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Electrophysiological properties of medium spiny neuron subtypes in the caudate-putamen of prepubertal male and female *Drd1a*-tdTomato line 6 BAC transgenic mice

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Abstract

The caudate-putamen is a striatal brain region essential for sensorimotor behaviors, habit learning, and other cognitive and premotor functions. The output and predominant neuron of the caudate-putamen is the medium spiny neuron (MSN). MSNs present discrete cellular subtypes that show differences in neurochemistry, dopamine receptor expression, efferent targets, gene expression, functional roles, and most importantly for this study, electrophysiological properties. MSN subtypes include the striatonigral and the striatopallidal groups. Most studies identify the striatopallidal MSN subtype as being more excitable than the striatonigral MSN subtype. However, there is some divergence between studies regarding the exact differences in electrophysiological properties. Furthermore, MSN subtype electrophysiological properties have not been reported disaggregated by biological sex. We addressed these questions using prepubertal male and female Drd1a-tdTomato line 6 BAC transgenic mice, an important transgenic line that has not yet received extensive electrophysiological analysis. We made acute caudate-putamen brain slices and assessed a robust battery of 16 relevant electrophysiological properties using whole-cell patch clamp recording, including intrinsic membrane, action potential, and miniature excitatory postsynaptic current (mEPSC) properties. We found that: (1) MSN subtypes exhibited multiple differential electrophysiological properties in both sexes, including rheobase, action potential threshold and width, input resistance in both the linear and rectified ranges, and mEPSC amplitude; (2) select electrophysiological properties showed interactions between MSN subtype and sex. These findings provide a comprehensive evaluation of mouse caudate-putamen MSN subtype electrophysiological properties across females and males, both confirming and extending previous studies.

Significance Statement

The findings presented here provide the most comprehensive evaluation of the electrophysiological properties of caudate-putamen medium spiny neuron (MSN) subtypes ever performed in a single study, both in terms of electrophysiological metrics and animal sex. These data selectively confirm, diverge from, and extend the findings of previous studies, providing a firm foundation upon which to pursue future studies of caudate-putamen MSNs.

Introduction

The most abundant neuron type in the mammalian caudate-putamen is the medium spiny neuron (MSN), also called the spiny projection neuron (Graveland and Difiglia, 1985; Gerfen and Surmeier, 2011). The MSN is the output neuron of the caudate-putamen and other striatal brain regions, and is implicated in a wide range of cognitive and sensorimotor behaviors and relevant striatal disorders (Kreitzer and Malenka, 2008; Koob and Volkow, 2010; Maia and Frank, 2011). In order to regulate these behaviors, MSNs integrate glutamatergic, dopaminergic, GABAergic, cholinergic, estrogenic, and other inputs to influence both internal and external targets. MSNs are phenotypically diverse, encompassing at least two different subtypes, which differ in neurochemistry, dopamine receptor expression, efferent targets, gene expression, functional roles, and electrophysiological properties (Gerfen et al., 1990; Cepeda et al., 2008; Gertler et al., 2008; Shuen et al., 2008; Ade et al., 2011; Kramer et al., 2011; Kreitzer and Berke, 2011; Chan et al., 2012; Kravitz et al., 2012; Ma et al., 2012; Ma et al., 2013; Planert et al., 2013; Fieblinger et al., 2014; Friend and Kravitz, 2014; Keeler et al., 2014; Gokce et al., 2016; Schier et al., 2017; Sebel et al., 2017; Ho et al., 2018).

These two MSN subtypes include the striatonigral and the striatopallidal. Striatonigral MSNs express D1 dopamine receptors, which are the product of the gene *Drd1a*, and contain the neuropeptides substance P and dynorphin. Striatopallidal MSNs express D2 dopamine receptors, which are the product of the gene *Drd2*, and contain the neuropeptide enkephalin. Previous studies exploring caudate-putamen MSN subtype-specific electrophysiological properties have generally identified the Drd2-expressing subtype as being more excitable compared to the Drd1a-expressing subtype. However, there is some divergence between studies regarding the exact differences in electrophysiological properties (Table 1), and few studies have comprehensively evaluated a wide variety of cellular electrophysiological properties in individual MSNs of identified subtypes. Furthermore, all previous studies of caudate-putamen MSN subtypes have been performed in rats or mice of either solely male or unreported sex, typical of the majority of neuroscience pre-clinical studies (Beery and Zucker, 2011; Shansky and Woolley, 2016; Will et al., 2017). This is problematic given that striatal-mediated behaviors and disorders exhibit sex differences and/or sex steroid hormone sensitivity in phenotype and/or incidence (Calhoun, 1962; Eckel et al., 2000; Zurkovsky et al., 2007; Hosseini-Kamkar and

Morton, 2014; Yoest et al., 2018), and that striatal region and developmental stage-influenced sex differences exist in MSN electrophysiological properties, at least in rats (Arnauld et al., 1981; Tansey et al., 1983; Mermelstein et al., 1996; Wissman et al., 2011; Dorris et al., 2015; Tozzi et al., 2015; Cao et al., 2016; Willett et al., 2016; Cao et al., 2018; Proano et al., 2018).

To address these gaps in knowledge, we employed female and male B6 *Cg-Tg (Drd1a-*tdTomato) line 6 Calak/J hemizygous mice, a bacterial artificial chromosome (BAC) transgenic mouse line initially developed in the laboratory of Dr. Nicole Calakos (Ade et al., 2011). This mouse line and many others are widely used for experiments targeting neuronal subtypes (Valjent et al., 2009; Ting and Feng, 2014). An advantage of this particular BAC transgenic line is that it expresses a sensitive and specific fluorescent reporter for the Drd1a-expressing MSN subtype, enabling accurate identification of MSNs subtypes within a single mouse. Other advantages of this mouse line compared to other candidates are that this line exhibits normal caudate-putamen-mediated behaviors and does not appear to show obvious cellular or physical confounds (Ade et al., 2011; Enoksson et al., 2012; Thibault et al., 2013). We made acute brain slices of male and female mouse caudate-putamen and then recorded individual MSN subtypes using whole-cell patch clamp. We analyzed a comprehensive battery of caudate-putamen MSN subtype electrophysiological attributes to test the hypothesis that MSN electrophysiological properties differs by subtype across both males and females, including action potential, excitability, passive membrane and input resistance properties, and miniature excitatory synaptic currents (mEPSCs).

