Female mice are resilient to age-related decline of substantia nigra dopamine neuron firing parameters

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Abstract

The degeneration of substantia nigra dopamine neurons is a central feature in the pathology associated with Parkinson's disease, which is characterized by progressive loss of motor and cognitive functions. The largest risk factors for Parkinson's Disease are age and sex; most cases occur after age 60 and males have nearly twice the incidence of Parkinson's as females. While much research in Parkinson's has focused on the mechanisms underlying the dopamine neuron degeneration, very little work has considered the influence of these two risk factors to disease risk and presentation. In this work, we performed whole cell patch clamp recordings in brain slices to study the alterations in intrinsic firing properties of dopamine neurons in C57BL/6 mice across ages and between sexes. We also performed a semiquantitative analysis of midbrain dopamine neuron populations, transcriptomic analysis of the substantia nigra, and an in vivo assessment of locomotor activity. We found a progressive decline in dopamine neuron function and number in males with age, while females remained largely unaffected. These impairments in dopamine neuron function in males may represent a vulnerability to further insults that could predispose these cells to neurodegenerative diseases such as in Parkinson's.

Introduction

Midbrain dopamine (DA) neurotransmission is involved in a wide array of functions including motor control, motivation, reward, and cognitive processes (Wise & Rompre, 1989; Crocker, 1997; Nieoullon, 2002; Wise, 2004). The dopaminergic cell bodies of substantia nigra (SN) and the ventral tegmental area (VTA) form the starting points for the nigrostriatal (Fuxe *et al.*, 1977) and mesolimbic pathways (Bjorklund & Dunnett, 2007), respectively. Degeneration of dopamine neurons in the SN is a central feature of the etiology of Parkinson's disease and is associated with a progressive loss of the ability to initiate movement (Hornykiewicz, 1975; Mazzoni *et al.*, 2012). Parkinson's disease is the result of multiple complex gene/environment interaction (Cannon & Greenamyre, 2013) and studies suggest that the incidence may be increasing (Savica *et al.*, 2016). Two of the most commonly observed risk factors for the development of Parkinson's are age and sex (Gillies *et al.*, 2014). Most reports show an increase in the incidence of Parkinson's disease after the age of 60 (de Lau & Breteler, 2006) and many also show a 1.5-2 times greater incidence in males compared with females (Baldereschi *et al.*, 2000; Wooten *et al.*, 2004; Moisan *et al.*, 2016). Despite the awareness of these two risk factors in the clinical literature, little basic research has considered the effects of age and sex on disease development and presentation.

Previous work in our lab identified a decrease in spontaneous firing frequency and a lack of pacemaker firing fidelity in DA neurons recorded in slices from male mice over 24 months of age (Branch *et al.*, 2014). Dopamine neurons show rhythmic, spontaneous pacemaking activity in brain slices (Grace & Onn, 1989), which is the result of the coordinated activity of at least a dozen ion channels (Bean, 2007), calcium homeostasis, and energy metabolism (Duda *et al.*, 2016). The maintenance of continuous activity renders dopamine neurons highly vulnerable to oxidative damage, which is thought to be a possible mechanism for their degeneration in Parkinson's disease (Surmeier *et al.*, 2011). Dysfunction in firing suggests deficits in the health of the cells (Branch *et al.*, 2016) and possibly impairment of the nigrostriatal circuit with consequences for motor function (Good *et al.*, 2011; Janezic *et al.*, 2013).

In this present study we sought to characterize these firing deficits, determine when during the mouse lifespan these deficits appear, and explore any potential effects of sex. To do this, we performed current clamp recordings of substantia nigra dopamine neurons across four age groups in both male and female C57Bl/6 mice. The age groups tested included 4, 12, 18, and 24-30 months, which correspond roughly to human ages 25, 42, 56, and 69-81 (Flurkey, 2007). We found that DA neurons from females show few obvious decrements with age, while males showed firing deficits by 18 months. We then assessed the overall population of dopamine neurons with a semiquantitative analysis of midbrain dopamine neuron populations in young and old, male and female mice as well as a transcriptomic analysis of isolated substantia nigra tissue, and an assessment of spontaneous locomotor activity across ages.

Materials and Methods

Animals: Male and female C57BL/6 mice were obtained from the National Institute on Aging aged rodent colony and housed in a humidity and temperature-controlled facility under a 12:12 light-dark cycle with food and water available *ad libitum*. Animals were used at 4 different ages: 4 months, 12 months, 18 months and 24-30 months. All experiments were reviewed and approved by the Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation.

