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Intrinsic excitability varies by sex in pre-pubertal striatal medium spiny neurons

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Abstract

Sex differences in neuron electrophysiological properties were traditionally associated with brain regions directly involved in reproduction in adult, post-pubertal animals. There is growing acknowledgement that sex differences can exist in other developmental periods and brain regions as well. This includes the dorsal striatum (caudate/putamen), which shows robust sex differences in gene expression, neuromodulator action (including dopamine and 17β-estradiol), and relevant sensorimotor behaviors and pathologies such as the responsiveness to drugs of abuse. Here we examine whether these sex differences extend to striatal neuron electrophysiology. We test the hypothesis that passive and active medium spiny neuron (MSN) electrophysiological properties in pre-pubertal rat dorsal striatum differ by sex. We made whole-cell recordings from male and females MSNs from acute brain slices. The slope of the evoked firing rate to current injection curve was increased in MSNs recorded from females compared to males. The initial action potential firing rate was increased in MSNs recorded from females compared to males. Action potential afterhyperpolarization peak was decreased and threshold was hyperpolarized in MSNs recorded from females compared to males. No sex differences in passive electrophysiological properties or miniature excitatory synaptic currents were detected. These findings indicate that MSN excitability is increased in pre-pubertal females compared to males, providing a new mechanism that potentially contributes to generating sex differences in striatal-mediated processes. Broadly, these findings demonstrate that sex differences in neuron electrophysiological properties can exist pre-puberty in brain regions not directly related to reproduction.

Keywords

Intrinsic excitability, sex differences, medium spiny neuron, dorsal striatum, mEPSC

Introduction

Neural sex differences are well established in many vertebrate brain regions, especially those directly involved in reproduction in adult, post-pubertal animals (Breedlove 2002; De Vries 2004; Yang and Shah 2014). Examples of these include the sexually-dimorphic nucleus of the preoptic area (SDN) (Gorski et al. 1978), the spinal nucleus of the bulbocavernosus (SNB) (Breedlove and Arnold 1981), and the telencephalic song control nuclei in sexually dimorphic songbirds (Nottebohm and Arnold 1976). These now famous brain regions all show robust sex differences in neuroanatomy and physiology and clear behavioral relevance to sex specific behaviors. What is less clear is the extent of sex differences in basic neurophysiological properties in brain regions without such dramatic sex differences in neuroanatomy, especially during the pre-pubertal period widely used for electrophysiological recordings. This is an important and timely question, given the current debate regarding how to properly account for sex in both basic and clinical biomedical science (Arnold and Lusis 2012; Beery and Zucker 2011; Cahill 2006; Geller et al. 2011; Prendergast et al. 2014; Woodruff et al. 2014), the known sex differences in many neural pathologies (Becker et al. 2013; Cosgrove et al. 2007; Giorgi et al. 2014; Ober et al. 2008), and the growing literature for sex differences in synaptic organization/neuromodulation across the nervous system (Babayan and Kramar 2013; Cooke and Woolley 2005; Huang and Woolley 2012; Mermelstein et al. 1996; Nunez and McCarthy 2008; Remage-Healey 2014; Srivastava et al. 2010).

We thus chose to investigate sex differences in the rat dorsal striatum (caudate/putamen). This brain region was targeted for both its prominence and the known sex differences in striatal-mediated behaviors and pathologies. These include significant sex differences in steroid sex hormone influences on sensorimotor function and behaviors, impulsivity, and striatal-mediated learning (Becker 2002; Calhoun 1962; Eckel et al. 2000; Hosseini-Kamkar and Morton 2014; Zurkovsky et al. 2007). Regarding pathologies, robust sex differences exist in the responsiveness to drugs of abuse in both humans and rats (Becker and Hu 2008; Becker et al. 2013; Bobzean et al. 2014; Carroll and Anker 2010; Fattore et al. 2014). Across models, females exhibit increased locomotor sensitivity, escalation, and motivation to take psychostimulants after initial exposure when compared to males, with estradiol playing a significant mechanistic role. Interestingly, the rat dorsal striatum and nucleus accumbens expresse little to no nuclear estrogen receptors, and instead express membrane-associated estrogen receptors α, β, and GPER-1 (Almey et al. 2012; Grove-Strawser et al. 2010; Kuppers and Beyer 1999; Mermelstein et al. 1996; Schultz et al. 2009).

Given the importance of these behaviors and pathologies, much research has established sex differences in adult striatum gene expression (Chen et al. 2009; Ghahramani et al. 2014; Trabzuni et al. 2013), estradiol sensitivity (Cummings et al. 2014; Grove-Strawser et al. 2010; Mermelstein et al. 1996; Schultz et al. 2009), catecholamine action (Becker and Hu 2008; Becker et al. 2013; Di Paolo 1994; Meitzen et al. 2013), ΔFosB expression (Sato et al. 2011), and GABA and dopamine release (Becker 1990; Hu et al. 2006; Walker et al. 2000; Xiao and Becker 1998).

In a related brain region, the nucleus accumbens, sex differences in synaptic organization have been found (Forlano and Woolley 2010; Wissman et al. 2012; Wissman et al. 2011). In contrast to regions like the SDN, SNB, and sexually dimorphic song control nuclei, striatal brain regions show no sex differences in neuron density or soma size (Meitzen et al. 2011), and the volume of the nucleus accumbens does not differ by sex (Campi et al. 2013). Most notably, it is unknown whether the basic electrophysiological properties of striatal neurons differ by sex.

Here we test the hypothesis that passive and active medium spiny neuron (MSN) electrophysiological properties in pre-pubertal rat dorsal striatum (caudate/putamen) differ by sex. The pre-pubertal period was chosen as it is widely used for electrophysiological recordings. We raised male and female rats and then recorded from MSNs using whole-cell current clamp configuration in acute brain slices of dorsal striatum. We found that the active electrophysiological properties varied by sex, with female MSNs exhibiting increased intrinsic excitability compared to male MSNs. No sex differences in passive electrophysiological properties or miniature excitatory synaptic currents (mEPSCs) were detected. These findings provide a new mechanism which potentially contributes to sex differences in striatal-mediated behavior and pathologies. Broadly, these findings demonstrate that sex differences in neuron electrophysiological properties can exist pre-puberty in brain regions not directly related to reproduction.