Materials

Animals

Male B6 *Cg-Tg (Drd1a*-tdTomato) line 6 Calak/J mice and female C57BL/6 background mice were purchased from the Jackson Laboratory (JAX stock number 16204). During the first week after arrival mice were individually housed. After the first week mice were housed in male and female pairs to enable breeding of hemizygous offspring. Offspring aged post-natal day (PND) 17-22 from F1 litters were used in experiments (n=25) and were matched between experimental groups (10 Drd1a male mice: PND 19.4 ± 0.2; 7 Drd1a female mice: PND 21.0 ± 0.2; 4 Drd2 male mice: PND 19.8 ± 0.5; 3 Drd2 female mice: PND 20.0 ± 0.7; P>0.05). Approximately 3

neurons were recorded from each mouse. Mice were not weaned before experimental use and female vaginal opening had not occurred before experimental use. Pups were ear punched for identification and genotyping. Mice were housed in a temperature- and light-controlled room (22 ± 1°C, 40-45% humidity, 12:12-hour light/dark cycle, lights on at 7:00 AM). All cages were washed polysulfone bisphenol A 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 (Markaverich et al., 2002; Mani et al., 2005; Villalon Landeros et al., 2012). Soy protein-free rodent chow (2020X; Teklad) and glass-bottle provided water were available *ad libitum.* All animals in these studies were maintained according to the applicable portions of the Animal Welfare Act and the U.S. Department of Health and Human Services *Guide for the Care and Use of Laboratory Animals*, and the study was approved by the Institutional Animal Care and Use Committee.

Animal Genotyping

Mice genotyping was performed by Celplor (Raleigh, NC), using the following primers according to the Jackson Laboratory suggested protocol: Transgene Forward (forward primer, 12153, 5-CTT CTG AGG CGG AAA GAA CC-3), Transgene Reverse (reverse primer, 12154, 5-TTT CTG ATT GAG AGC ATT CG-3), PCR product length is 750 base pairs. The internal control was: Internal Positive Control Forward (oIMR7338) CTA GGC CAC AGA ATT GAA AGA TCT, Internal Positive Control Reverse (oIMR7339) GTA GGT GGA AAT TCT AGC ATC ATC C), PCR product length is 324 base pairs. PCR was performed according to the suggested protocol from Jackson Laboratory: 1 cycle of 94°C for 2 min, 5 cycles of 94°C for 30 sec, 60–55°C touchdown ramp for 30 sec and 72°C for 30 sec, 25 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, followed by 1 cycle of 72°C for 5 min.

Acute Brain Slice Preparation

Brain slices for electrophysiological recordings were prepared following a previously published protocol (Dorris et al., 2014). Briefly, mice were deeply anesthetized with isoflurane gas and killed by decapitation. The brain was then dissected rapidly into ice-cold, oxygenated sucrose artificial cerebellum spinal fluid (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. The osmolarity of the s-

ACSF was between 295-305 mOsm, and pH was between 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 30 ± 1 ºC, and then 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

Slices were allowed to rest at least 1 hour after sectioning, and were then placed in a Zeiss Axioscope equipped with IR-DIC and fluorescent optics, a Dage IR-1000 video camera, and 10X and 40X lenses with optical zoom. Slices were superfused with oxygenated ACSF heated to 28 ± 0.2 ºC. Whole-cell patch-clamp recordings were used to record the electrical properties of fluorescently labeled Drd1a and unlabeled Drd2 MSNs in the caudate-putamen (Figure 1). Caudate-putamen gross regional volume and cell density and soma size do not grossly vary by sex in rodents and humans (Meitzen et al., 2011; Wong et al., 2016). Glass patch electrodes contained the following solution (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 (Dorris et al., 2015), recordings were first made in current clamp to assess neuronal action potential and passive membrane 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 membrane potential more negative than -65 mV, inward rectification, and prominent spike after hyperpolarization (O'Donnell and Grace, 1993; Belleau and Warren, 2000). After MSN identification and current clamp recording, oxygenated ACSF containing both the $GABA_A$ receptor antagonist Picrotoxin (PTX,150 µM; Fisher) and the voltage-gated sodium channel blocker tetrodotoxin (TTX, 1 µm, Abcam Biochemicals) was applied to the bath solution to abolish GABAergic inhibitory post-synaptic current events and action potentials, respectively. Following an established protocol (Cao et al., 2016), once depolarizing current injection no longer generated an action potential after exposure to TTX and PTX, MSNs were voltage

clamped at -70 mV and miniature excitatory post-synaptic currents (mEPSCs) were recorded for at least five minutes. In all experiments input/series resistance was monitored for changes and cells were excluded if resistance changed more than 25%.

Data Analysis

Intrinsic electrophysiological properties, action potential and mEPSC characteristics were recorded and analyzed using pClamp 10. After break-in, the resting membrane potential was first allowed to stabilize for ~1-2 minutes, as in (Mu et al., 2010). Then, at least three series of depolarizing and hyperpolarizing current injections were applied to elicit basic neurophysiological properties. The electrophysiological properties measured followed previously described definitions (Dorris et al., 2015; Cao et al., 2016; Willett et al., 2016; Willett et al., 2018), which were based upon those of Perkel and colleagues (Farries and Perkel, 2000; Farries and Perkel, 2002; Farries et al., 2005; Meitzen et al., 2009). For each neuron, measurements were made of at least three action potentials generated from the minimum current injection necessary to elicit one or two action potentials. These measurements were then averaged to generate the reported action potential measurements for that neuron. For action potential measurements, only the first generated action potential was analyzed. 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). The slope of the linear range of the evoked firing rate to positive current curve (FI slope) was calculated from the first current stimulus which evoked an action potential to the first current stimulus that generated an evoked firing rate that persisted for at least two consecutive current stimuli. Input resistance in the linear, non-rectified range was calculated from the steady-state membrane potential in response to −0.02 nA hyperpolarizing injected current. Rectified range input resistance, inward rectification, and percent inward rectification (RRIR/IR x 100) was calculated using the most hyperpolarizing current injected into the MSN, as previously described (Belleau and Warren, 2000). 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. mEPSCs frequency, amplitude, and decay were analyzed off-line using Mini Analysis (Synaptosoft, http://www.synaptosoft.com/MiniAnalysis/). Threshold was set as 5 pA, noise filter was set at 1000 Hz, and accurate event detection was validated by visual inspection.