Ex vivo electrophysiology: Mice were deeply anesthetized with isoflurane before rapid dissection of the brain, which was immediately placed in an ice-cold oxygenated cutting solution containing the following in mM: 250 sucrose, 26 $NaHCO_3$, 2 KCl, 1.2 NaH_2PO_4, 11 glucose, 7 $MgCl_2$, 0.5 $CaCl_2$. Coronal slices (200 µm) containing ventral midbrain were collected using a VF-200 Compresstome (Precisionary, Cambridge, MA, USA). Slices were incubated at 34°C for 20

minutes, after which they were incubated at room temperature in an artificial cerebrospinal fluid (aCSF) solution containing the following in mM: 126 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 26 $NaHCO_3$, 11 glucose, 1.3 $MgCl_2$, 2 $CaCl_2$ and with 0.01 MK-801. During recording, slices were superfused with aCSF at 34-36°C with a flow rate of 2 ml/min in the presence of 100 µM picrotoxin and 10 µM DNQX to antagonize any spontaneous GABAergic and glutamatergic synaptic activity that could influence the cell firing parameters that were measured.

DA neurons were patched using an upright microscope (Nikon, Melville, NY, USA) and using thin wall glass pipettes (1.5-3 MΩ, World Precision Instruments, Sarasota, FL, USA) filled with internal solution containing the following in mM: 142 K-gluconate, 2 KCl, 4 $MgCl_2$, 10 HEPES, 0.5 EGTA, 4 ATP-Mg, 0.5 Na-GTP, 280-300 mOsm, pH adjusted to 7.2 to 7.4 with KOH. In addition, 0.05% neurobiotin (Dufour et al., 2014) was added to the patch pipette to allow for *post hoc* verification of the DA identity of neurons recorded, through immunolabeling for tyrosine hydroxylase (see below). Current clamp recordings were made with a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), filtered at 4 kHz and digitized at 50 kHz. Data were collected using Axograph software (www.axograph.com). Voltages were corrected for a liquid junction potential of 11.1 mV between the internal and external solution. Electrophysiological data were analyzed with IgorPro (Wavemetrics, Lake Oswego, OR, USA).

Immunohistochemistry: Animals (n = 4-5, per age and sex) were deeply anesthetized with Tribromoethanol (250 mg/kg, i.p.) and perfused by intracardiac puncture with 20 ml of phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM phosphate buffer, pH 7.4), followed by 20 ml of 4 % paraformaldehyde (PFA) solution in 0.1 M phosphate buffer (PB, pH 7.4). The brain was removed, post-fixed overnight in 4 % PFA solution at 4 °C, washed in PBS and immerse in 30% sucrose at 4°C for 3 days. After this, the brain was embedded in OCT, stored at -80 °C and processed as follow below.

Cryostat cutting: Consecutive coronal sections (40 µm thick) were collected throughout the whole extension of the ventral midbrain containing the VTA and the SN, between stereotaxic coordinates 2.9 to 3.88 posterior to Bregma (Franklin and Paxinos, 2012). The sections were collected in wells containing PBS for further processing.

Tyrosine hydroxylase (TH) immunolabeling: Polyclonal rabbit anti-tyrosine hydroxylase antibody (Millipore-Sigma, Burlington) was used as our primary antibody and goat anti-rabbit Alexa Fluor 594 (Abcam, Cambridge) was used as secondary antibody. Normal goat serum (Jackson ImmunoResearch, West Grove) was used in all blocking steps.

Brain sections were equilibrated to room temperature (RT) for 30 minutes, rinsed in PBS and incubated in a blocking solution (BS; 5% normal goat serum in 0.2% PBST) for 4 hours at RT in a platform shaker at low speed. Then, sections were incubated overnight with primary TH antibody (1:750 dilution in 0.5% normal goat serum in PBS) at 4 °C with gentle shaker agitation. After rinsing with 0.2% PBST, sections were incubated with the secondary antibody Alexa Fluor 594 (1:200 dilution in PBS) for 2 hours at RT and protected from light with tin foil. After rinsing with PBST and PBS, sections were mounted onto glass slides using Shur/Mount Aqueous (EMS, Hatfield) coverslip and sealed with nail polish.

For neurobiotin filled cells, brain slices from electrophysiology experiments were fixed in 4% PFA overnight and then processed for TH immunolabeling as described above. Natural streptavidin protein Dylight 488 (1:500 dilution in PBS, Abcam, Cambridge) was added to label neurobiotin. 100 ng/ml DAPI in 0.2% PBST (1.5 hrs. incubation) was added in the last rinse after secondary antibodies incubation. This was followed by three rinse steps (1-hour, 30 min, 30 min) with PBST to remove staining excess. Brain slices were then mounted on glass slides as described above.