Materials and Methods

Animals

All animal protocols were approved by Institutional Animal Care and Use Committee at North Carolina State University or the Marine Biological Laboratory. Experiments took place at both institutions. Female (n=18) and male (n=18) Sprague-Dawley CD IGS rats were born from timed-pregnant females purchased from Charles River (Raleigh, NC). Rats were housed with their littermates and dam until weaning on postnatal day 21 (P21), and afterward with same-sex littermates. Age at experimental use ranged from P11 to P23, and was matched between sexes (Mean ± SEM: Male: P15 ± 1; Female: P15 ± 1). All cages were washed polysulfone (BPA-free) and were filled with bedding manufactured from virgin hardwood chips (Beta Chip, NEPCO, Warrensburg, NY) to avoid the endocrine disruptors present in corncob bedding (Mani et al. 2005; Markaverich et al. 2002; Villalon Landeros et al. 2012). Rooms were temperature, humidity and light-controlled (23 ºC, 40% humidity, 12h light:12h darkness cycle). Soy protein-free rodent chow (2020X, Teklad, Madison, WI USA) and glass-bottle provided water were available *ad libitum.*

Electrophysiology

Preparation of brain slices

Methods for preparing brain slices for electrophysiological recordings were as previously described (Dorris et al. 2014). Rats were deeply anesthetized with isoflurane gas and killed by decapitation. The brain was dissected rapidly into ice-cold, oxygenated sucrose artificial CSF (s-ACSF) containing (in mM): 75 sucrose, 1.25 NaH_2PO_4, 3 $MgCl_2$, 0.5 $CaCl_2$, 2.4 Na pyruvate, 1.3 ascorbic acid from Sigma-Aldrich, St. Louis, MO, and 75 NaCl, 25 $NaHCO_3$, 15 dextrose, 2 KCl from Fisher, Pittsburg, PA; osmolarity 295-305 mOsm, pH 7.2-7.4. Coronal brain slices (300 µm) were prepared using a vibratome and then incubated in regular ACSF containing (in mM): 126 NaCl, 26 $NaHCO_3$, 10 dextrose, 3 KCl, 1.25 NaH_2PO_4, 1 $MgCl_2$, 2 $CaCl_2$, 295-305 mOsm, pH 7.2-7.4) for 30 minutes at 35ºC, and at least 30 minutes at room temperature (21-23 ºC). Slices were stored submerged in room temperature, oxygenated ACSF for up to 5 hours after sectioning in a large volume bath holder.

Electrophysiological recording

After resting for ≥1 hour after sectioning, slices were placed in a Zeiss Axioscope equipped with IR-DIC optics, a Dage IR-1000 video camera, and 10X and 40X lenses with optical zoom. Slices were superfused with oxygenated ACSF heated to 27±1 ºC (Male: 27 ± 1 ºC; Female: 27 ± 1 ºC, P>0.05). In some experiments, ACSF contained the $GABA_A$ receptor antagonist Picrotoxin (150 µM; Fisher), the NMDA receptor antagonist D-AP5 (10 µM, Sigma-Aldrich), and the AMPA receptor antagonist DNQX (25 µM, Tocris, Minneapolis, MN). Whole-cell patch-clamp recordings were made from medium spiny neurons (MSNs) in the dorsal striatum using glass electrodes (4-8 MΩ) containing (in mM): 115 K D-gluconate, 8 NaCl, 2 EGTA, 2 $MgCl_2$, 2 MgATP, 0.3 NaGTP, 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 software. Membrane potentials were corrected for a calculated liquid junction potential of -13.5 mV. Using previously described procedures (Farries et al. 2005; Meitzen et al. 2009), recordings were 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, MSNs were then voltage-clamped at -70 mV and mEPSCs recorded in the presence of tetrodotoxin (TTX; 1 µM; Abcam Biochemicals) and picrotoxin (PTX, 150 µM). These recording parameters make the recorded mEPSCs to be most likely AMPA receptor mediated. mEPSCS were recorded for at least five minutes, with the exception of one female neuron which was recorded for three minutes. Input and series resistance was monitored for possible changes, and cells were discarded if input or series resistance changed more than 15%.

Data analysis

Basic electrophysiological properties and action potential characteristics were analyzed using pClamp 10. After break-in, the resting membrane potential was first allowed to stabilize ~1-2 minutes, as in (Mu et al. 2010). We then injected at least three series of depolarizing and hyperpolarizing current injections to elicit basic neurophysiological properties (Meitzen et al. 2009). For most properties measured, we followed the definitions of (Meitzen et al. 2009), which were drawn from those of Farries and colleagues (Farries et al. 2005; Farries and Perkel 2000; 2002). Following Farries, for each neuron, measurements were made of at least five action potentials generated from minimal current injections. These measurements were then averaged to generate the reported action 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 five action potentials per neuron. We used different methods from Farries to calculate action potential threshold, steady state firing rate, rectified range input resistance, inward rectification, and percent inward rectification. Action potential threshold was defined as the first point of sustained positive acceleration of voltage ($\delta^2V/\delta t^2$) that was also more than three times the SD of membrane noise before the detected threshold (Baufreton et al. 2005). We defined steady-state firing rate as the mean firing rate over the last 300 ms of the current pulse (Gale and Perkel 2006). Initial firing rate was defined as the inverse of the first interspike interval. Rectified range input resistance, inward rectification, and percent inward rectification was calculated as described previously (Belleau and Warren 2000). The slope of the evoked firing rate to positive current curve (FI slope) was calculated from the first current which evoked an action potential to the first current that generated the maximum evoked firing rate (Meitzen et al. 2009). Input resistance in the linear, non-rectified range was calculated from the steady-state membrane potential in response to −0.02 nA hyperpolarizing pulses. The membrane time constant was calculated by fitting a single exponential curve to the membrane potential change in response to −0.02 nA hyperpolarizing pulses. Membrane capacitance was calculated using the following equation: capacitance = membrane time constant/input resistance. Sag index was used to assess possible sex differences in hyperpolarization-induced "sag" (i.e., I_H current) (Farries et al. 2005). Sag index is 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. Thus, a cell with no sag would have a sag index of 0, whereas a cell whose maximum voltage deflection is twice that of the steady-state deflection would have a sag index of 1. Cells with considerable sag typically have an index of ≥0.1.

mEPSCs frequency, amplitude, and decay were analyzed off-line using Mini Analysis (Synaptosoft, http://www.synaptosoft.com/MiniAnalysis/). Threshold was set at 2.5 the value of the root mean square of 10 blocks of the baseline noise with a minimum value of 5 pA, and accurate event detection was validated by visual inspection. There were no differences in root mean square noise between sexes (Male: 1.1 ± 0.01, Female: 1.5 ± 0.02, $t_{(19)}$=1.923; P>0.05).

Statistics

Experiments were analyzed using two-tailed *t* tests, Mann Whitney tests, Kolmogorov-Smirnov tests, one or two way ANOVAs, linear regressions or ANCOVAs (Excel 2010; Microsoft, Redmond, WA or Prism version 5.00; GraphPad Software, La Jolla, CA). Distributions were analyzed for normality using the D'Agostino & Pearson omnibus normality test, and t tests or Mann Whitney tests were employed as appropriate. The use of t tests or Mann Whitney tests did not alter overall experimental conclusions. P values < 0.05 were considered *a priori* as significant. Data are presented as mean ± SEM.

Results

We recorded from 33 medium spiny neurons (MSNs) from 18 pre-pubertal male rats and 32 MSNs from 18 pre-pubertal female rats. MSNs are the predominant neuron type in the dorsal striatum, projecting both within and outside the brain region. MSN electrophysiological properties closely resembled those reported in earlier studies that used males or animals of undetermined sex, including the presence of a slow ramping subthreshold depolarization in response to low-magnitude positive current injections, a hyperpolarized resting potential, inward rectification, and prominent spike afterhyperpolarization (Figure 1A; Table 1) (Belleau and Warren 2000; Farries et al. 2005; O'Donnell and Grace 1993; Shen et al. 2004).