Statistics

Experiments were analyzed via a two-way ANOVA with a Tukey's multiple comparisons post-hoc test (Excel version 2010; Microsoft, Redmond, WA; Prism version 6.07, GraphPad Software, La Jolla, CA). P values < 0.05 were considered *a priori* as significant. Values 6 standard deviations away from the mean were *a priori* excluded from analysis. Effect size was assessed using Cohen's *d* value (Calin-Jageman, 2018). *d* values are reported numerically and were classified *a priori* as small (>0.20), medium (>0.50) and large (>0.80) (Cohen, 1977). Data are presented as mean ± standard error of the mean (SEM).

Results

A total of 86 MSNs from the caudate-putamen of male and female B6 *Cg-Tg (Drd1a*-tdTomato) 6 Calak/J hemizygous mice were recorded for this study. Recorded MSNs were *a priori* sorted into four experimental groups: male tdTomato-labeled Drd1a-positive MSNs, female tdTomato-labeled Drd1a-positive MSNs, male tdTomato-unlabeled MSNs, and female tdTomato-unlabeled MSNs. MSNs unlabeled by tdTomato fluorescence nearly exclusively comprise the Drd2-positive MSN subtype, including during the developmental age and striatal region assessed in this study (Ade et al., 2011; Enoksson et al., 2012; Thibault et al., 2013). tdTomato-unlabeled MSNs have rare (~1.6%) contamination with Drd1a-positive MSNs. Thus, for convenience in this study we refer to all tdTomato-unlabeled MSNs as Drd2 MSNs, with the full acknowledgment that this designation is putative.

Action potential properties

To test the hypothesis that action potential properties differed across MSN subtype and animal sex, MSNs were current-clamped and injected with increasing amounts of depolarizing current to elicit action potential generation (Figure 2A). The resting membrane potential, rheobase, action potential threshold, width, amplitude, action potential afterhyperpolarization peak amplitude and time to afterhyperpolarization peak amplitude were assessed (Table 2). MSNs exhibited differences between subtypes or interactions between subtype and sex in several attributes, including the resting membrane potential (Figure 2B). Compared between groups, the resting membrane potential of male Drd1a MSNs was hyperpolarized compared to male Drd2 MSNs ($P<0.01$; $d=0.86$), but not between female Drd1a MSNs compared to female Drd2 MSNs

(P>0.05, *d*=0.42). Rheobase, or the minimum current sufficient for eliciting action potential generation, was increased in Drd1a MSNs compared to Drd2 MSNs (Figure 2C). Compared between groups, the rheobase of male Drd1a MSNs differed from male and female Drd2 MSNs (P<0.05, *d*=0.74; P<0.05, *d*=0.77; respectively). The action potential threshold was hyperpolarized in Drd1a MSNs compared to Drd2 MSNs (Figure 2D). Compared between groups, the action potential threshold of female Drd1a MSNs differed from female Drd2 MSNs (P<0.01, *d*=1.00), but not between male Drd1a MSNs and male Drd2 MSNs (P>0.05, *d*=0.05). The action potential width of Drd1a MSNs was longer compared to Drd2 MSNs (Figure 2E). Compared between groups, the action potential width of male Drd1a MSNs was increased compared to male Drd2 MSNs (P<0.01, *d*=1.24), but not between female Drd1a MSNs and female Drd2 MSNs (P>0.05, *d*=0.03). Considering other passive properties, no differences were detected between MSN subtype or sex in action potential amplitude, action potential afterhyperpolarization peak amplitude and time to afterhyperpolarization peak amplitude (Table 2). These differences in action potential properties indicate that Drd1a MSNs are less likely to generate an action potential at low magnitudes of injected depolarizing current than are Drd2 MSNs.

Intrinsic excitability and action potential generation rates

These differences between MSN subtype rheobase, action potential threshold, width, and time to first action potential properties indicate that overall MSN excitability may also differ by subtype (Figure 3A). To assess this, we began by analyzing the frequency of action potentials evoked by depolarizing current injections. Action potential firing rates evoked by depolarizing current injections were visibly decreased in Drd1a compared to Drd2 MSNs in both males and females (Figure 3B). To further probe the relationship between MSN subtype and action potential generation, we quantified the slope of the evoked firing rate to positive current curve (FI slope). FI slope differed by subtype but not sex, with Drd1a MSNs exhibiting decreased excitability compared to Drd2 MSNs (Figure 3C). Compared between groups, the FI slope of male Drd1a MSNs differed from male Drd2 MSNs (P<0.01, *d*=0.96). Female Drd1A MSNs differed from male Drd2 MSNs (P<0.0001, *d*=1.25) and female Drd2 MSNs (P<0.05, *d*=1.10). These data indicate that excitability robustly differs between MSN subtypes.

