Resource Facility of North Carolina State University. All cages were washed with polysulfone Bisphenol A (BPA) free and were filled with bedding manufactured from virgin hardwood chips (Beta Chip; NEPCO, Warrensburg, NY) to avoid endocrine disruptors present in corncob bedding (Mani et al. 2005; Markaverich et al. 2002; Villalon Landeros et al. 2012). Soy protein-free rodent chow (2020X; Teklad, Madison, WI) and glass bottle-provided water were available ad libitum. For experiments employing gonadectomized rats, female (n = 13) and male (n = 21) Sprague-Dawley CD IGS rats were purchased from Charles River Laboratories and gonadectomized at P60. Age at recording ranged from P70 to P80 and was matched between sexes (mean \pm SE: female: P73 \pm 1; male: P73 \pm 1). Rats were housed at the Biological Resource Facility of North Carolina State University as described above.

Estrous Cycle Assessment

Estrous cycle assessment was performed with a wet mount preparation as previously described (Hubscher et al. 2005). Briefly, females (P60 or older) were swabbed with potassium phosphate buffer solution at ~10:00 AM. Slides were visualized under a microscope to determine estrous cycle stage according to cell morphology as previously described (Westwood 2008). Estrous cycle stage was confirmed via assessment of plasma concentrations of progesterone, estradiol, and testosterone (Table 1). At death (~10:30 AM), trunk blood was collected from each subject and centrifuged within 30 min. Harvested plasma was stored at -20° C until assessment at the Ligand Assay and Analysis Core at the University of Virginia with commercially available ELISA kits manufactured by Calbiotech (estradiol) or IBL (progesterone, testosterone). All protocols were validated based on the recommendations of the Endocrine Society (Wierman et al. 2014). Samples were run in duplicates. Intra- and interassay percent coefficients of variation were estradiol: 8.3%, 9.9%; progesterone: 5.6%, 10.2%; and testosterone: 5.4%, 7.8%, respectively. The minimum detectable plasma estradiol, progesterone, and testosterone concentrations were 3 pg/ml, 0.15 ng/ml, and 10 ng/dl, respectively. The maximum detectable plasma concentrations were 300 pg/ml, 40 ng/ ml, and 1,600 ng/dl, respectively. Plasma estradiol levels differed across estrous cycle phase in females, as expected (Butcher et al. 1974). Estradiol was significantly elevated in proestrus compared with diestrus and estrus phases (Table 1). Progesterone levels did not significantly differ across phases, indicating that animals in the proestrus phase were used before manifestation of peak progesterone levels. Overall, plasma hormone levels matched estrous cycle phase identi-

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Hormone	Diestrus	Proestrus	Estrus	Males	Statistics (F/KW, P)
17β -Estradiol, pg/ml Progesterone, ng/ml	$5.345 \pm 1.856 (11)^{a_{*}}$ 27.27 $\pm 3.144 (11)$	$16.09 \pm 2.469 \ (8)^{b}$ 23.17 $\pm 5.092 \ (8)$	$2.657 \pm 1.021 \ (7)^{a*}$ 27.61 ± 5.317 (7)*	_	12.05, 0.0003 0.31, 0.74
Testosterone, ng/ml	_	_	_	8.144 ± 1.096 (6)	

Table 1. Plasma hormone concentrations in gonad-intact adult rats

Notes: Values are means \pm SE for numbers of animals in parentheses. —, Not measured. ^{a,b}Superscript letters indicate statistically significant differences across groups. KW, Kruskal-Wallis. *Nonnormal distribution.

fication from vaginal cytology assessment, confirming the validity of this method for estrous cycle phase identification.

Acute Brain Slice Preparation

Brain slices for electrophysiological recordings were prepared as previously described (Dorris et al. 2014). Rats were deeply anesthetized with isoflurane gas and killed by decapitation. The brain was then dissected rapidly into ice-cold oxygenated sucrose artificial cerebrospinal fluid containing (in mM) 75 sucrose, 1.25 NaH₂PO₄, 3 MgCl₂, 0.5 CaCl₂, 2.4 Na pyruvate, and 1.3 ascorbic acid from Sigma-Aldrich (St. Louis, MO) and 75 NaCl, 25 NaHCO₃, 15 dextrose, and 2 KCl from Fisher (Pittsburgh, PA). The osmolarity of the sucrose ACSF was 295-305 mosM, and the pH was between 7.2 and 7.4. Coronal brain slices (300 μ m) were prepared with a vibratome and then incubated in regular ACSF containing (in mM) 126 NaCl, 26 NaHCO₃, 10 dextrose, 3 KCl, 1.25 NaH₂PO₄, 1 MgCl₂, and 2 CaCl₂ (295-305 mosM, pH 7.2-7.4) for 30 min at 30-35°C and then for at least 30 min at room temperature (22-23°C). Slices were stored submerged in room-temperature oxygenated ACSF for up to 5 h after sectioning in a large-volume bath holder.

Electrophysiological Recording

Slices rested for at least 1 h after sectioning. They were then placed in a Zeiss Axioscope equipped with IR-DIC optics, a Dage IR-1000 video camera, and ×10 and ×40 lenses with optical zoom and superfused with oxygenated ACSF heated to ~22°C. Whole cell patch-clamp recordings were used to record the electrical properties of MSNs in the AcbC (estrous cycle experiments, Fig. 1A; gonadectomized experiments, Fig. 1B). Glass electrodes (6–23 M Ω) contained (in mM) 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 MgCl₂, 2 MgATP, 0.3 NaGTP, and 10 phosphocreatine from Sigma-Aldrich and 10 HEPES from Fisher (285 mosM, pH 7.2-7.4). Signals were amplified, filtered (2 kHz), and digitized (10 kHz) with a MultiClamp 700B amplifier attached to a Digidata 1550 system and a personal computer using pCLAMP 10.7 software. Membrane potentials were corrected for a calculated liquid junction potential of -13.5 mV. With previously described procedures (Dorris et al. 2015), recordings were first made in current clamp to assess neuronal electrophysiological properties. MSNs were identified by their medium-sized somas, the presence of a slow-ramping subthreshold depolarization in response to low-magnitude positive current injections, a hyperpolarized resting potential more negative than -65 mV, inward rectification, and prominent spike afterhyperpolarization (Belleau and Warren 2000; O'Donnell and Grace 1993).