Female medium spiny neurons show increased evoked firing rates compared to male neurons

We then tested the hypothesis that MSN electrophysiological properties varied between males and females by injecting MSNs with a series of positive and negative currents and assessing standard electrophysiological properties (Table 1). Several electrophysiological properties related to intrinsic excitability varied by sex, collectively indicating that female MSNs showed increased excitability compared to male MSNs. Action potential firing rates evoked by depolarizing current injection were visibly increased in MSNs recorded from female rats compared to male rats (Figure 1A, 1B). We quantified this by comparing the slope of the evoked firing rate to positive current curve (FI slope) between male and female MSNs (Figure 1C). MSNs from female animals showed a steeper FI slope compared to MSNs recorded from male animals ($t_{(61)}$=3.026; P<0.004). This indicates that female neurons fire more action potentials per ampere of injected current throughout the linear range, reaching maximum rates more quickly. Supporting this finding, the cumulative distribution of female MSN FI slopes also differed from that recorded from male MSNs (Figure 1D; $D_{(61)}$=0.3909; P<0.02). These data indicate that MSNs show increased excitability in females compared to males.

There was no difference in maximum firing rate between MSNs recorded from males and females (Male: 16.7 ± 1.7 Hz, Female: 15.2 ± 1.2 Hz, $t_{(61)}$=0.70; P>0.05), suggesting that the sex difference in action potential generation likely involves either the delay to first spike or the initial firing rate (initial: firing rate of the first interspike interval, early in the current injection). No sex differences were detected in the delay to first spike at either the minimum current injection necessary for action potential generation (Table 1), or at Δ10 pA from the minimum current

injection necessary for action potential generation (Male: 99 ± 7 msec, Female: 99 ± 9, P>0.05). Instead, female MSNs showed increased initial firing rates compared to male MSNs (Figure 1E, Sex: $F_{(1, 259)}$=7.014; P<0.009, Current: $F_{(5, 259)}$=24.22; P<0.0001, Interaction: $F_{(5, 259)}$=0.1556; P>0.05). We note that one outlier (>+4 standard deviations from the mean) in the female group was not included in analysis of the instantaneous firing rate. Removal of this outlier did not change experimental conclusions (Statistics including outlier: Sex: $F_{(1, 265)}$=9.251; P<0.003, Current: $F_{(5, 265)}$=3.687; P<0.004, Interaction: $F_{(5, 265)}$=0.0852; P>0.05). Conversely, while the overall magnitude of the steady state firing rate (steady state: mean firing rate over the last 300 ms of the current injection) was increased in female MSNs compared to male MSNs across current injections, this did not reach statistical significance (Figure 1F, Sex: $F_{(1, 265)}$=2.616; P>0.05, Current: $F_{(5, 265)}$=8.187; P<0.0001, Interaction: $F_{(5, 265)}$=0.1030; P>0.05).

Female medium spiny neurons show decreased afterhyperpolarization peak and hyperpolarized action potential threshold compared to male neurons

A sex difference in evoked firing rate suggests that there may be differences in either action potential (AP) properties or the passive membrane properties of the MSN. Regarding action potential properties, female MSNs showed decreased magnitude of the afterhyperpolarization (AHP) peak compared to male MSNs (Figure 1G; $t_{(61)}$=3.035; P<0.004). The cumulative distribution of AHP peaks recorded from female MSNs also differed from those recorded from male MSNs (Figure 1H; $D_{(61)}$=0.3212; P<0.005). The AP threshold also differed by sex, with female MSNs showing a hyperpolarized AP threshold compared to male MSNs (Figure 1I; $t_{(61)}$=2.099; P<0.041). The cumulative distribution of female MSN AP thresholds also differed from those recorded from male MSNs (Figure IJ; $D_{(61)}$=0.2697; P<0.05). No other AP property differed by sex (Table 1). These data support the hypothesis that decreased AHP peak and hyperpolarized AP potentials comprise part of the mechanism underlying increased excitability in female MSNs, especially given that both of these properties are associated with changes in MSN excitability in other contexts (Mu et al. 2010; Shen et al. 2005).

We reasoned that if these sex differences in AP properties contribute to sex differences in FI slope, then these properties should be correlated. We thus calculated linear regressions between MSN FI slopes and, respectively, AHP peaks and AP thresholds. Increased FI slopes strongly associated with decreased AHP peaks (Figure 2A; Slope: 0.02, r^2=0.35, P<0.001). Likewise, FI slope and AP threshold also correlated, with increased FI slopes associating with hyperpolarized AP thresholds (Figure 2B; Slope: -0.02, r^2=0.08, P<0.03). To validate this methodology, we also calculated a linear regression between AHP peak and AP threshold, finding that decreased AHP peaks associated with hyperpolarized thresholds (Figure 2C, Slope: -1.22, r^2=0.35, P<0.0001). This makes sense given that female MSNs exhibit both of these properties and increased FI slope. These data indicate that decreased AHP peak and hyperpolarized AP threshold are associated with increased neuron excitability.

In addition to AP properties, other mechanisms that could potentially drive differences in MSN excitability are changes in passive membrane properties such as the input resistance or the membrane time constant. Passive membrane properties were not different between male and female MSNs (Table 1). This included input resistance in both the linear and rectified ranges (Figure 3; $F_{(3,570)}$=0.02657, P>0.05), the membrane time constant, and capacitance (Table 1). The lack of sex differences in capacitance is consistent with a previous report showing no sex difference in medium spiny neuron soma size in rat dorsal striatum (Meitzen et al. 2011). These results support the hypothesis that sex differences in MSN excitability are driven by differences in action potential properties and not passive membrane properties.

Sex differences are not blocked by glutamate and GABA receptor antagonists

Our working model regarding these particular sex differences in MSN electrophysiological properties is that they are intrinsic to the neuron and not driven by external synaptic input. If this interpretation is correct, then sex differences should be preserved during blockade of glutamatergic and GABAergic receptors. To test this hypothesis, we exposed a subset of MSNs to a cocktail of the NMDA receptor antagonist D-AP5 (10 µM), the AMPA receptor antagonist DNQX (25 µM), and the $GABA_A$ receptor antagonist Picrotoxin (PTX; 150 µM). This drug combination eliminated spontaneous post-synaptic potentials (Figure 4A), indicating effective blockade of glutamatergic and GABAergic receptors. We then assessed standard electrophysiological properties as described above. Exposure to D-AP5, DNQX, and PTX did not eliminate sex differences in either FI slope (Figure 4B, Sex: $F_{(1, 8)}$=13.92; P<0.03, Drug: $F_{(2, 8)}$=1.78; P>0.05), AHP peak (Figure 4C; Sex: $F_{(1, 8)}$=13.92; P<0.045, Drug: $F_{(2, 8)}$=1.78; P>0.05) or AP threshold (Figure 4D; $F_{(1, 8)}$=13.92; P<0.02, Drug: $F_{(2, 8)}$=0.07; P>0.05). Exposure to D-AP5, DNQX, and PTX did not alter any other measured electrophysiological property (data not shown). We also note that the sampled male neurons by chance exhibited decreased FI Slopes and increased magnitudes of the AHP peak compared to the overall data set (analysis not shown). Overall, these data support the hypothesis that sex differences in MSN electrophysiological properties are intrinsic, and not driven by glutamatergic or GABAergic synaptic input.