Image acquisition and cell counting: Images were obtain using at Zeiss LSM-710 confocal microscope with a 10X magnification objective. High resolution images were stitched to include the range of ventral midbrain containing the VTA and SN. Semi-quantitative cell counting was done using Image J software.

RNA-Seq: The substantia nigra was dissected from mice 4 months old (5 males, 5 females) and 30 months old (5 males and 5 females). Samples were immediately snap- frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from frozen tissue using RNeasy plus mini kit (Qiagen, CA, USA). The amount of total RNA was quantified using Nanodrop 2000 spectrophotometer and RNA quality and purity was evaluated using Agilent 2200 TapeStation system (Agilent Technologies, CA, USA). One sample failed quality control leaving 4 mice in the aged female group.

Library Preparation

Polyadenylated RNA was captured from 25 ng total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). Once enriched for mRNA, RNA-Sequencing libraries were made using NEBNext Ultra II Directional RNA Library Kit for Illumina (NEB #E7760), according to manufacturer's instructions. Briefly, oligo-dT captured RNA was fragmented and primed prior to first strand cDNA synthesis and subsequent second strand cDNA synthesis. cDNA libraries were end-prepped and adaptor-ligated. PCR enrichment of adaptor-ligated cDNA libraries was used to incorporate indices from NEBNext Multiplex (NEB #E7335, #E7500) Oligos for Illumina, using 15

cycles of PCR. cDNA libraries were then cleaned with SPRISelect Reagent (Beckman Coulter #B23318) and eluted in 23 µL of 0.1X TE buffer. After eluting, libraries were sized using 2 µL of cDNA libraries to run HSD1000 ScreenTape (Agilent #5067-5584) on the Agilent Technologies 2200 TapeStation. Qubit dsDNA HS Assay Kit (Thermofisher #Q32854) was used to quantify the concentration of the cDNA libraries. cDNA libraries were normalized to 4 nM and pooled for sequencing.

Sequencing and bioinformatic analysis

A 4 nM pool was submitted to the Clinical Genomics Center at OMRF for a NextSeq500 High PE75 sequencing run. After sequencing, raw fastq filed were uploaded into StrandNGS for adaptor-trimming, alignment, de-duplication, and quantification. Reads were quality filtered for Q>30 and adapter sequences were trimmed. Reads were aligned to the Mm10 genome build and RefSeq annotations and duplicate reads removed.

Locomotor activity: Spontaneous locomotor activity was assessed in terms of distance traveled and average velocity in animals across the four age groups including 4 months (n=15, males; n=13, females), 12 months (n=11, males; n=8, females), 18 months (n=10, males; n=8, females), and 24-30 months (n=7, males; n=8, females). For this, mice were placed in locomotor chambers (44.5 x 44.5 cm; Columbus Instruments, Columbus, OH, USA) equipped with infrared photosensors. Spontaneous locomotor activity was quantified for 30 minutes using the Autotrack 5.5 software (Columbus Instruments, Columbus).

Statistical analysis: Data are represented in box and whisker plots, to show distributions of data, or line graphs with means ± standard error of the mean. Data were analyzed by two-way ANOVA (factors: sex, age) and two-way repeated measures (RM) ANOVA, followed by Bonferroni t-test post hoc (Sigma Stat, San Jose, CA, USA). Student's t-tests were used where indicated. Alpha was set to 0.05.

Results

DA neuron pacemaking becomes disrupted in males, but not females, by 18 months of age

DA neurons were identified in the ventral half of the substantia nigra of coronal midbrain slices based on their large cell bodies, characteristic slow firing rate (<7Hz) (Grace & Onn, 1989) and the presence of a large voltage sag as a result of the hyperpolarization-activated cation current, I_H (Neuhoff *et al.*, 2002; Dufour *et al.*, 2014). Recorded cells were also filled with neurobiotin in order to confirm their dopaminergic identify through post *hoc* immunostaining of slices for tyrosine

hydroxylase (TH), the rate limiting enzyme of dopamine synthesis (Pickel *et al.*, 1975; Figure 1A). DA neurons were recorded from male and female mice across four age groups: 4 months (n=21 cells from 6 male mice; n=23 from 3 females), 12 months (n=20, from 8 males; n=17 from 6 females), 18 months (n=21 from 4 males; n=19 from 4 females) and 24-30 months (n=31 from 10 males; n=26 from 7 females). No change in input resistance with age was observed, as previously reported (Branch *et al.*, 2014), however DA neurons from females overall showed lower input resistance compared with males ($F(1,170)=14.453$, $p<0.001$; Fig 1C). A range of spontaneous firing frequencies were recorded in current clamp for at least 2 minutes each, across all ages in DA neurons from males (from 0.6 to 4.425 Hz) and females (0.76 to 5.3 Hz). An age-sex interaction was detected in firing frequency ($F(3,170)=2.707$, $p=0.047$; Fig 1D), with males showing lower firing frequencies than females at ages 18 months and older. Males showed a slight decrease in firing frequencies with age, compared with 4-month-old males, that was more pronounced at 18 months (33.96% decrease) than at 24-30 months (16.53% decrease). This may be a result of a greater number of dysfunctional dopamine cells still living at 18 months that could be gone from the neuronal population by 24 months of age or unhealthy mice that are no longer represented in the population by 24 months.