In a subset of recordings, oxygenated ACSF containing both the GABA_A receptor antagonist picrotoxin (PTX, 150 µM; Fisher) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 μ M; Abcam Biochemicals) was applied to the bath to abolish inhibitory postsynaptic current events and action potentials, respectively. Once depolarizing current injection no longer generated an action potential, MSNs were voltage clamped at -70 mV and mEPSCs were recorded for at least 5 min. These settings enable recordings from almost exclusively AMPA glutamate receptors (Nowak et al. 1984). To confirm that mEPSCs were generated from AMPA glutamate receptors under the present experimental conditions, we exposed two MSNs to oxygenated ACSF containing the AMPA receptor antagonist 6,7dinitroquinoxaline-2,3-dione disodium salt (DNQX, 25 µM; Tocris) in addition to PTX and TTX. DNQX exposure eliminated mEPSCs, lowering mEPSC frequency to $0.01 \pm 0.00\%$ of pre-DNQX exposure values. In all experiments input/series resistance was monitored for changes, and cells were excluded if resistance changed >25%.

Data Recording and Analysis

Intrinsic electrophysiological properties and action potential characteristics were analyzed with pCLAMP 10.7. After break-in, the resting membrane potential was first allowed to stabilize ~1–2 min, as in Mu et al. (2010). After stabilization, resting membrane potential was calculated from the average of at least 8,900 ms of recording in the absence of injected current. At least three series of depolarizing and hyperpolarizing current injections were applied to elicit basic neurophysiological properties. Most properties measured followed the definitions of Cao et al. (2016), Dorris et al. (2015), and Willett et al. (2018, 2016), which were based on those of Perkel and colleagues (Farries et al. 2005; Farries and Perkel 2000, 2002; Meitzen et al. 2009). For each neuron, measurements were made of at least three action potentials generated from minimal current injections. These measurements were then averaged to generate the reported action



Fig. 1. Location of whole cell patch-clamped medium spiny neurons (MSNs) in rat nucleus accumbens (Acb) core. A: gonad-intact females in differing estrous cycle phases and males. B: gonadectomized females and males. AC, anterior commissure; LV, lateral ventricle.

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potential measurement for that neuron. For action potential measurements, only the first generated action potential was used unless more action potentials were required to meet the standard three action potentials per neuron. Action potential threshold was defined as the first point of sustained positive acceleration of voltage $(\delta^2 V / \delta t^2)$ that was also >3 times the SD of membrane noise before the detected threshold (Baufreton et al. 2005). The delay to first action potential is the average time in milliseconds of the time from the initial deflection generated by the current step function to the action potential threshold of the first spike. Action potential width at half peak is the width of the action potential halfway between action potential peak and threshold in milliseconds. The action potential amplitude is the change in millivolts between action potential threshold and peak. Afterhyperpolarization peak amplitude is the difference in millivolts between action potential threshold and the most hyperpolarized voltage point after action potential peak. Afterhyperpolarization time to peak amplitude is the time measured in milliseconds between the action potential threshold voltage point on the descending phase of the action potential and the afterhyperpolarization peak amplitude. Rheobase, measured in nanoamps, is the lowest amplitude of injected positive current needed to produce an initial action potential. The slope of the linear range of the evoked action potential firing rate-to-positive injected current curve (FI slope) was calculated from the first current stimulus that evoked an action potential to the first current stimulus that generated an evoked firing rate that persisted for at least two consecutive current stimuli. Minimum firing rate was defined as the action potential rate generated in response to a 600-ms rheobase current. Maximum firing rate was defined as the action potential rate generated in response to a 600-ms +0.1-nA injected current. Input resistance in the linear, nonrectified range was calculated from the steady-state membrane potential in response to -0.02-nA hyperpolarizing pulses. Rectified range input resistance, inward rectification, and percent inward rectification were calculated as described previously, with rectified range input resistance measured using the most hyperpolarizing current injected into the MSN (Belleau and Warren 2000). Inward rectification is the input resistance of the -0.02-nA step minus the rectified range input resistance. Percent inward rectification is defined as rectified range input resistance/ input resistance \times 100. The time constant of the membrane was calculated by fitting a single exponential curve to the membrane potential change in response to -0.02-nA hyperpolarizing pulses. Possible sex differences in hyperpolarizationinduced "sag" (i.e., I_H current) were assessed with the "sag index" (Farries et al. 2005). Briefly, the sag index is defined as the difference between the minimum voltage measured during the largest hyperpolarizing current pulse and the steady-state voltage deflection of that pulse, divided by the steady-state voltage deflection. A cell with no sag would exhibit a sag index of 0, whereas a cell whose maximum voltage deflection is twice that of the steady-state deflection would exhibit a sag index of 1. Cells with considerable sag typically have an index of >0.1. Frequency, amplitude, and decay of mEPSCs were analyzed off-line with Mini Analysis (Synaptosoft, http://www.synaptosoft.com/MiniAnalysis/), and mEPSC decay τ calculated at 10-90% of the mEPSC amplitude was analyzed off-line with Clampfit 10.7 (Molecular Devices). mEPSC threshold was set at a minimum value of 5 pA, and accurate event detection was validated by visual inspection. mEPSC frequency was defined as the number of detected mEPSC events per second (Hz). mEPSC amplitude was calculated as the difference between the averaged baseline 10 ms before initial mEPSC rise and peak mEPSC amplitude. mEPSC decay was calculated as the time required for peak mEPSC amplitude to return to baseline.

Statistics

Experiments were analyzed as appropriate with either one-way ANOVAs with Newman-Keuls post hoc tests or Kruskal-Wallis tests with Dunn's correction for multiple comparisons as well as linear regression tests. Distributions were analyzed for normality with the D'Agostino and Pearson omnibus normality test (Prism). An a priori outlier analysis was performed, and values falling >4 SDs from the mean were excluded from analysis. A total number of four cells were excluded from analysis. *P* values < 0.05 were considered a priori as significant. Data are presented as means \pm SE.

RESULTS

Here we comprehensively tested the hypothesis that the estrous cycle influences female rat MSN electrophysiology, including both excitatory synaptic properties and intrinsic membrane properties. To accomplish this goal, we performed two separate experiments. First, we tested how the different phases of the estrous cycle with concomitant cyclical changes in hormone concentrations modulated MSN electrophysiological properties in gonad-intact rats. Second, we gonadectomized female and male rats to test whether sex differences in MSN electrophysiological properties remained after abolition of the estrous cycle. In this section, we first address the effects of the estrous cycle in gonad intact rats and then follow with experiments in gonadectomized rats.