Sex differences are present across all examined ages

A priori, we hypothesized that sex differences in electrophysiological properties would be stable throughout the recorded pre-pubertal period. This hypothesis was chosen given that MSNs reach electrophysiological maturity after the initial critical period for organizational steroid sex hormone action, which may program sex differences in the dorsal striatum and relevant behaviors/pathologies. In order to test the interaction of sex differences in MSN electrophysiological properties with age at recording, MSNs were analyzed across a sex-matched age range that encompassed the late pre-pubertal period (Male: P15 ± 1, Female: P15 ± 1, $t_{(65)}$=0.36; P>0.05). The age range began with the onset of the presence of mature MSNs (~P11) to prior to weaning (~P20) to just before the beginning of the peri-pubertal period (~P22-P24).

We then calculated linear regressions between age and male and female MSN FI slope, AHP peak and AP threshold. We first analyzed whether the slopes of the linear regressions were significantly non-zero to determine whether the measured property differed by age. We then analyzed whether the slopes and elevations/intercepts differed by sex.

FI Slope decreased with age in both males and females (Figure 4E, Male: slope: -11.82, r^2=0.14, P<0.05, Female: slope: -12.24, r^2=0.13, P<0.05), similar to previously reported results in MSNs recorded from rat nucleus accumbens of unknown sex (Belleau and Warren 2000). The slopes of the linear regressions did not differ by sex ($F_{(1,59)}$=0.00, P>0.05). However, the intercept of the regression did vary by sex, with females showing increased FI Slope across all ages (Male: intercept: 496, Female: intercept: 571, $F_{(1,60)}$=6.84, P<0.02). AHP peak amplitude remained stable with age in females but not in males (Figure 4F, Male: slope: -0.453, r^2=0.14, P<0.03, Female: slope: 0.04, r^2=0.00, P>0.05). Thus, the slopes of the linear regressions differed by sex ($F_{(1,59)}$=4.14, P<0.05). AP threshold did not change across age in either males or females (Figure 4G, Male: slope: -0.06, r^2=0.00, P>0.05, Female: slope: -0.36, r^2=0.03, P>0.05), similar to previously reported results of accumbal and dorsal striatal MSNs of unknown sex (Belleau and Warren 2000; Tepper et al. 1998). Accordingly, the slopes of the linear regressions did not differ by sex ($F_{(1,59)}$=0.30, P>0.05). The intercept did differ by sex, with females showing hyperpolarized AP threshold across all ages compared to males (Male: intercept: -53.08, Female: intercept: -52.62, $F_{(1,60)}$=4.78, P<0.035). We note that sex differences in the linear regressions of FI Slope, AHP peak, and AP threshold are maintained when post-weaning animals are excluded from analysis (data not shown). Collectively, these results indicate that sex differences in MSN electrophysiological properties are determined early in development and are already present in the pre-pubertal period analyzed here.

No sex differences are detected in mEPSC properties

In a subset of recordings, we voltage clamped 10 male and 11 female MSNs to -70 mV and recorded mEPSCs in the presence of TTX and PTX (Figure 5A). We then analyzed mEPSC frequency, amplitude, and decay (Table 2). This was spurred by the recognition that intrinsic electrophysiological properties act in concert with synaptic properties to generate neuronal output, and by a previous report of sex differences in mEPSC properties in adult rat nucleus accumbens core (Wissman et al. 2011). No sex differences were detected in mEPSC frequency (Figure 5B; $t_{(19)}$=1.632; P>0.05), mEPSC amplitude (Figure 5C; $t_{(19)}$=0.5259; P>0.05), or mEPSC decay (Figure 5D; $t_{(19)}$=1.078; P>0.05). To further test this conclusion, we then analyzed mEPSC properties by age at recording. To do this, we calculated linear regressions between age and male and female MSN mEPSC frequency, amplitude, and decay. We first analyzed whether the slopes of the linear regressions were significantly non-zero to determine whether the measured property differed by age. We found no slopes that were significantly non-zero, indicating little change over the analyzed age range (data not shown). We then analyzed whether the slopes and elevations/intercepts of mEPSC frequency, amplitude, and decay differed by sex.

No differences were detected (data not shown). Overall, these data indicate that sex differences in pre-pubertal MSN do not include mEPSC properties.

Discussion

There are five general findings of these experiments. First, the slope of the evoked firing rate to current injection curve was increased in MSNs recorded from females compared to males. Second, the initial action potential firing rate was increased in MSNs recorded from females. Third, action potential afterhyperpolarization peak was decreased and action potential threshold hyperpolarized in MSNs recorded from females. Fourth, no sex differences in passive electrophysiological or mEPSC properties were detected. Fifth, these sex differences were not attenuated by GABAergic or glutamatergic receptor blockade and were present across all ages examined. These findings indicate that MSN intrinsic membrane excitability in the dorsal striatum is increased in pre-pubertal females compared to males. Broadly, these findings show that sex differences in neuron electrophysiological properties can occur in brain regions not directly related to reproduction, and during the pre-pubertal developmental period in which many neuron electrophysiological recordings take place.

To our knowledge this is the first report of a sex difference in an intrinsic electrophysiological property of the medium spiny neuron, providing a new potential mechanism for the known sex differences in striatal-mediated behavior and pathologies. Intrinsic membrane excitability regulates action potential generation in response to synaptic input (Hille 2001), making intrinsic excitability a key player in determining the functional output of striatal circuitry and a final common convergence point of all striatal sex differences. Multiple ion channels have been implicated in generating differences in intrinsic excitability, with particular targets in MSNs including soma-expressed sodium channels, calcium-activated potassium channels, and L-type calcium channels (Hille 2001; Kole and Stuart 2012; Kourrich and Thomas 2009; Mermelstein et al. 1996; Mu et al. 2010; Zhang et al. 1998). Determining which of these drive sex differences in intrinsic excitability is an important future extension of this study.