Passive membrane properties

To test the hypothesis that MSN passive electrophysiological properties differed by subtype and sex, a series of increasingly negative current pulses were injected into individual neurons (Figure 4A). MSN subtypes exhibited differences in input resistance across both the linear and rectified ranges (Figure 4B). Input resistance in the linear and rectified ranges, % inward rectification, the time constant of the membrane, and capacitance were analyzed (Table 2). Linear range input resistance was largely decreased in Drd1a MSNs compared to Drd2 MSNs (Figure 4C). Compared between groups, the linear range input resistance of male Drd1a MSNs differed from male Drd2 MSNs ($P<0.05$. $d=0.67$), but did not differ between female Drd1a MSNs and female Drd2 MSNs ($P>0.05$, $d=0.34$). Male Drd2 MSNs also differed from female Drd1a MSNs ($P<0.05$, $d=0.83$). Rectified range input resistance did not differ between MSN subtypes (Figure 4D) or other measures of inward rectification (Table 2). Considering other passive properties, no differences were detected between MSN subtype or sex in the time constant of the membrane, and capacitance (Table 2). Collectively, these analyses indicate that input resistance varies between MSN subtypes, with no differences in other passive membrane properties.

mEPSC properties

We voltage-clamped 18 male and 17 female Drd1a MSNs and 10 male and 6 female Drd2 MSNs at -70 mV and recorded mEPSCs in the presence of TTX and PTX (Figure 5A). mEPSC frequency, amplitude and decay were analyzed (Table 2). mEPSC amplitude was increased in Drd1a MSNs compared to Drd2 MSNs (Figure 5B). Compared between groups, the mEPSC amplitude of male Drd1a MSNs differed from female Drd2 MSNs ($P<0.01$, $d=0.82$) but not male Drd2 MSNs ($P>0.05$, $d=0.67$). The mEPSC amplitude of female Drd1a MSNs differed from female Drd2 MSNs ($P<0.001$, $d=1.74$). mEPSC decay did not differ between MSN subtype or sex (Figure 5C). Likewise, mEPSC frequency did not differ between MSN subtype or sex (Figure 5D). These data indicate that mEPSC amplitude differs between MSN subtypes.

Discussion

MSNs form at least two major pathways depending on their dopaminergic receptor and neuropeptide expression, electrophysiological properties, where they project, and ultimately their effect on behavior. The caudate-putamen MSNs of the direct pathway predominantly express D1-dopamine receptors, contain substance P and dynorphin, project to the basal ganglia output

nuclei, and stimulate downstream behavioral output. Indirect pathway MSNs express D2-dopamine receptors, contain encephalin, project to the lateral globus pallidus, and leads to inhibition of downstream behavioral output. This study comprehensively evaluates mouse caudate-putamen MSN subtype electrophysiological properties, extending previous studies that targeted a smaller battery of electrical properties and that were performed solely in males or mice of unknown sex (Table 1). Electrophysiological properties differed between MSN subtypes, with Drd2 MSNs exhibiting increased intrinsic excitability compared to Drd1a MSNs, indicated most notably by differences in rheobase, action potential threshold, input resistance in the linear range, and increased FI Slope. Interestingly, this robust set of properties exhibits varying degrees of consistence with previous literature on MSN subtype electrophysiology, and select electrophysiological properties showed statistical interactions between subtype and biological sex.

The detected increase in excitability in Drd2 MSNs is generally consistent with previous studies of MSN subtypes in rodents across striatal regions, although there are subtle differences depending on the assessed electrophysiological metric and perhaps age (Onn et al., 1994; Onn et al., 1994; Onn et al., 1994; Venance and Glowinski, 2003; Kreitzer and Malenka, 2007; Ade et al., 2008; Cepeda et al., 2008; Gertler et al., 2008; Chan et al., 2012; Ma et al., 2012; Planert et al., 2013; Reig and Silberberg, 2014; Maurice et al., 2015; Cao et al., 2018; Goodliffe et al., 2018). Perhaps the most consistent metric indicating increased excitability in Drd2 MSNs compared to Drd1a MSNs is the decreased rheobase in Drd2 MSNs. Every study that has assessed this property has detected this difference, despite utilizing varying protocols and electrophysiological methods. From an electrophysiological perspective, differences in rheobase are rarely the sole electrophysiological difference between neuron types. Generally, a shift in rheobase is accompanied by concomitant changes in properties such as resting membrane potential, input resistance in the linear range, and/or action potential threshold. For instance, Gertler and colleagues detected changes in rheobase accompanied by changes in resting membrane potential and input resistance, but not action potential threshold (Gertler et al., 2008). Planert and colleagues detected changes in rheobase accompanied by a change in input resistance, but not action potential threshold or resting membrane potential in rats but not mice (Planert et al., 2013). Cepeda and colleagues did not assess rheobase, but did detect a difference in action potential threshold (Cepeda et al., 2008). The current study detected a difference in

rheobase accompanied by changes in action potential threshold and input resistance, supporting a model where the decreased rheobase values in Drd2 MSN subtypes is largely driven by a hyperpolarized action potential threshold and an increased input resistance. The increase in excitability observed in Drd2 MSNs could ultimately translate to a decrease in behavioral output. However, this particular interpretation is highly tentative given that MSNs make complex calculations between dopamine, glutamate, intrinsic properties, and other neuromodulators. Interestingly, we detected an interaction between MSN subtype and sex in resting membrane potential and action potential threshold. Thus, it is possible that the reason why the results of our study differ from those of Gertler and colleagues is because of the use of animals of undetermined sex in that study.

Regarding excitatory synaptic input, the current study detected an increase mEPSC amplitude in Drd2 MSN subtypes compared to Drd1a MSN subtypes. To our knowledge, this is the first indication that mEPSC amplitude can differ by MSN subtype (Table 1). This may be due to a variance and power interaction, although previous studies of MSN subtypes employed similar experimental N. This finding does align with previous research which detected large amplitude AMPA-mediated synaptic events in Drd2 MSNs that were not seen in Drd1a MSNs (Cepeda et al., 2008). The recording conditions under which the mEPSCs were assessed in this study eliminate non-AMPA-mediated currents (Proano et al., 2018). A number of factors could potentially mediate this difference in mEPSC amplitude, including morphological differences and/or differences in AMPA receptor number or subunit composition (Tallaksen-Greene and Albin, 1996; Vorobjev et al., 2000). Supporting this, there is evidence that the size of corticostriatal presynaptic terminals is larger on Drd2 MSN spines compared to Drd1a MSN spines (Lei et al., 2004). One possibility why previous studies did not detect a significant difference in mEPSC amplitude is because the effect size for this particular attribute is larger in females compared to males. Previous studies either only tested males or did not report sex, or sex-specific findings. Following this, two previous studies detected a greater sEPSC and mEPSC frequency in caudate putamen Drd2 MSNs compared to Drd1a MSNs that is not accompanied by differences in amplitude or decay (Kreitzer and Malenka, 2007; Cepeda et al., 2008). However, this literature is mixed as Cepeda and colleagues detected a difference in mEPSC rise time, and Day et al. did not detect a difference in mEPSC frequency in prepubertal animals, and Goodliffe et al. did not detect a difference in mEPSC frequency in adult animals (Day et al., 2006; Cepeda

et al., 2008; Goodliffe et al., 2018). It is possible that this variability in findings is in some part explained by the neglect or overrepresentation of one sex compared to another.