Experiment 1: Gonad-Intact Rats

mEPSC frequency decreases in diestrus compared with proestrus and estrus. Previous work has identified sex differences in mEPSC properties in AcbC MSNs. Most notably, mEPSC frequency was increased in prepubertal females compared with prepubertal males and in adult females of unknown estrous cycle phase compared with adult males (Cao et al. 2016; Wissman et al. 2011). Thus it is possible that mEPSC properties may vary across the estrous cycle. To test this hypothesis, we voltage clamped MSNs to -70 mV and recorded mEPSCs while exposing MSNs to 1 μ M TTX and 150 μ M PTX to block sodium-dependent action potentials and GABA_A receptors, respectively (Fig. 2A). We assessed mEPSC frequency, amplitude, and decay. Complete statistical information can be found in Table 2. mEPSC frequency was significantly elevated in the proestrus and estrus phases in females compared with diestrus-phase females and males (Fig. 2B). mEPSC amplitude was significantly decreased in estrusphase females compared with males (Fig. 2C). mEPSC amplitude was also significantly decreased in diestrus-phase females compared with males (Fig. 2C). mEPSC decay was significantly decreased in estrus-phase females compared with all other groups, with mEPSC decay being the longest in males (Fig. 2D). mEPSC decays can be longer or shorter because of changes in decay rate or because of changes in mEPSC amplitude while decay rate remains constant. To differentiate between these possibilities, we plotted mEPSC decay vs. mEPSC amplitude (Fig. 2E). We then analyzed the data with linear regressions, which found significant correlations between mEPSC amplitude and mEPSC decay in diestrus- and proestrus-phase females and a trend in estrus-phase females (diestrus: R^2 : 0.57, F: 22.22, P = 0.0002; proestrus: R^2 : 0.66, F: 19.44, P = 0.0013; estrus: R^2 : 0.23, F: 3.51, P < 0.086; males: R^2 : 0.075, F: 0.65, P = 0.44). This correlation-based analysis indicates that the differences in mEPSC decay in most female estrous cycle stages are primarily driven by changes in mEPSC amplitude and not differences in decay rate. This lack of difference in mEPSC decay rate in females is in contrast to males, which exhibit uncorrelated changes in mEPSC decay



Fig. 2. Medium spiny neuron (MSN) miniature excitatory postsynaptic current (mEPSC) properties: gonad-intact females and males. A: representative examples of mEPSCs recorded in diestrus, proestrus, and estrus female and male MSNs. B–D: mEPSC frequency (B), mEPSC amplitude (C), and mEPSC decay (D) varied across estrous cycle phases in females and/or from males. E: mEPSC amplitude was significantly correlated with mEPSC decay in diestrus, proestrus, and estrus females but not males. Horizontal line superimposed upon scatterplots in B–D indicates the mean. Lines situated above scatterplots indicate statistical significance. *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001. Complete statistical information is in Table 2.

and amplitude. This lack of correlation suggests that males display independent differences in both mEPSC decay rate and amplitude compared with females. To further probe this phenomenon, we calculated a normalized mEPSC decay τ value taken at 10–90% of mEPSC amplitude for each experimental

group (Kruskal-Wallis = 14.62, P = 0.022; diestrus females: 1.00 ± 0.09; proestrus females: 0.93 ± 0.10; estrus females: 0.88 ± 0.08; males: 0.54 ± 0.05). Normalized mEPSC decay τ did not differ between diestrus-, proestrus-, and estrus-phase females (P > 0.05 for all comparisons), but males differed

	1 1 5	1 5	0		
Property	Diestrus	Proestrus	Estrus	Male	Statistics (F/KW, P)
Frequency, Hz Amplitude, pA Decay, ms	$\begin{array}{l} \textbf{1.05 \pm 0.23} \ (19)^{a,b,*} \\ \textbf{10.37 \pm 0.69} \ (19)^{a,*} \\ \textbf{4.17 \pm 0.46} \ (19)^{a} \end{array}$	$\begin{array}{l} \textbf{2.07 \pm 0.39} \ (12)^{a,c} \\ \textbf{11.40 \pm 0.88} \ (12)^{a,b} \\ \textbf{4.79 \pm 0.55} \ (12)^{a} \end{array}$	$\begin{array}{l} \textbf{2.31} \pm \textbf{0.42} \ (14)^{c*} \\ \textbf{9.11} \pm \textbf{0.30} \ (14)^{a} \\ \textbf{2.66} \pm \textbf{0.31} \ (14)^{b} \end{array}$	$\begin{array}{l} \textbf{0.65 \pm 0.25 (10)^{b}} \\ \textbf{14.10 \pm 1.04 (10)^{b}} \\ \textbf{6.78 \pm 0.48 (10)^{c}} \end{array}$	16.11, 0.001 13.85, 0.003 11.38, <0.0001

Table 2. mEPSC properties recorded from medium spiny neurons in gonad-intact adult rat nucleus accumbens core

Values are means \pm SE for numbers of recorded neurons in parentheses. Values in boldface are statistically significant. ^{a,b,c}Superscript letters indicate statistically significant differences across groups. KW, Kruskal-Wallis. *Nonnormal distribution.

from females in diestrus (P < 0.01), proestrus (P < 0.05), and estrus (P < 0.05). This analysis supports the conclusion that females primarily show differences in mEPSC frequency across estrous cycle phases and differ from males but not from each other in both mEPSC decay rate and amplitude. It also supports the conclusion that differences in mEPSC decay between females and males are not solely due to differences in mEPSC amplitude. Overall, these findings show that excitatory synaptic input as assessed by mEPSC properties varies robustly across the estrous cycle.

Most individual action potential properties do not differ between estrous cycle phases. We also tested whether intrinsic MSN electrophysiological properties differed between estrous cycle phases, including individual action potential properties, excitability, and passive membrane properties. To accomplish this, we injected MSNs with a series of positive and negative current injections and analyzed a battery of electrophysiological attributes. These electrophysiological attributes and related statistical information are provided in Table 3. Regarding action potential properties (Fig. 3A), individual action potentials recorded from MSNs in diestrus-phase females trended toward a shorter delay to first action potential in comparison to proestrus- and estrus-phase females and to males (Fig. 3B; Table 3; P = 0.0545). This delay is a canonical feature of MSNs and reflects the underlying slow inactivating A current (Nisenbaum et al. 1994). Action potential width was significantly longer in diestrus- and proestrus-phase females compared with males, in which the overall average change was less than a millisecond (Fig. 3C). No differences were detected in action potential threshold (Fig. 3D), action potential amplitude

(Fig. 3E), action potential afterhyperpolarization time to peak amplitude (Fig. 3F), or action potential afterhyperpolarization peak amplitude (Fig. 3G). These findings indicate that most individual action potential properties do not differ across the estrous cycle, except for a relatively minor change in action potential width.