Intrinsic excitability is strongly implicated in both normal and pathological striatal function (Ishikawa et al. 2009; Kourrich and Thomas 2009; Mu et al. 2010; Wolf 2010; Zhang et al. 1998). Indeed, previous research has not only found differences in MSN intrinsic membrane excitability related to homeostatic plasticity (Ishikawa et al. 2009) and drugs of abuse (Kourrich and Thomas 2009; Mu et al. 2010; Wolf 2010; Zhang et al. 1998), but also differences in intrinsic excitability between D1- and D2-dopamine receptor expressing MSNs (Gertler et al. 2008; Planert et al. 2013). These differences in intrinsic excitability between MSN subtypes are already present in rats during the pre-pubertal period examined here. One possibility is that sex differences in MSN intrinsic excitability were generated by differential sampling of D1- or D2-dopamine receptor expressing MSNs. We believe this to be unlikely. In rats of the same age range employed in the current study, D1- versus D2-dopamine receptor expressing MSNs showed differences in passive membrane properties including input resistance, the membrane

time constant, and rheobase (Planert et al. 2013). We found no sex differences in passive membrane properties, only in active properties such as the slope of the FI curve and AP threshold and AHP peak amplitude. There were also no signs of bimodality in any property. Thus, the recorded sex differences in MSN intrinsic excitability do not match those reported for rat MSN subtypes, and are not likely driven by differential sampling.

A related question is whether both MSN subtypes show sex differences in intrinsic excitability. Future studies should directly test this question. In the meantime, we sorted the present dataset for each sex to find neurons with the 10 lowest and 10 highest values for three attributes that differ between rat MSN subtypes: input resistance, membrane time constant, and rheobase (Planert et al. 2013). We did this to bias each dataset towards containing unequal proportions of MSN subtypes. We then used 2 way ANOVAs to analyze whether the FI slope of these neurons significantly differed in both the low value and high value groups of input resistance, membrane time constant, and rheobase (analysis not shown). For all three attributes sex was a significant source of variation. However there was no interaction between sex and attribute amplitude. None of the attributes demonstrated a bimodal distribution. This is consistent with the hypothesis that sex differences may be present in both MSN subtypes. Supporting this, both MSN subtypes express non-nuclear, membrane-associated, estrogen receptor α and β expression in the dorsal striatum (Almey et al. 2012; Grove-Strawser et al. 2010; Kuppers and Beyer 1999; Mermelstein et al. 1996; Schultz et al. 2009; Toran-Allerand et al. 1992). Large area estradiol infusions into female dorsal striatum modulates sensorimotor performance (Becker et al. 1987), paced mating behavior (Xiao and Becker 1997), and learning and memory tasks (Zurkovsky et al. 2007). This indicates that estradiol activation of both MSN subtypes does not appear to compromise estradiol-induced changes in striatal-mediated behaviors. Furthermore, the dorsal striatum as a whole is necessary for aspects of maternal behavior (Henschen et al. 2013; Keer and Stern 1999). We thus tentatively speculate that sex differences in intrinsic excitability are present in both D1- and D2-dopamine receptor expressing MSNs with the full acknowledgement that this prediction needs to be empirically tested.

Changes in intrinsic membrane excitability can occur either independently or in concert with other neuronal attributes such as changes in extracellular non-synaptic glutamate levels and/or synaptic input (Otaka et al. 2013; Schulz 2006; Suska et al. 2013; Wolf 2010; Zakon 1998). In the current study, no sex differences in mEPSC properties were detected. Possible sex differences in excitatory or inhibitory synaptic input has not yet been examined in adult dorsal striatum or in pre-pubertal nucleus accumbens. Interestingly, Woolley and colleagues found increased mEPSC frequency and spine density in female rat adult MSNs located in the nucleus accumbens core and to a lesser extent in the nucleus accumbens shell, and that these properties are modulated by cocaine exposure (Forlano and Woolley 2010; Wissman et al. 2012; Wissman et al. 2011). Increased spine density has since been detected in female medium spiny neurons in human nucleus accumbens (Sazdanovic et al. 2013). MSN spine density is modulated by estradiol in adult female hamsters and rats in the nucleus accumbens core but not in other striatal

regions (Peterson et al. 2014; Staffend et al. 2011). Given that changes in synaptic input are often linked with changes in intrinsic excitability (Ishikawa et al. 2009; Wolf 2010), this raises the possibility that estradiol or cocaine exposure also differentially modulates intrinsic excitability by sex. While no sex differences in excitatory synaptic input were found by the current study, we do note that both intrinsic excitability and synaptic input may be reorganized during puberty, similar to glutamatergic synaptic input in the medial amygdala (Cooke 2011; Cooke and Woolley 2009), or modulated by adult hormone profile (Woolley 2007; Wu et al. 2011). These questions all represent outstanding avenues of research, in addition to determining ionic mechanism, the possible roles of dopamine and dopamine receptors, and whether the sex differences reported here are generated through a genetic/epigenetic mechanism or via the organizational influences of early steroid sex hormone exposure/absence (McCarthy and Arnold 2011).

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The authors declare that this research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflict of interests.

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Figure Legends

Figure 1. Evoked firing rate is increased in MSNs recorded from pre-pubertal rat females compared to males in dorsal striatum, and this is driven by sex differences in action potential properties. A) Response of a medium spiny neuron (MSN) from a male (left) and from a female (right) to depolarizing current injections. B) Evoked firing rate and injected current (FI) curves of individual MSNs differ by sex. C) MSNs recorded from females show an increased slope of the evoked firing rate to injected current curve (FI slope). D) The cumulative frequency distribution of MSN FI slopes is shifted to the right in females. E) Mean initial firing rate (the reciprocal of the first interspike interval) is increased in MSNs recorded from females. F) Mean steady-state firing rate (mean firing rate over the last 300 ms of the current injection) does not significantly differ by sex. However we do note that the mean steady state firing rate is consistently higher in females than in males. G) Action potential (AP) afterhyperpolarization (AHP) peak amplitude is decreased in females. H) The cumulative frequency distribution of MSN AHP peak amplitude is shifted to the right in females. I) AP threshold is hyperpolarized in females. J) The cumulative frequency distribution of MSN AP threshold is shifted to the left in females. The *P* value within each subpanel indicates statistical significance; complete statistical information is in Results.

Figure 2. Sex differences in AP properties correlate with sex differences in FI slope. A) Decreased AHP peaks associate with increased FI slopes. B) Hyperpolarized AP thresholds associate with increased FI slopes. C) Hyperpolarized AP thresholds associate with decreased AHP peaks. The *P* value within each subpanel indicates statistical significance; complete statistical information is in Results.

Figure 3. No sex difference is detected in input resistance in either the linear or rectified range. See Results section and Table 1 for statistical analysis.

Figure 4. Sex differences are not driven by synaptic input or by age at recording. A) Example blockade of miniature post-synaptic potentials via simultaneous application of PTX, DNQX, and AP-5 to block GABAergic and glutamatergic synaptic activity. B) Sex differences in FI Slope were not blocked by drug application. C) Sex differences in AHP peak were not blocked by drug application. D) Sex differences in AP threshold were not blocked by drug application. E) Relationship of FI Slope and age of the animal at recording. The linear regression of the female data set shows an increased intercept compared to the male. F) Relationship between AHP peak and age. The linear regression of the female data set shows a different slope compared to the male. G) Relationship of AP Threshold and age. The linear regression of the female data set shows a hyperpolarized intercept compared to the male. The *P* value within each subpanel indicates statistical significance; complete statistical information is in Results.