There are other factors which may also play a role, including animal age. Most studies of MSN subtype properties used mice that were between P17 and P30 (Table 1). During these periods, various levels of MSN synaptic maturation are occurring, which could contribute to variance in mEPSC properties (Tepper et al., 1998; Uryu et al., 1999). Gertler and colleagues employed a wide variety of ages to demonstrate that MSN subtype intrinsic properties differ before puberty, but the study did not assess excitatory synaptic properties (Gertler et al., 2008). Goodliffe and colleagues assessed at a much older age (~P365), and found differences in intrinsic properties but not mEPSC properties (Table 1). Thus, our assessment of the literature is that there is ample evidence for differences in MSN subtype electrophysiological properties prepuberty, but that there is a real need for further studies in adult animals, especially within the context of excitatory synapse properties, sex-specific hormone dynamics, and animal sex, especially since the current study is the only available analysis by sex in mouse caudate-putamen.

Similarly, Drd1a and Drd2 MSN subtypes display different sensitivities to neuromodulators such as dopamine during prepubertal development (Lieberman et al., 2018). Indeed, it has been documented in multiple striatal regions that dopamine receptor expression and/or action shows sex-specific effects during puberty (Andersen et al., 1997; Andersen et al., 2002; Kopec et al., 2018). Interestingly, excitatory currents generated by pyramidal tract stimulation show an increased amplitude in Drd1a MSNs compared to Drd2 MSNs in adult male and female mice that were not analyzed with regard to sex (Kress et al., 2013). In *in vivo* experiments in adult female mice, Drd1a MSNs were found to be more responsive to excitatory glutamatergic input compared to Drd2 MSNs (Escande et al., 2016). However, this study employed the line 5 Drd1a-tdTomato BAC-transgenic mice, which express properties such as an X-linked inheritance pattern and undefined mammary glands that reduces this strain's utility for assessing interactions between MSN subtypes and sex (Shuen et al., 2008; Ade et al., 2011). Subtype-specific differences in the development of glutamateric and dopaminergic inputs onto MSNs in the caudate-putamen require further research, especially in the context of biological sex, environmental stimuli, and animals beyond mice.

The strain and/or species is also a relevant factor in explaining differences in MSN subtype electrophysiological properties across studies. For instance, multiple strains of transgenic mice have been employed across studies which have employed a variety of means to determine MSN subtype identity. Cepeda et al., Gertler et al., and the current study all targeted Drd1a or Drd2 expression to identify MSN subtypes (albeit with different transgenic strategies), whereas other studies have employed different targets such as the muscarinic M4 receptor locus which labels striatonigral MSNs (Kreitzer and Malenka, 2007). There is some evidence that there is incomplete overlap between M4 and Drd1a dopamine receptors which may contribute to variance in detected electrophysiological properties, including sEPSC frequency between M4 and D1 cells (Bernard et al., 1992; Cepeda et al., 2008). Here we employed the B6 *Cg-Tg (Drd1a*-tdTomato) 6 Calak/J hemizygous mice (line 6). We chose this strain because of its established high specificity, in that tdTomato-labeled MSNs almost exclusively consist of Drd1a MSNs, and that tdTomato-unlabeled MSNs exhibit only ~1.6% contamination with Drd1a MSNs (Ade et al., 2011; Enoksson et al., 2012; Thibault et al., 2013). Furthermore, the tdTomato label is easily detected using standard fluorescent microscopy, optimizing differentiation for whole-cell patch clamp. While the line 6 version of this transgenic mouse line does not show the obvious confounds for sex research that the line 5 version displays, including the X-linked inheritance pattern and undefined mammary glands, caution is always necessary with any transgenic mouse line that targets dopamine receptors. It is possible that transgenes targeting dopamine receptors subtly disrupt sexual differentiation, especially given the long documented and recently re-affirmed sex differences and hormone-sensitivity of the dopaminergic system in both rats and mice (Di Paolo, 1994; Becker, 1999; Calipari et al., 2017). Independent of sex, other mouse lines with transgenic manipulations of the dopamine system by attaching fluorophores have shown aberrant striatal-mediated behaviors, especially when strain and genetic homozygosity were not carefully monitored (Kramer et al., 2011; Chan et al., 2012). We raise this possibility neither to argue that transgenic mice in general are not useful for neuroscience research nor for understanding the effects of sex and steroid sex hormones. Several mouse models have made critical contributions to our understanding of sexual differentiation, most notably the four core genotypes (De Vries et al., 2002), including as applied to the caudate-putamen (Chen et al., 2009). Rather, we argue that the specific disadvantages and advantages of each research animal should be thoughtfully considered, especially for studies of the impact of

natural variables such as sex upon individual neuron function. There is no "one size fits all" mouse strain, just as there is no "one size fits all" strain of rat or any other research animal, echoing arguments presented by many investigators in diverse contexts (Beach, 1950; Krebs, 1975; Young et al., 2013; Brenowitz and Zakon, 2015; Ellenbroek and Youn, 2016; Klinck et al., 2017; Remage-Healey et al., 2017). Regarding species differences, to our knowledge, there is only one study which has assessed MSN subtype electrophysiological properties in the caudate putamen of a species other than mice. Similar to the current study in mice, Planert and colleagues found a difference in rheobase and related properties in prepubertal rats of unreported sex (Planert et al., 2013). Synaptic properties were not assessed.