Rheobase, the amount of current required for action potential initiation, decreases in diestrus compared with proestrus and estrus. To test the hypotheses that action potential initiation and generation vary across estrous cycle phases, we injected a series of positive current injections into MSNs and analyzed the initiation and number of evoked action potentials (Fig. 4A). Regarding action potential initiation, the minimum amount of excitatory current necessary to trigger an action potential significantly varied between estrous cycle phases (Fig. 4B). Rheobase was drastically reduced in MSNs recorded in diestrus-phase females compared with proestrus-phase females and males. Changes in rheobase are sometimes concomitant with changes in action potential threshold, resting membrane potential, and/or input resistance. Since there were no changes in action potential threshold between estrous cycle phases (Fig. 3D), we next focused on analyzing resting membrane potential. Resting membrane potential was depolarized in MSNs recorded from diestrus-phase females compared with proestrus- and estrus-phase females and males (Fig. 4D). Interestingly, after action potential initiation, the frequency of action potentials evoked by depolarizing current injection did not differ by estrous cycle phase, including minimum firing rate (Fig. 4F), maximum firing rate (Table 3), and slope of the frequency of action potentials evoked by depolarizing current

Table 3. Electrophysiological properties of medium spiny neurons in adult gonad-intact rat nucleus accumbens core

Property	Diestrus	Proestrus	Estrus	Male	Statistics (F/KW, P)
Resting membrane potential, mV	$-83.81 \pm 1.14 (26)^{a}$	-87.37 ± 0.89 (20) ^b	-88.13 ± 1.51 (16) ^b	$-88.34 \pm 0.56 (16)^{b}$	4.17, 0.009
Delay to first AP, ms	402.30 ± 15.59 (18)	438.90 ± 13.15 (18)	465.90 ± 14.63 (15)	438.20 ± 20.70 (15)	2.68, 0.0545
Rheobase, nA	$0.08 \pm 0.01 (26)^{a_{*}}$	$0.13 \pm 0.01 (19)^{b,c}$	$0.11 \pm 0.01 (16)^{c}$	$0.13 \pm 0.01 (15)^{b,c}$	23.15, <0.0001
AP threshold, mV	-51.81 ± 1.86 (26)*	-47.44 ± 2.02 (19)	-50.45 ± 1.97 (16)	-46.86 ± 1.62 (15)	6.97, 0.07
AP width at half peak amplitude, ms	$3.78 \pm 0.17 (26)^{a_*}$	$3.44 \pm 0.13 (17)^{a,b}$	$3.63 \pm 0.16 (16)^{a}$	$3.06 \pm 0.16 (15)^{b}$	11.23, 0.01
AP amplitude, mV	54.79 ± 3.01 (26)	54.55 ± 3.47 (19)	54.99 ± 3.47 (16)	50.03 ± 2.48 (15)	0.46, 0.71
AHP peak amplitude, mV	-7.20 ± 0.53 (25)	-7.66 ± 0.58 (19)	-7.06 ± 0.49 (16)	-9.13 ± 0.77 (14)	2.16, 0.10
AHP time to peak, ms	25.82 ± 3.14 (25)*	23.21 ± 2.13 (19)*	26.28 ± 3.33 (16)	21.41 ± 2.28 (15)	0.70, 0.87
FI slope, Hz/nA	298.70 ± 17.85 (26)	241.30 ± 14.84 (19)	297.10 ± 21.20 (16)	258.60 ± 16.23 (15)	2.58, 0.06
Time constant of membrane, ms	$19.07 \pm 1.45 (26)^{a}$	$14.83 \pm 1.23 (20)^{a,b*}$	$16.42 \pm 2.38 (16)^{a,b*}$	$12.29 \pm 1.22 (16)^{b*}$	11.31, 0.01
Linear range input resistance, $M\Omega$	$299.10 \pm 18.67 (26)^{a}$	$203.90 \pm 16.01 (20)^{\text{b}}$	$258.60 \pm 29.36 (16)^{a,b}$	$220.80 \pm 30.63 (16)^{\text{b}}$	3.92, 0.01
Rectified range input resistance, $M\Omega$	217.50 ± 12.72 (26)	164.80 ± 12.60 (20)	189.50 ± 17.88 (16)	165.90 ± 20.66 (16)	2.94, 0.04
Percent inward rectification, %	74.92 ± 3.03 (26)	81.36 ± 1.99 (20)	$77.92 \pm 4.10 (16)$	78.98 ± 3.44 (16)*	1.90, 0.59
Inward rectification, $M\Omega$	81.65 ± 14.08 (26)*	39.03 ± 5.91 (20)*	69.05 ± 19.00 (16)*	54.83 ± 16.18 (16)*	6.10, 0.11
Sag index (unitless)	$0.004 \pm 0.004 (26)^*$	0.003 ± 0.001 (20)	$0.005 \pm 0.004 (16)^*$	0.008 ± 0.004 (16)*	1.48, 0.69
Minimum firing rate, Hz	4.57 ± 0.53 (25)*	3.52 ± 0.27 (20)	$3.43 \pm 0.29 (17)^*$	3.21 ± 0.21 (15)	3.54, 0.32
Maximum firing rate, Hz	17.70 ± 1.12 (25)	18.16 ± 1.11 (20)	21.10 ± 1.51 (17)*	17.62 ± 1.52 (15)	4.01, 0.26

Values are means \pm SE for numbers of recorded neurons in parentheses. Values in boldface are statistically significant. AP, action potential; AHP, afterhyperpolarization; FI, evoked firing rate-to-positive current curve. KW, Kruskal-Wallis.^{a,b,c}Superscript letters indicate statistically significant differences across groups. *Nonnormal distribution.



Fig. 3. Medium spiny neuron (MSN) individual action potential properties: gonad-intact females and males. A: voltage responses of diestrus, proestrus, and estrus female and male MSNs to a single depolarizing current injection. B: delay to first action potential varies across estrous cycle and/or vs. males but remained a statistical trend. C: action potential width measured at half peak amplitude varied across estrous cycle and/or vs. males. D-G: action potential threshold (D), action potential amplitude (E), action potential afterhyperpolarization time to peak amplitude (F), and action potential afterhyperpolarization peak amplitude (G) did not vary across estrous cycle phases in females and/or vs. males. Horizontal line superimposed upon scatterplots in B-G indicates the mean. Lines situated above scatterplots indicate statistical significance. *P < 0.05. Complete statistical information is in Table 3. AP, action potential; AHP, afterhyperpolarization.

injection curve (FI curve) (Fig. 4*E*). Thus MSNs in diestrus exhibit increased excitability in the sense that action potential rheobase was decreased, indicating that less depolarizing current is required for initial action potential generation.