Figure 5. No sex differences are detected in mEPSC properties. A) Representative examples of mEPSCs recorded in male and female dorsal striatum MSNs. MSNs were voltage clamped at -70

mV and recorded in the presence of TTX and PTX to block voltage-gated sodium channels and GABAergic synaptic activity, respectively. B) Left: No sex difference was detected in MSN mEPSC frequencies. Right: No sex differences were detected in the cumulative frequency distribution of MSN mEPSC frequencies. C) Left: No sex difference was detected in MSN mEPSC amplitude. Right: No sex difference was detected in the cumulative frequency distribution of MSN mEPSC amplitudes. D) Left: No sex difference was detected in MSN mEPSC decay time. Right: No sex difference was detected in the cumulative frequency distribution of MSN mEPSC decay times. The *P* value within each subpanel indicates statistical significance; complete statistical information is in Results.

730 **Tables (please note that Table 1 legend is on the following page)**

731 Table 1. Electrophysiological properties of male and female medium spiny neurons

Property	Male	Female	Statistics (*t*/U, P)
Resting Potential (mV)	-81.2 ± 0.9 (33)	-80.0 ± 1.2 (31)	0.52, 0.40
Input Resistance (MΩ)	448 ± 42 (33)	465 ± 42 (31)	484, 0.72
Time Constant of the Membrane (ms)	23 ± 1 (33)	27 ± 2 (31)	1.74, 0.08
Capacitance (pF)	61 ± 4 (33)	69 ± 7 (31)	476, 0.64
Rectified Range Input Resistance (MΩ)	289 ± 29 (33)	291 ± 26 (31)	499, 0.87
Inward Rectification (MΩ)	156 ± 18 (33)	175 ± 19 (31)	0.72, 0.47
% Inward Rectification (%)	67 ± 2 (33)	65 ± 2 (31)	0.86, 0.39
Sag Index	0.01 ± 0.00 (33)	0.01 ± 0.00 (31)	501, 0.89
AP Threshold (mV)	**-54 ± 1 (33)**	**-58 ± 1 (30)**	**2.09, 0.040**
AP Amplitude (mV)	72 ± 2 (33)	75 ± 2 (30)	1.26, 0.21
AP width at half-peak (ms)	2.6 ± 0.1 (33)	2.6 ± 0.l (30)	0.27, 0.78
AHP Peak (mV)	**-10.8 ± 0.7 (33)**	**-8.3 ± 0.4 (30)**	**3.04, 0.004**
AHP Time to Peak (ms)	52.9 ± 2.9 (33)	47.9 ± 4.1 (30)	1.18, 0.24
Delay to first spike (ms)	313 ± 15 (33)	307 ± 19 (30)	0.24, 0.81
Rheobase (nA)	0.040 ± 0.008 (33)	0.025 ± 0.004 (30)	356, 0.053
FI Slope (Hz/nA)	**312.1 ± 18.5 (33)**	**395.2 ± 20.4 (30)**	**3.03, 0.004**

Notes: Values are mean ± SEM. Numbers in parentheses indicate sample size. The sag index is unitless. None of these neurons fired spontaneous action potentials. Statistical differences between groups are depicted with bold font. Data were analyzed with t tests or Mann Whitney U tests as appropriate. Action Potential, AP; Afterhyperpolarization, AHP; Frequency of Evoked spikes to injected depolarization current, FI.

739 Table 2. mEPSC properties of male and female medium spiny neurons

mEPSC Property	Male	Female	Statistics (*t*, P)
Frequency (Hz)	1.7 ± 0.4 (10)	3.1 ± 0.9 (11)	1.36, 0.19
Amplitude (pA)	9.5 ± 0.6 (10)	9.5 ± 0.6 (11)	0.53, 0.61
Decay (ms)	5.9 ± 0.7 (10)	4.9 ± 0.7 (11)	1.08, 0.29

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741 Notes: Values are mean ± SEM. Numbers in parentheses indicate sample size. No significant
742 differences were detected.

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Figure 1.

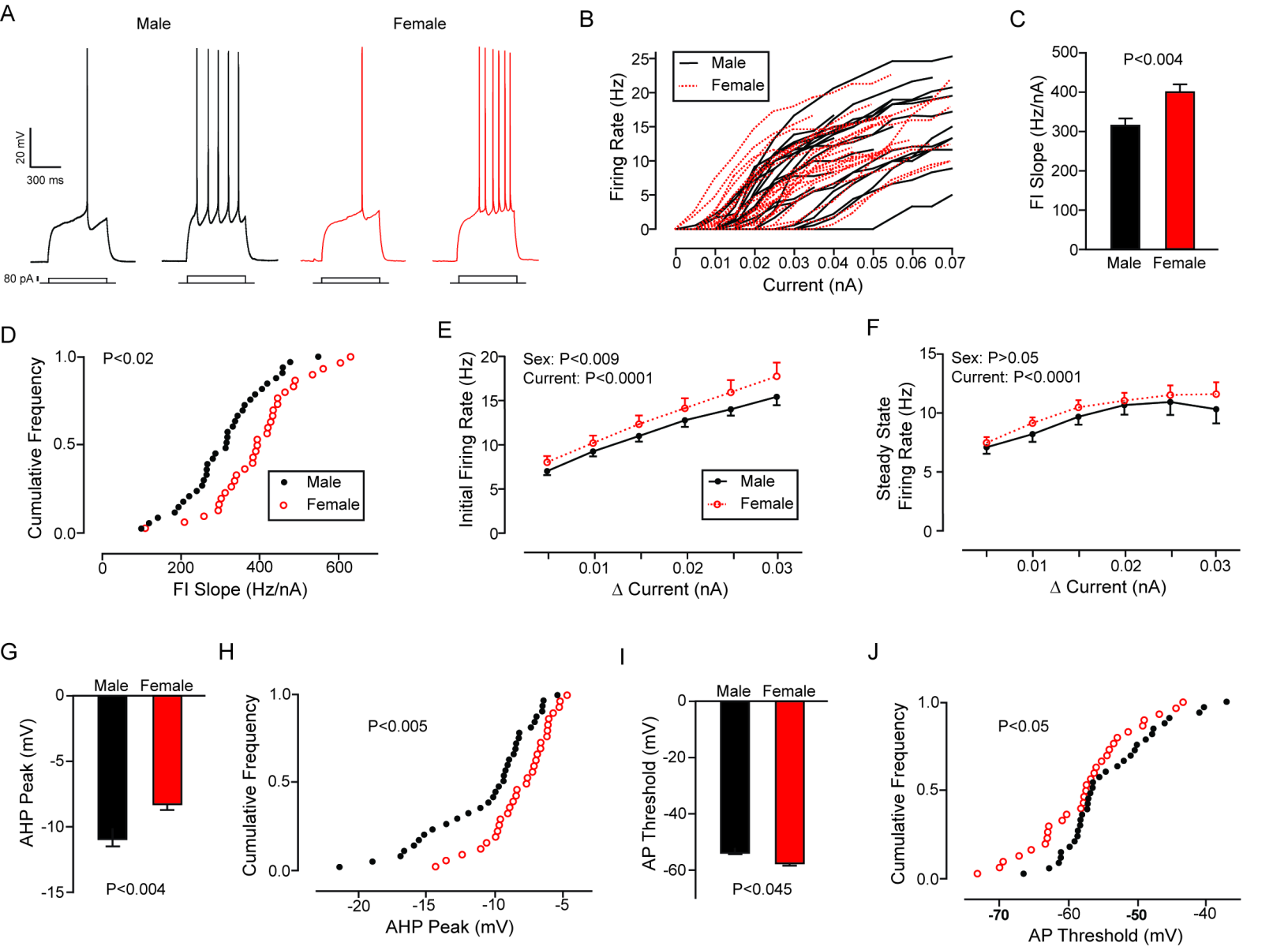
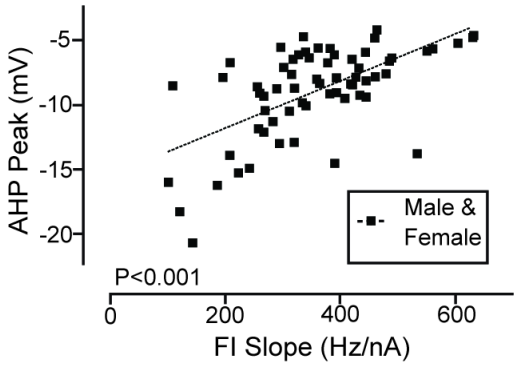
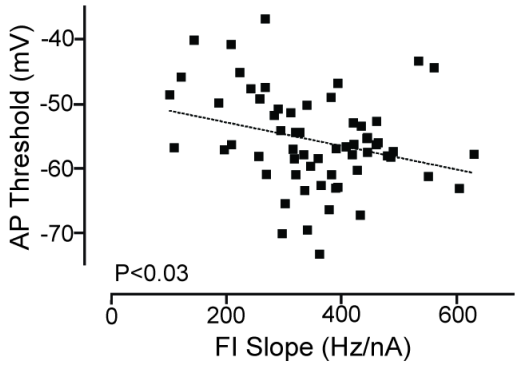