Further complicating interpretation was the number of interactions between MSN subtype and sex detected by the current study. Given that animals were assessed before pubertal onset but after the perinatal critical period for hormone-induced organization of the neural substrate, it is possible that these sex differences were generated through some combination of masculinizing/defeminizing hormone action, genes, or epigenetics. All three of these mechanisms are potentially at work in the caudate-putamen and could contribute to the MSN subtype and sex interactions observed here (Chen et al., 2009; Cao et al., 2016). Previous studies in mice employed only males, animals of unreported sex, or animals of both sex sexes that were pooled for data analysis. This lack of consideration of biological sex is problematic given the long-known sex differences in striatal mediated behaviors, disorders, MSN properties, and neuromodulator systems such as dopamine (Mermelstein et al., 1996; McLean and Anderson, 2009; Carroll and Anker, 2010; Young and Korszun, 2010; Becker and Chartoff, 2018; Meitzen et al., 2018). While this work has predominantly been performed in rats, importantly, adult mice exhibit sex differences in striatal gene expression and function in both the caudate putamen and nucleus accumbens (Chen et al., 2009; Calipari et al., 2017). The current study detected an interaction in action potential threshold, which was also found to differ by sex in prepubertal rats (Dorris et al., 2015). Other electrophysiological properties also differed by sex in rats, including the frequency of evoked action potentials to injected current and the action potential afterhyperpolarization, but were not found to differ by the current study in mice. This difference between mice and rats may be due to a number of potential factors, including but not limited to variance between inbred and outbred rodent strains, overall species differences and the effects of domestication, MSN subtype sampling bias, developmental trajectory, environmental factors

such as stress, and/or location within the caudate-putamen or striatum as a whole. For a phylogenetically ancient and highly conserved brain region such as the caudate-putamen, it will be particularly interesting to investigate the intersecting roles of subtype, development, and biological sex in influencing MSN electrophysiological properties across a wide range of animals with divergent reproductive behaviors.

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Figure Legends

Figure 1. Whole-cell patch clamped medium spiny neuron (MSNs) location in the caudate-putamen of female and male Drd1a-tdTomato line 6 BAC transgenic mice. “Drd1a” males and females represent recordings from fluorescently-labeled Drd1a-positive MSNs. “Drd2 ” males and females represent recordings from non-fluorescently labeled MSNs. Acronyms: LV, lateral ventricle; AC, anterior commissure; ACB, nucleus accumbens.

Figure 2. Action potential rheobase, threshold, and width vary by MSN subtype. A) Voltage response of male and female Drd1a and Drd2 MSN subtypes to a depolarizing rheobase current injection. B) Resting membrane potential exhibited greater diversity in male Drd1A MSNs. C) Action potential rheobase is increased in Drd1a MSNs compared to Drd2 MSNs. D) Action potential threshold is depolarized in Drd1a MSNs compared to Drd2 MSNs, and interacts with sex. E) Action potential width is longer in Drd1a MSNs compared to Drd2 MSNs, and interacts with sex. Acronyms: AP, action potential; *, $P<0.05$; **, $P<0.01$.

Figure 3. Action potential firing rates evoked by depolarizing current injections vary by MSN subtype. A) Voltage response of male and female Drd1a and Drd2 MSN subtypes to a depolarizing post-rheobase current injection. B) Drd1a MSNs exhibited decreased action potential firing rates evoked by depolarizing current injections compared to Drd2 MSNs. C) The slope of the evoked action potential to depolarizing current injection curve (FI slope) differed by MSN subtype, with Drd2 MSNs exhibiting increased excitability compared to Drd1a MSNs. Acronyms: FI Slope: slope of the evoked action potential to depolarizing current injection curve; *, $P<0.05$; **, $P<0.01$; ****, $P<0.0001$.

Figure 4. Input resistance varies by MSN subtype. A) Voltage response of male and female Drd1a and Drd2 MSN subtypes to a series of increasingly negative current injections (-0.01 nA current steps). B) Injected negative current to steady-stage voltage deflection curve (IV curve). Legend: red solid circles with red line: Drd1a males; black solid circles with black line: Drd2 males; red open circles with red line: Drd1a females; black open circles with black line: Drd2 females. C) Input resistance in the linear range is moderately decreased in Drd1a MSNs compared to Drd2 MSNs. D) Input resistance in the rectified range does not differ between subtypes. Acronyms: *, $P<0.05$.

743 **Figure 5.** mEPSC properties vary by MSN subtype. A) mEPSCs recorded from male and female
744 Drd1a and Drd2 MSN subtypes. MSNs were voltage clamped at -70 mV and mEPSCs were
745 recorded in the presence of tetrodotoxin (TTX) and picrotoxin (PTX) to block voltage-gated
746 sodium channels and GABAergic synaptic activity, respectively. B) mEPSC amplitude was
747 increased in Drd1a MSNs compared to Drd2 MSNs. C) mEPSC decay did not differ by subtype
748 or sex. D) mEPSC frequency did not differ by subtype or sex. Acronyms: mEPSC, miniature
749 excitatory postsynaptic current; **, $P<0.01$; ***, $P<0.001$.

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752 **Tables (2)**

753 **Table 1**. Drd1a and Drd2 Caudate-putamen Medium Spiny Neuron Properties Compared Across
754 Studies.