Input resistance increases in diestrus compared with proestrus females and males. The above-mentioned decrease in rheobase in diestrus compared with other estrous cycle phases and males may also be driven by an increase in input resistance. To investigate input resistance and other passive membrane properties, we injected a series of negative current injections in MSNs across female estrous cycle phases and in males (Fig. 5A). When we plotted the steady-state voltage deflection evoked by injected hyperpolarizing current curve (IV curve) (Fig. 5B), MSNs recorded from females in diestrus phase showed increased voltage deflections in response to higher-magnitude hyperpolarizing current injections compared with males and females in other estrous cycle phases. We further evaluated this by measuring input resistance in both the linear and rectified ranges. Input resistance in the linear range was increased in MSNs recorded from diestrus-phase females compared with proestrus-phase females and males (Fig. 6A). Input resistance in the rectified range was also increased in MSNs recorded from diestrus-phase females compared with proestrus-phase females and males (Fig. 6B). Additionally, the time constant of the membrane was longer in MSNs recorded from diestrus-phase females compared with males (Fig. 6C). This increase in input resistance and time constant of the membrane in MSNs recorded from diestrus-phase females compared with males (Fig. 6C). This increase in input resistance and time constant of the membrane in MSNs recorded from diestrus-phase females is consistent with the above-mentioned decreased rheobase in this phase.

Experiment 2: Gonadectomized Rats

If these changes in MSN excitatory synapse properties and intrinsic electrophysiological properties are driven by the naturally occurring estrous cycle, then elimination of the estrous



 $\stackrel{\circ}{\circ}$

00

Males

Fig. 4. Medium spiny neuron (MSN) action potential initiation and generation: gonad-intact females and males. A: voltage responses of diestrus, proestrus, and estrus female and male MSNs to a series of depolarizing current injections. B: action potential firing rates evoked by depolarizing current injections. C and D: rheobase (C) and resting membrane potential (D) varied across estrous cycle phase in females and/or vs. males: E and F: slopes of the evoked firing rate-to-positive current curve (FI slope) in individual MSNs (E) and minimum evoked firing rate (F) did not vary across estrous cycle and/or vs. males. Horizontal line superimposed upon scatterplots in C-F indicates the mean. Lines situated above scatterplots indicate statistical significance. *P < 0.05, ***P < 0.001. Complete statistical information is in Table 3.

Diestrus Proestrus Estrus

cycle should likewise eliminate sex differences in MSN electrophysiology. Therefore, to test the hypothesis that the estrous cycle is necessary for sex differences in AcbC MSN electrophysiological properties, we gonadectomized adult female and male rats by removing the ovaries and testes, respectively. After surgery, we waited 2 wk and then recorded MSNs. All MSN electrophysiological properties and statistics can be found in Table 4 and Table 5.

FI Slope (Hz/nA)

Males

400

200

0

Α

.79 28 m\ -85.50 m\ -95.79 m\

25

20

15

10.

5

0

-60

-70

-80

-90

-100-

-110

Diestrus Proestrus Estrus

0.0

В

Evoked Firing Rate (Hz)

Resting Membrane Potential (mV) **U**

Estrous cycle is necessary for sex differences in mEPSC properties. We first present excitatory synaptic input properties, given that these differed by estrous cycle in gonad-intact animals (Fig. 2). MSNs from gonadectomized females and males were voltage clamped to -70 mV, and mEPSCs were recorded during exposure to 1 μ M TTX and 150 μ M PTX. mEPSC frequency, amplitude, and decay were analyzed to evaluate excitatory synaptic input (Table 4). mEPSC frequency

(Fig. 7B), mEPSC amplitude (Fig. 7C), and mEPSC decay (Fig. 7D) did not differ between females and males as they did in intact animals. Thus the presence of the estrous cycle is necessary for sex differences in mEPSC properties.

Diestrus Proestrus Estrus

Males

10

0

Individual action potential properties do not differ by sex in gonadectomized rats. In gonad-intact animals, no sex or estrous cycle phase differences were detected in individual action potential properties apart from a relatively small difference in action potential width (Fig. 3C). To further corroborate that AcbC MSNs exhibit minimal differences in individual action potential properties, action potentials were evoked from MSNs from gonadectomized females and males by injecting positive current (Fig. 8A). No differences were detected in delay to first action potential (Fig. 8B), action potential threshold (Fig. 8C), and action potential width measured at half peak amplitude (Fig. 8D). Gonadectomized male MSNs showed a small but



Fig. 5. Medium spiny neuron (MSN) passive electrophysiological properties: gonad-intact females and males. *A*: voltage response of diestrus, proestrus, and estrus female and male MSNs to a series of hyperpolarizing current injections. *B*: the injected current-to-steady-stage voltage deflection curve (IV curve) varied across estrous cycle in females and/or vs. males.

significant increased mean action potential amplitude compared with gonadectomized female MSNs (Fig. 8*E*; P = 0.04). Given that the ranges of gonadectomized female and male action potential amplitudes were similar (female: 10% percentile 38.65 mV, 90% percentile 73.2 mV; male: 10% percentile

 Table 4.
 mEPSC properties recorded from medium spiny neurons

 in adult gonadectomized rat nucleus accumbens core

Property	Female	Male	Statistics (t/U, P)
Frequency, Hz Amplitude, pA Decay, ms	$\begin{array}{c} 1.50 \pm 0.31 \ (23)^{*} \\ 11.67 \pm 0.31 \ (23) \\ 2.58 \pm 0.33 \ (23) \end{array}$	$\begin{array}{c} 1.59 \pm 0.23 \; (30)^{*} \\ 12.71 \pm 0.41 \; (30) \\ 3.27 \pm 0.23 \; (30) \end{array}$	310, 0.54 1.91, 0.06 1.77, 0.08

Values are means \pm SE; numbers in parentheses indicate sample size. mEPSC, miniature excitatory postsynaptic current. No comparisons reached statistical significance. *Nonnormal distribution.

40.18 mV, 90% percentile 78.99 mV), we further assessed the distributions of action potential amplitudes (Fig. 8*F*). Action potential amplitude distributions did not differ between MSNs recorded from gonadectomized females and males (P = 0.0880). This lack of statistical significance between action potential amplitude distributions suggests that this is not a robust sex difference. Action potential afterhyperpolarization peak magnitude (Fig. 8*H*) and time to afterhyperpolarization peak magnitude (Fig. 8*G*) also did not differ between gonadectomized females and males. Overall, this analysis indicates that the MSN individual action potential properties are comparable across sex, similar to findings from gonad-intact rats.

Estrous cycle is necessary for sex differences in rheobase, the amount of current required for action potential initiation. In gonad-intact animals, MSN action potential initiation and generation varied across the estrous cycle, with MSNs recorded from diestrus-phase females exhibiting decreased rheobase compared with MSNs recorded from proestrus females and males. To test whether this difference persisted in gonadectomized animals, we injected a series of positive current injections into MSNs and analyzed the resulting number of evoked action potentials (Fig. 9A). We found that MSN action potential initiation and generation in the AcbC did not differ by sex in gonadectomized animals (Fig. 9B). Consistent with this conclusion, no sex differences were detected in rheobase (Fig. 9C), the slope of the linear range of the evoked firing rate per positive current injection curve (FI slope) (Fig. 9D), or minimum firing rate (Fig. 9E). No sex difference was detected in resting membrane potential (Table 5). These findings do not exhibit a sex difference in gonadectomized rats, indicating that MSN action potential ignition and generation are regulated by the estrous cycle.