A
Male
Female
20 mV
300 ms
80 pA
B
Male
Female
Firing Rate (Hz)
Current (nA)
C
P<0.004
FI Slope (Hz/nA)
Male
Female
D
P<0.02
Cumulative Frequency
Male
Female
FI Slope (Hz/nA)
E
Sex: P<0.009
Current: P<0.0001
Initial Firing Rate (Hz)
Male
Female
Δ Current (nA)
F
Sex: P>0.05
Current: P<0.0001
Steady State Firing Rate (Hz)
Δ Current (nA)
G
Male
Female
AHP Peak (mV)
P<0.004
H
P<0.005
Cumulative Frequency
AHP Peak (mV)
I
Male
Female
AP Threshold (mV)
P<0.045
J
P<0.05
Cumulative Frequency
AP Threshold (mV)

Figure 2.

A


AHP Peak (mV)
-5
-10
-15
-20
Male &
Female
P<0.001
0
200
400
600
FI Slope (Hz/nA)

B


AP Threshold (mV)
-40
-50
-60
-70
P<0.03
0
200
400
600
FI Slope (Hz/nA)

C

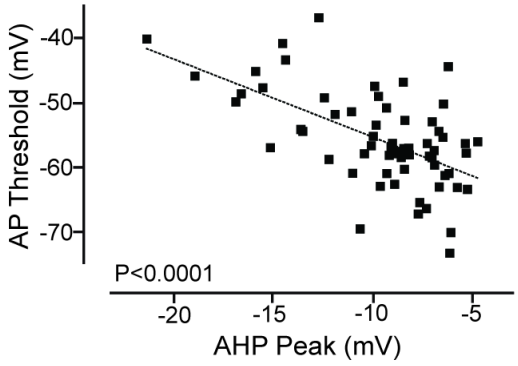

AP Threshold (mV)
-40
-50
-60
-70
P<0.0001
-20
-15
-10
-5
AHP Peak (mV)

Figure 3.

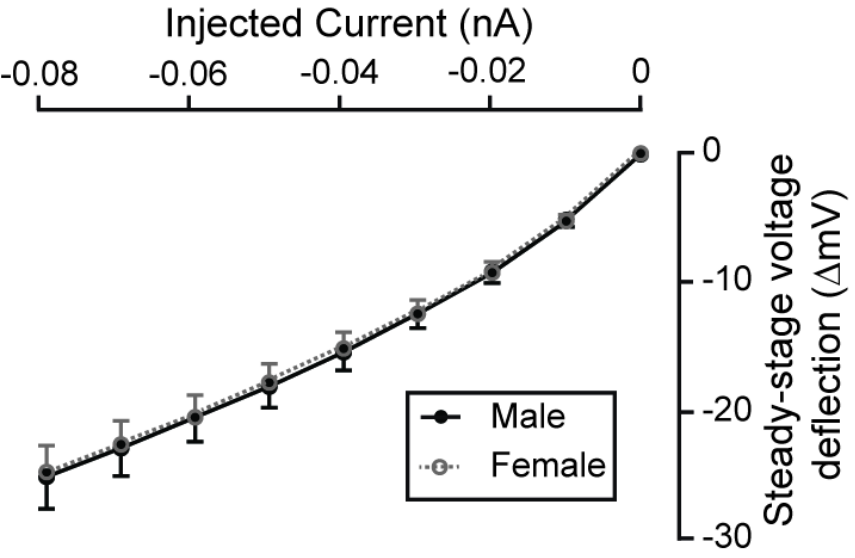

Injected Current (nA)
-0.08
-0.06
-0.04
-0.02
0
0
-10
-20
-30
Steady-stage voltage deflection (ΔmV)
Male
Female

Figure 4.