Property	Kreitzer and Malenka, 2007[@]	Gertler et al., 2008	Cepeda et al., 2008	Ade et al., 2008[%]	Chan et al., 2012	Planert et al., 2013	Planert et al., 2013	Goodliffe et al., 2018	Current study, 2019
Animal	Mice	Mice	Mice	Mice	Mice	Mice	Rats	Mice	Mice
MSN Subtype Identification	M4- or D2-eGFP BAC transgenic mice	D1 and D2 receptor-eGFP BAC transgenic mice on an FVB background	D1 and D2 receptor-eGFP BAC transgenic mice	D1 and D2 receptor-eGFP BAC transgenic mice on a C57BL/6J background	D1 and D2 receptor-eGFP BAC transgenic mice on either a FVB/NJ or C57BL/6J background	D1 receptor-eGFP BAC transgenic mice	Retrograde labeling of striatonigral MSNs	D1 and D2 receptor-eGFP BAC transgenic mice on a C57BL/6J background	B6 Cg-Tg (Drd1a-tdTomato) 6 Calak/J hemizygous mice ona C57BL/6J background
Animal Age	P20-25	P17-70	P39.7 $\pm$ 1.6	P16-25	P21-35	P15, 21-32	P14-19	~P365	P17-22
Animal Sex	Not reported	Not reported	Not reported	Male and female data pooled regardless of sex	Male	Not reported	Not reported	Male and Female data pooled regardless of sex	Male and Female data analyzed by sex
Resting Membrane Potential	D1=D2	D1<D2	---	D1=D2	---	D1=D2	D1=D2	D1=D2	D1=D2
Rheobase	---	D1>D2	---	---	---	D1>D2[#]	D1>D2	D1>D2	D1>D2
AP Threshold	---	D1=D2	D1>D2	---	---	D1=D2	D1=D2	D1>D2	D1>D2
AP Amplitude	---	---	D1=D2	---	---	----	---	D1=D2	D1=D2
AP Amplitude change from first to second AP	---	---	---	---	---	D1=D2	D1>D2	---	---
AP width	---	---	D1=D2	---	---	D1=D2	D1=D2	---	D1>D2
AHP Peak	---	---	D1=D2	---	---	---	---	---	D1=D2
AHP Time to Peak	---	---	---	---	---	---	---	---	D1=D2
Frequency of evoked action potentials/FI slope	D1<D2	D1<D2	---	D1<D2	D1<D2	D1=D2	D1=D2	---	D1<D2
Linear Range Input Resistance	D1=D2	D1<D2	D1=D2	D1=D2	---	D1=D2	D1<D2	D1=D2	D1<D2
Rectified	---	---	---	---	---	D1=D2	D1=D2	---	D1=D2

Range Input Resistance									
% Inward Rectification	---	---	---	---	---	---	---	---	D1=D2
Time Constant of the Membrane	---	D1>D2	D1=D2	---	---	D1=D2	D1<D2	D1<D2	D1=D2
Capacitance	---	D1>D2	D1=D2	---	---	---	---	---	D1=D2
sEPSC Frequency	---	---	D1<D2	---	---	---	---	---	---
sEPSC Amplitude	---	---	D1=D2	---	---	---	---	---	---
sEPSC Kinetics	---	---	D1=D2	---	---	---	---	---	---
mEPSC Frequency	D1<D2	---	D1<D2*	---	---	---	---	D1=D2	D1=D2
mEPSC Amplitude	D1=D2	---	D1=D2	---	---	---	---	D1=D2	D1<D2
mEPSC Decay	D1=D2	---	D1=D2	---	---	---	---	---	D1=D2
mEPSC Rise Time	---	---	D1<D2	---	---	---	---	---	D1=D2
s/mIPSC Frequency	---	---	---	D1=D2	---	---	---	---	---
s/mIPSC Amplitude	---	---	---	D1=D2	---	---	---	---	---
s/mIPSC Decay	---	---	---	D1=D2	---	---	---	---	---
s/mIPSC Rise Time	---	---	---	D1=D2	---	---	---	---	---
Probability of occurrence of spontaneous membrane depolarization after $GABA_A$ blockade	---	---	D1<D2	---	---	---	---	---	---
Paired-Pulse Ratio	D1>D2	---	D1<D2	---	---	---	---	---	---
AMPA-induced current amplitude	---	---	D1>D2	---	---	---	---	---	---
NMDA/AMPA Ratio	D1<D2	---	---	---	---	---	---	---	---
Endocannabinoid-mediated LTD	D1<D2	---	---	---	---	---	---	---	---
Tonic $GABA_A$ current and sensitivity to $GABA_A$ current	---	---	D1<D2	D1<D2	---	---	---	---	---

Only caudate-putamen MSN subtype electrophysiology studies in acute brain slice preparation experiments independent of variables such as dopamine depletion and psychostimulant exposure are included. This criteria *a priori* excludes studies that analyzed MSN subtype electrophysiological properties but did not directly compare D1 and D2 subtype groups, for example (Day et al., 2006; Ade et al., 2011), or were performed in regions such as the nucleus accumbens, for example (Ma et al., 2012; Cao et al., 2018). Acronyms: ---, did not measure; AP, action potential; AHP, afterhyperpolarization, FI: Frequency of evoked spikes to injected depolarization current; s/mEPSC, spontaneous/miniature excitatory post-synaptic current. IPSC, inhibitory post-synaptic current; LTD: long term depression. *This finding significant in some but not all analyses within this study. #Planert and colleagues assessed rheobase using multiple analyses. The conclusion of all analyses was similar and is

thus condensed here. [@]The use of M4 eGFP labeling as equivalent to the D1 MSN subtype has been cautioned (Cepeda et al., 2008). [%]A number of studies from Vicini and colleagues have investigated GABA conductance between MSN subtypes; here we feature the initial report.

766 **Table 2.** Electrophysiological Properties of Male and Female Drd1a and Drd2 Mouse Caudate-
767 putamen Medium Spiny Neurons