Fig. 6. Medium spiny neuron (MSN) input resistance and time constant of the membrane properties: gonad-intact females and males. Input resistance in the linear range (A), input resistance in the rectified range (B; overall ANOVA for input resistance in rectified range was significant, but no differences were detected by post hoc test), and time constant of the membrane (C) varied across estrous cycle in females and/or vs. males. Horizontal line superimposed upon scatterplots in A-C indicates the mean. Lines situated above scatterplots indicate statistical significance. *P < 0.05. Complete statistical information is in Table 3.

Table 5. Electrophysiological properties of medium spiny neurons in adult gonadectomized rat nucleus accumbens core

Property	Female	Male	Statistics (t/U, P)
Resting membrane potential, mV	-86.32 ± 0.60 (28)	-87.00 ± 0.63 (39)	0.76, 0.45
Delay to first AP, ms	422.90 ± 15.63 (27)*	413.20 ± 12.26 (39)*	475, 0.51
Rheobase, nA	0.10 ± 0.01 (28)	0.10 ± 0.01 (39)	0.09, 0.92
AP threshold, mV	-50.30 ± 1.49 (28)	-53.04 ± 1.02 (39)	1.58, 0.12
AP width at half peak amplitude, ms	3.49 ± 0.12 (28)*	3.36 ± 0.11 (39)	495, 0.52
AP amplitude, mV	$53.02 \pm 2.51 (28)^{a}$	$60.11 \pm 2.24 (39)^{b}$	2.09, 0.04
AHP peak amplitude, mV	-7.78 ± 0.48 (28)	-7.42 ± 0.43 (39)	0.56, 0.58
AHP time to peak, ms	31.01 ± 2.99 (28)	27.38 ± 3.08 (38)*	432, 0.20
FI slope, Hz/nA	272.50 ± 14.09 (28)*	272.5 ± 12.27 (37)	509, 0.91
Time constant of membrane, ms	16.88 ± 1.40 (28)*	$18.23 \pm 1.22 \ (39)^*$	469, 0.33
Linear range input resistance, MΩ	260.20 ± 17.2 (28)*	227.2 ± 15.94 (39)*	420, 0.11
Rectified range input resistance, $M\Omega$	189.40 ± 9.11 (28)	167.80 ± 10.03 (39)	1.51, 0.14
Percent inward rectification, %	78.85 ± 2.73 (28)	76.28 ± 1.67 (39)	0.85, 0.40
Inward rectification, $M\Omega$	59.35 ± 10.61 (28)*	59.32 ± 7.58 (39)*	511, 0.66
Sag index (unitless)	0.008 ± 0.003 (28)*	0.006 ± 0.001 (39)	485, 0.44
Minimum firing rate, Hz	3.24 ± 0.23 (28)	3.19 ± 0.14 (39)	0.13, 0.95

Values are means \pm SE for numbers of recorded neurons in parentheses. Values in boldface are statistically significant. AP, action potential; AHP, afterhyperpolarization; FI, evoked firing rate-to-positive current curve. ^{a,b}Superscript letters indicate statistically significant differences across groups. *Nonnormal distribution.

Input resistance does not differ between gonadectomized females and males. Input resistance in diestrus-phase females was increased compared with proestrus females and males (Fig. 6A). To assess whether this difference is associated with the estrous cycle, we injected a series of negative current injections into gonadectomized female and male MSNs (Fig. 10A). When the steady-state voltage deflection evoked by injected current curve (IV curve) was plotted, female MSNs showed no robust differences compared with male MSNs (Fig. 10B). Consistent with this, input resistance in the linear range (Fig. 11A) or the rectified range (Fig. 11B) did not differ by sex. Other passive membrane properties also did not differ by

sex, including the time constant of the membrane (Fig. 11*C*). In total, this collection of evidence indicates that input resistance does not differ between gonadectomized females and males, congruent with the hypothesis that input resistance is modulated by the estrous cycle.

DISCUSSION

This study demonstrates that MSN electrophysiological properties robustly change across the adult female estrous cycle and, depending on the cycle phase, significantly differ from males as well (Fig. 12). Specific discoveries include I)



Fig. 7. Medium spiny neuron (MSN) miniature excitatory postsynaptic current (mEPSC) properties: gonadectomized females and males. A: representative examples of mEPSCs recorded in gonadectomized female and male MSNs. B-D: mEPSC frequency (B), mEPSC amplitude (C), and mEPSC decay (D) did not vary between gonadectomized females and males. Horizontal line superimposed upon scatterplots in B-D indicates the mean. Complete statistical information is in Table 4.

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Fig. 8. Medium spiny neuron (MSN) individual action potential properties: gonadectomized females and males. A: voltage responses of gonadectomized female and male MSNs to a single depolarizing current injection. B-D: delay to first action potential (B), action potential threshold (C), and action potential width measured at half peak amplitude (D) did not vary between gonadectomized females and males: E and F: action potential amplitude appeared to vary between gonadectomized females and males (E); however, a cumulative frequency plot of action potential amplitude data distributions (F) failed to reach significance, indicating that action potential amplitude (H) did not vary between gonadectomized females and males. Horizontal line superimposed upon scatterplots in B-E, G, and H indicates the mean. Lines situated above scatterplots indicate statistical significance. *P < 0.05. Complete statistical information is in Table 5. AP, action potential; AHP, afterhyperpolarization.

MSN excitatory synaptic input as assessed via mEPSC properties was augmented in females during the proestrus and estrus phases compared with females in diestrus phase and males; 2) intrinsic neuronal excitability was decreased in females during proestrus and estrus compared with females in diestrus phase and males; and 3) removal of the estrous cycle via gonadectomy eliminated sex differences in MSN electrophysiological characteristics. Collectively, these findings demonstrate that MSNs are highly sensitive to the estrous cycle, a natural, endogenous hormone cycle that ensures successful

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Fig. 9. Medium spiny neuron (MSN) action potential initiation and generation: gonadectomized females and males. A: voltage responses of gonadectomized female and male MSNs to a series of depolarizing current injections. B: action potential firing rates evoked by depolarizing current injections. C-E: rheobase (C), slopes of the evoked firing rate-to-positive current curve (FI slope) in individual MSNs (D), and minimum firing rate (E) did not vary between gonadectomized females and males. Horizontal line superimposed upon scatterplots in C-E indicates the mean. Complete statistical information is in Table 5.

reproduction. These data provide a new framework for understanding how changes at the level of MSN electrophysiology potentially mediate hormone cycle influences on AcbC functions.