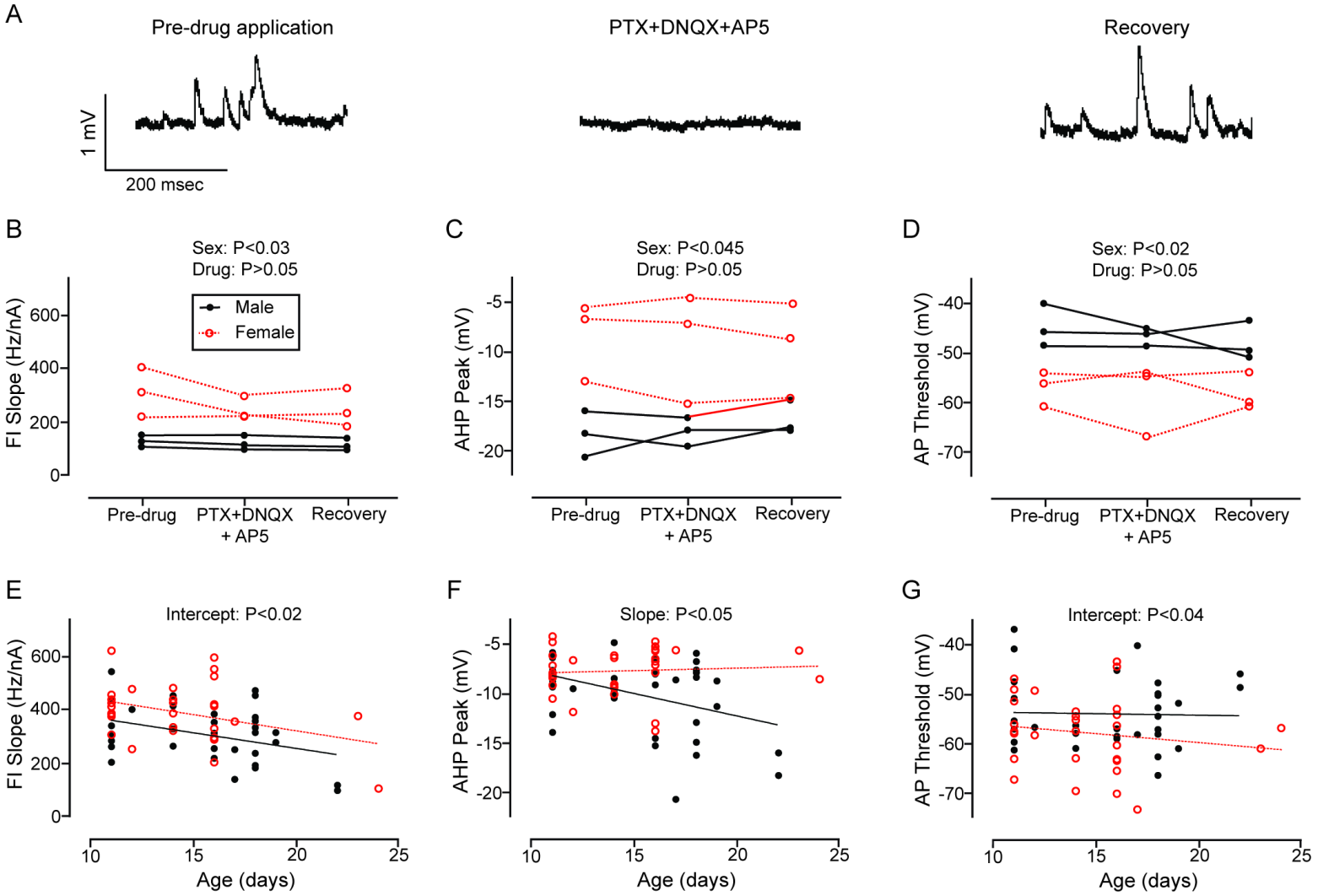
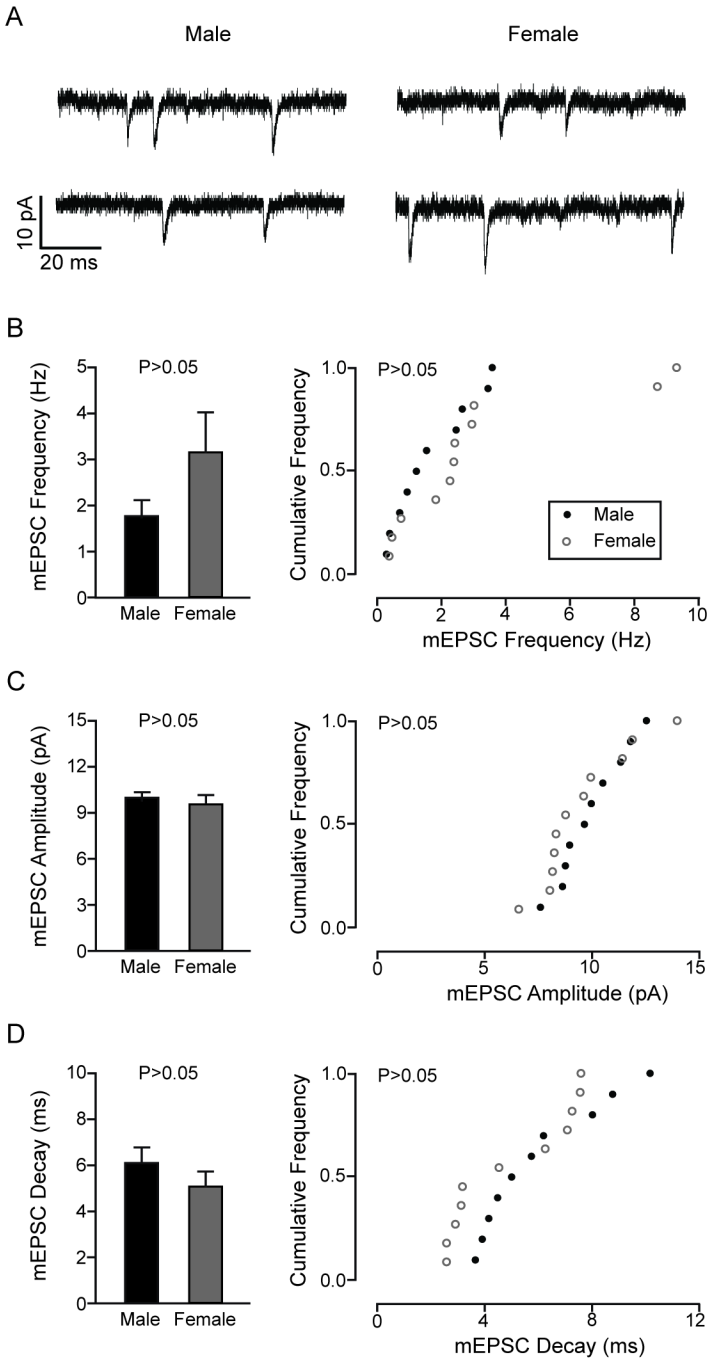

A
Pre-drug application
PTX+DNQX+AP5
Recovery
1 mV
200 msec
B
Sex: P<0.03
Drug: P>0.05
Male
Female
FI Slope (Hz/nA)
Pre-drug
PTX+DNQX + AP5
Recovery
C
Sex: P<0.045
Drug: P>0.05
AHP Peak (mV)
D
Sex: P<0.02
Drug: P>0.05
AP Threshold (mV)
E
Intercept: P<0.02
Age (days)
F
Slope: P<0.05
G
Intercept: P<0.04

Figure 5.


A
Male
Female
10 pA
20 ms
B
mEPSC Frequency (Hz)
P>0.05
Male
Female
Cumulative Frequency
P>0.05
Male
Female
mEPSC Frequency (Hz)
C
mEPSC Amplitude (pA)
P>0.05
Male
Female
Cumulative Frequency
P>0.05
mEPSC Amplitude (pA)
D
mEPSC Decay (ms)
P>0.05
Male
Female
Cumulative Frequency
P>0.05
mEPSC Decay (ms)