Property	Drd1a	Drd2	Statistics (*F*, P)
Resting Potential (mV)	Male: -90.9 ± 1.2[a] Female: -87.5 ± 0.7[a,b]	Male: -85.0 ± 1.8[b] Female: -89.0 ± 1.2[a,b]	**Interaction: $F_{(1,82)}$=8.7; P=0.004** Sex: $F_{(1,82)}$=0.1; P=0.75 Subtype: $F_{(1,82)}$=2.8; P=0.10 **Post-hoc: Tukey's**
Rheobase (nA)	Male: 0.16 ± 0.01[a] Female: 0.16 ± 0.01[a]	Male: 0.10 ± 0.02[b] Female: 0.12 ± 0.02[a,b]	Interaction: $F_{(1,82)}$=0.4; P=0.52 Sex: $F_{(1,82)}$=0.4; P=0.54 **Subtype: $F_{(1,82)}$=12.3; P=0.0007** **Post-hoc: Tukey's**
AP Threshold (mV)	Male: -58.3 ± 1.0[a,b] Female: -54.6 ± 1.2[a]	Male: -58.6 ± 0.7[a,b] Female: -62.6 ± 2.9[b]	**Interaction: $F_{(1,81)}$=7.2; P=0.0087** Sex: $F_{(1,81)}$=0.01; P=0.90 **Subtype: $F_{(1,81)}$=8.0; P=0.0058** **Post-hoc: Tukey's**
AP Amplitude (mV)	Male: 68.8 ± 2.1 Female: 69.5 ± 2.9	Male: 69.3 ± 2.8 Female: 79.1 ± 3.1	Interaction: $F_{(1,81)}$=2.3; P=0.13 Sex: $F_{(1,81)}$=3.1; P=0.08 Subtype: $F_{(1,81)}$=3.0; P=0.09
AP width at half-peak (ms)	Male: 1.63 ± 0.05[a] Female: 1.45 ± 0.05[a,b]	Male: 1.31 ± 0.06[b] Female: 1.43 ± 0.15[a,b]	**Interaction: $F_{(1,80)}$=4.7; P=0.0339** Sex: $F_{(1,80)}$=0.1; P=0.7052 **Subtype: $F_{(1,80)}$=5.3; P=0.0234** **Post-hoc: Tukey's**
AHP Peak (mV)	Male: -8.9 ± 0.4 Female: -11.0 ± 0.7	Male: -9.8 ± 0.8 Female: -9.2 ± 0.7	Interaction: $F_{(1,80)}$=3.5; P=0.0654 Sex: $F_{(1,80)}$=0.8; P=0.37 Subtype: $F_{(1,80)}$=0.2; P=0.69
AHP Time to Peak (ms)	Male: 32.2 ± 1.3 Female: 26.3 ± 1.6	Male: 31.8 ± 3.4 Female: 32.5 ± 3.2	Interaction: $F_{(1,81)}$=2.1; P=0.15 Sex: $F_{(1,81)}$=1.4; P=0.24 Subtype: $F_{(1,81)}$=1.6; P=0.21
FI Slope (Hz/nA)	Male: 192.5 ± 10.4[a] Female: 164.0 ± 12.8[a]	Male: 278.7 ± 28.9[b] Female: 242.0 ± 23.0[b]	Interaction: $F_{(1,81)}$=0.1; P=0.82 Sex: $F_{(1,81)}$=3.4; P=0.0708 **Subtype: $F_{(1,81)}$=21.2; P<0.0001** **Post-hoc: Tukey's**
Linear Range Input Resistance (MΩ)	Male: 102.1 ± 5.8[a] Female: 93.6 ± 6.6[a]	Male: 141.0 ± 19.2[b] Female: 109.8 ± 18.3[a,b]	Interaction: $F_{(1,78)}$=1.1; P=0.30 Sex: $F_{(1,78)}$=2.9; P=0.09 **Subtype: $F_{(1,78)}$=6.3; P=0.0138** **Post-hoc: Tukey's**
Rectified Range Input Resistance (MΩ)	Male: 83.4 ± 4.7 Female: 83.9 ± 8.6	Male: 112.0 ± 17.7 Female: 83.1 ± 11.7	Interaction: $F_{(1,79)}$=2.0; P=0.16 Sex: $F_{(1,79)}$=1.9; P=0.17 Subtype: $F_{(1,79)}$=1.8; P=0.18
% Inward Rectification (%)	Male: 82.3 ± 1.3 Female: 81.0 ± 1.8	Male: 80.8 ± 4.3 Female: 79.2 ± 3.3	Interaction: $F_{(1,79)}$=0.0; P=0.93 Sex: $F_{(1,79)}$=0.3; P=0.57 Subtype: $F_{(1,79)}$=0.4; P=0.52
Time Constant of the Membrane (ms)	Male: 10.3 ± 0.8 Female: 19.6 ± 1.2	Male: 14.6 ± 3.2 Female: 9.9 ± 1.9	Interaction: $F_{(1,82)}$=2.9; P=0.09 Sex: $F_{(1,82)}$=1.2; P=0.28 Subtype: $F_{(1,82)}$=1.6; P=0.20
Capacitance (pF)	Male: 109.6 ± 10.0 Female: 99.0 ± 7.0	Male: 94.4 ± 11.2 Female: 91.2 ± 8.5	Interaction: $F_{(1,78)}$=0.1; P=0.74 Sex: $F_{(1,78)}$=0.4; P=0.55 Subtype: $F_{(1,78)}$=1.0; P=0.31
mEPSC Frequency (Hz)	Male: 1.9 ± 0.2 Female: 1.9 ± 0.2	Male: 2.0 ± 0.5 Female: 1.4 ± 0.4	Interaction: $F_{(1,47)}$=0.9; P=0.33 Sex: $F_{(1,47)}$=1.2; P=0.29 Subtype: $F_{(1,47)}$=0.3; P=0.58
mEPSC Amplitude (pA)	Male: 15.9 ± 0.4[a] Female: 15.2 ± 0.6[a]	Male: 17.7 ± 1.1[a,b] Female: 20.7 ± 1.5[b]	**Interaction: $F_{(1,47)}$=5.2; P=0.0270** Sex: $F_{(1,47)}$=2.1; P=0.1498 **Subtype: $F_{(1,47)}$=20.4; P<0.0001** **Post-hoc: Tukey's**
mEPSC Decay (ms)	Male: 1.9 ± 0.2 Female: 1.9 ± 0.2	Male: 2.0 ± 0.5 Female: 1.4 ± 0.4	Interaction: $F_{(1,47)}$=2.6; P=0.11 Sex: $F_{(1,47)}$=0.1; P=0.76 Subtype: $F_{(1,47)}$=0.0; P=0.95

768 Values are mean ± SEM. Bold font indicates statistical significance. Different superscript letters denote significant
769 differences detected by a Tukey's post-hoc test. Abbreviations: MSN, medium spiny neuron; AP, action potential;
770 AHP, afterhyperpolarization; FI, Frequency of evoked spikes to injected depolarization current; mEPSC, miniature
771 excitatory post-synaptic current.

Figure 1