Interestingly, female MSNs in the proestrus and estrus phases show contrasting changes in excitatory synaptic input and intrinsic excitability. During proestrus and estrus, mEPSC frequency increases, as do mEPSC amplitude and decay, broadly indicating an augmentation of excitatory synaptic function. In contrast, during proestrus and estrus, resting membrane potential hyperpolarizes, action potential rheobase increases, and input resistance decreases. These attributes and others (Fig. 12) indicate a decrease in intrinsic neuronal excitability, suggesting that large-scale changes in neuromodulator

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Fig. 10. Medium spiny neuron (MSN) passive electrophysiological properties: gonadectomized females and males. *A*: voltage response of gonadectomized female and male MSNs to a series of hyperpolarizing current injections. *B*: the injected current-to-steady-stage voltage deflection curve (IV curve) did not vary between gonadectomized females and vs. males.

action or potassium or sodium ion channel distribution or function may be occurring in MSNs between estrous cycle phases. These findings are reminiscent of homeostatic plasticity, which encompasses electrophysiological phenomena, such as synaptic scaling, that stabilize individual neuron and neural circuit activity (Turrigiano 2012) and typically occur over long time periods such as days. Homeostatic plasticity has been demonstrated in MSNs in the nucleus accumbens (Ishikawa et al. 2009). However, we hesitate to label the estrous cycleinduced changes in MSN function detected by this study as homeostatic plasticity, primarily because the precise hormone mechanism and temporal order of changes in MSN electrophysiological properties are unknown.

One mechanistic model that explains estrous cycle-induced changes in MSN electrophysiology is hormone and activity dependent. In this hormone- and activity-dependent model, during proestrus sex steroid hormones act on excitatory synapses in the AcbC and potentially its afferent brain regions to

augment excitatory synaptic input onto MSNs. This augmentation of excitatory input includes, at a minimum, increase in excitatory synapse number, as elegant electron microscopy, immunocytochemistry, and electrophysiological studies have shown that excitatory synapse number increases in proestrusphase females compared with male rats (Forlano and Woolley 2010; Wissman et al. 2011, 2012). Additionally, these glutamatergic synapses are sensitive to estradiol exposure in both adults and neonate rats as measured via mEPSC properties or dendritic spine density, a neuroanatomical correlate of excitatory synapse formation (Cao et al. 2016; Martinez et al. 2014; Peterson et al. 2015, 2016; Staffend et al. 2011). The experiments demonstrating estradiol sensitivity in AcbC MSN dendritic spines detected that estradiol induced a decrease in dendritic spine density and that this effect was blocked by metabotropic glutamate receptor inhibitors and endocannabinoid pathways (Peterson et al. 2015, 2016; Staffend et al. 2011). At first glance, this decrease in spine density appears contradictory to the present study's findings with increased mEPSC frequency. However, in the dendritic spine studies female rats were killed 24 h after exposure to estradiol, potentially placing these animals in a diestrus-like time period, after estradiol levels have dropped and when mEPSC frequency is low. Thus these results are likely consistent with the findings presented here.

The estradiol-sensitive changes observed in spine density reflect differences in excitatory synaptic transmission. Thus increases in spine density could, in turn, induce differences in behavioral output such as locomotor activity and other behaviors influenced by the AcbC observed in proestrus-phase female rats and other animal species, including humans during the follicular phase of the menstrual cycle (Jackson et al. 2006). Over a period of hours, this increase in excitation, in turn, could then induce compensatory changes in MSN intrinsic excitability that ultimately reduces Acb-related locomotor behavior. This activity- and hormone-dependent model is reminiscent of hormone-induced seasonal changes in songbird song control neuron electrophysiological and cellular anatomical properties in the robust nucleus of the arcopallium and the basal ganglia region area X (Brenowitz and Remage-Healey 2016; Cohen et al. 2016).



Fig. 11. Medium spiny neuron (MSN) input resistance and time constant of the membrane properties: gonadectomized females and males. Input resistance in the linear range (A), input resistance in the rectified range (B), and time constant of the membrane (C) did not vary between gonadectomized females and males. Horizontal line superimposed upon scatterplots in A-C indicates the mean. Complete statistical information is in Table 5.

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Fig. 12. Schematic of nucleus accumbens core medium spiny neuron excitatory synaptic input and intrinsic excitability changes between estrous cycle phases in the adult female rat. Excitatory synaptic input properties are decreased in the diestrus phase of the cycle while intrinsic excitability is increased, as measured by changes in rheobase, resting membrane potential, and input resistance. In the proestrus and estrus phases of the cycle, excitatory synaptic input is augmented, potentially through both an increase in excitatory synapse number and other presynaptic modifications and postsynaptic AMPA receptor number and/or other properties. In contrast, intrinsic excitability is decreased. LH, luteinizing hormone; FSH, follicle-stimulating hormone.

Alternatively, different hormones such as estradiol and progesterone could be synergistically acting to modulate specific aspects of MSN electrophysiology independent of direct hormone action. For example, since dopaminergic terminals in nucleus accumbens MSNs express estrogen receptors such as G protein-coupled estrogen receptor 1, membrane-associated estrogen receptor α , and membrane-associated estrogen receptor β (Almey et al. 2015), estradiol alone may directly regulate both excitatory synapse properties and intrinsic properties, perhaps in part by altering dopamine action. It is also possible that progesterone regulates changes in MSN electrophysiology as circulating plasma levels of progesterone change with the estrous cycle. Consistent with this, progesterone has been demonstrated to modulate AcbC-mediated behaviors related to drugs of abuse (Becker 1999), and a membrane progesterone receptor has been identified in rat striatal tissue (Ke and Ramirez 1990; Ramirez et al. 1996). Although nuclear progesterone and estrogen receptors are not detected in abundance in the AcbC, many brain regions that project to the AcbC express nuclear estrogen and progesterone receptors and could play an instrumental role in modulating MSN electrophysiology. We also note that testosterone may be acting in males to modulate AcbC properties, since it has been documented that long-term testosterone exposure in males also modulates nucleus accumbens dendritic spine density (Wallin-Miller et al. 2016).

The present experiments generate but cannot differentiate these two not necessarily mutually exclusive models, creating multiple future avenues of investigation. It will be critical for future experiments to differentiate between early proestrus and late proestrus. More specifically, a future experiment will need

to test the hypothesis as to whether changes in excitatory synapse properties that occur during late diestrus/early proestrus, when estradiol levels begin to rise, are unaccompanied by changes in intrinsic excitability. Furthermore, another critical next experiment will be to employ exogenous systemic exposures to estradiol, progesterone, and a combination of estradiol and progesterone in ovariectomized females to determine the relative contribution of each hormone toward inducing specific changes in MSN electrophysiological properties. Other future directions include establishing the MSN subtypes and AcbC interneurons that are sensitive to the estrous cycle and testing whether estrous cycle sensitivity is present in striatal regions beyond the AcbC. We suspect that this is the case. In adult caudate-putamen, for example, experiments published in the 1980s demonstrated that systemic estradiol exposure in ovariectomized adult female rats elevated in vivo spontaneous action potential generation and dopamine sensitivity (Arnauld et al. 1981). Later experiments elucidated that nigrostriatal MSNs increased in vivo spontaneous action potential generation during proestrus and estrus phases in gonad-intact adult female rats (Tansey et al. 1983) and in response to exogenous estradiol exposure in ovariectomized adult female rats. However, other MSN subtypes and striatal interneurons were not examined.

One difference between this study and two previous studies of sex differences in AcbC MSN is in regard to mEPSC properties. Previous studies of adult MSNs in females of unreported estrous cycle phase and of prepubertal MSNs detected increased mEPSC frequency in females compared with males (Cao et al. 2016; Wissman et al. 2011), similar to the present study. However, these previous studies did not detect sex differences in mEPSC amplitude or decay, unlike the present study. Several possibilities exist that could explain these differences. First, previous experiments did not separate female animal data by estrous cycle and also, by necessity, employed animals exposed to either cocaine or vehicle injections before analysis, unlike the present work (Wissman et al. 2011). Second, the other relevant experiment employed prepubertal animals (Cao et al. 2016). It is possible that activational hormone effects during puberty modulate the excitatory synaptic inputs onto the AcbC, reminiscent of changes in dopamine receptor complement in the AcbC during puberty (Andersen et al. 2002; Brenhouse et al. 2015; Teicher et al. 1995). Furthermore, the properties of excitatory synapse in the nucleus accumbens shell have been shown to be sensitive to the effects of environmental stimuli such as drugs of abuse and stress (Brancato et al. 2017; Hodes et al. 2015), reminiscent of classical research in the hippocampus (Shors et al. 2001). Although it remains formally possible that the lack of sex differences in the gonadectomized animals is because of surgical stress instead of hormone cycle removal, this explanation is unlikely because stress generally augments sex differences in excitatory synapse genetic markers in the nucleus accumbens, at least as demonstrated to date (Brancato et al. 2017), a finding consistent with the sex differences in incidence and phenotype of stress-linked disorders such as depression and anxiety (Altemus et al. 2014).

We also acknowledge that the temperature of mEPSC recordings differed between the present study and previous studies. This study recorded mEPSC properties at an average of ~22°C, whereas Wissman and colleagues recorded at ~34°C and Cao and colleagues recorded at $\sim 28^{\circ}$ C. Thus it is possible that a higher recording temperature would have eliminated differences in mEPSC amplitude and decay. Housing conditions also differed between previous studies. Cao and colleagues and the present study both employed glass water bottles, BPA-free caging, soy-free animal diets, and corncobfree bedding to help control for the potential confounding influences of endocrine disruptors. Wissman and colleagues, on the other hand, do not report employing any of these specialized housing conditions. Thus it is possible that uncontrolled exposure to endocrine disruptors may decrease or otherwise alter the magnitude of naturally occurring sex differences (Patisaul 2017). However, mitigating this speculation are the sex differences detected in mEPSC frequency by the present study, which are similar in magnitude to previous studies. In addition, the conditions under which mEPSCs were recorded (-70 mV, in the presence of PTX and TTX), along with the control experiment employing an AMPA receptor antagonist reported in METHODS, ensure that AMPA-mediated activity encompasses the majority of if not all mEPSC activity. This suggests that estrous cycle-induced changes in mEPSC properties are generated by a combination of underlying factors, including increases in excitatory synapse number (Cao et al. 2016; Wissman et al. 2011), along with potential changes in AMPA receptor number, composition, or phosphorylation, on the postsynaptic side of the synapse. Given that MSNs in the AcbC and other striatal regions express membrane-associated estrogen receptors, it is possible that hormones are acting in the AcbC to directly manipulate excitatory synapse function, as in

other brain regions such as the hippocampus (Oberlander and Woolley 2016).

The present study focuses on the changes in circulating plasma sex steroid hormone levels across the estrous cycle. Sex steroid hormones can also be manufactured in the brain independent of gonad activity. While there are strong species differences in estradiol levels in the telencephalon, rat AcbC contains aromatase, and estradiol has been measured in the nucleus accumbens of ovariectomized rats (Morissette et al. 1992). Furthermore, in the caudate-putamen, it has been reported that at least one form of long-term potentiation associated with excitatory synapse onto male MSNs is dependent on aromatase activity (Tozzi et al. 2015). Thus, although gonadectomy and the consequent elimination of changes in circulating sex steroid hormones are sufficient for eliminating estrous cycle-induced sex differences in MSN electrophysiology, it remains possible that local hormone synthesis could be playing a role in other aspects of AcbC function. More broadly, given that environmental stimuli such as exposure to drugs of abuse can manipulate other excitatory synapse properties such as NMDA-to-AMPA ratio (Andersen et al. 2002; Brenhouse et al. 2015; Jedynak et al. 2016; Kourrich et al. 2007; Rothwell et al. 2011; Teicher et al. 1995), it is likely that many sex differences in AcbC synaptic physiology remain undiscovered. Collectively, these sex differences and hormone sensitivity may not only set the stage for sex differences in AcbC-influenced behaviors but also prepare this brain region for the rewardrelated activities related to reproduction (Bradley et al. 2005).

Beyond the AcbC, it is notable that relatively few brain regions, much less neuron types, have been tested for estrous cycle differences in neuron function (Alreja 2013; Blume et al. 2017; Salvatore et al. 2018; Terasawa and Timiras 1968; Wang et al. 2016; Woolley et al. 1990). We suspect that many other neuron types and perhaps even glia, given their sensitivity to estradiol, also exhibit differential properties across cycle phases in sexually mature females. This potential has profound implications for our understanding of the plasticity inherent in neuron physiology in both females and males.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.P. and J.M. conceived and designed research; S.P. performed experiments; S.P., H.J.M., L.M.K., D.M.D., and J.M. analyzed data; S.P. and J.M. interpreted results of experiments; S.P., D.M.D., and J.M. prepared figures; S.P. and J.M. drafted manuscript; S.P., D.M.D., and J.M. edited and revised manuscript; S.P., H.J.M., L.M.K., D.M.D., and J.M. approved final version of manuscript.

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