The coefficient of variation of the inter-spike interval (CV_{ISI}) in spontaneous firing was measured during the most stable 30 seconds of firing of the minute-long recordings from each cell, to assess the pacemaker function. An age-sex interaction was detected ($F(3,170)=6.247$, $p<0.001$; Fig 1E), with males showing an increase in variability of firing by 18 months that remained through 24-30 months. Even though the average CV_{ISI} was much higher in males ages 18 months and older, it was evident from the range of data (3.83% to 89.48%) that there was still a population of cells present that showed regular “young-seeming” pacemaking ability. A least 1/3 of the cells recorded from males ages 18 to 30 months had a CV_{ISI} less than 15% (23.8% of cells from 18 months and 38.7% of cells from 24 to 30 months). Those healthy cells came from half of the animals in each the 18 month and 24-30 month age groups. Of those animals, 30.95±1.94% of the cells had low CV_{ISI} in the 18 month old group and 57.67±10.16% of the cells from the 24-30 month old group. This suggests variability in the animal population at the oldest age group, with some animals having mainly healthy cells. Females did not show any change across age and still maintained low variability in pacemaking at ages 18 months and older (1.95% to 28.94%), with 73.33% of cells having a CV_{ISI} below 15%.

Action potential waveforms show divergent effects across sex and age

To further assess the underlying changes in firing activity in males and females across age, the waveforms of the action potentials during spontaneous firing activity were averaged and analyzed for the following parameters: spike height (measured from peak to trough), half width (measured at the midpoint of the spike) and afterhyperpolarization potential (AHP; raw value taken at the trough). The action potential threshold and maximum rise and decay velocities were also measured by using phase plane plots of the action potential averaged for each cell. Age-sex interactions were detected for spike height ($F(3,170)=3.207$, $p=0.025$; Fig 2C), half width ($F(3,170)=3.351$, $p=0.02$), AHP ($F(3,170)=3.467$, $p=0.018$), and maximum rise velocity ($F(3,170)=5.344$, $p=0.002$). Across the four age groups, males showed a biphasic evolution in spike height, half width, and maximum rise and decay velocities. Between 4 and 12 months across all four parameters there was an increase in spike height and rise/decay velocities and a narrowing of the half width, while after 12 months there was a steady decline in spike height, velocities and increasing half width. In contrast, females showed a more linear trajectory in these parameters with age, starting at 4 months with much higher spike heights, velocities, and narrower half widths than males, which progressively declined with age. For maximum decay velocity, only main effects of age and sex were detected (age: $F(3,170)=6.778$, $p<0.001$; sex: $F(1,170)=12.346$, $p<0.001$), where females showed overall higher velocities than males and both sexes showed decreases in velocities starting at 18 months. The AHP remained steady across ages in males and females until decreasing at 24-30 months in males only. No differences were detected in action potential threshold across ages and between sexes.

DA neurons from males, but not females, are more prone to depolarization block by 18 months of age

The membrane excitability of neurons was measured through frequency-current (F-I) curves in response to increasing step current injections of 20pA (20 to 400pA total). DA neurons were recorded across four age groups including 4 months (n=24 from 9 males; n=21 from 3 females), 12 months (n=17 from 7 males; n=18 from 7 females); 18 months (n=22 from 7 males; n=18 from 5 females), and 24-30 months (n=29 from 9 males; n=25 from 10 females). The frequency was determined from the first three action potentials. In males and females, no differences were detected for frequency between ages nor were any differences between males and females across ages (Fig. 3E).

Males showed a robust age-current interaction ($F(57,1672)=21.142$, $p<0.001$). 18-month old males showed decreases in spike numbers by 200 pA current injection and 24-30 months old

males showed decreases by 300 pA injection. Females did not show any change in the number of spikes across ages. Overall, females showed higher numbers of spikes at all ages compared with males ($F(1,166)=7.378$, $p=0.007$) and higher maximum numbers of spikes ($F(1,166)=6.666$, $p=0.011$). Similarly, males 18 months and older showed fewer spikes for the last current injection (400pA) compared with females ($F(3,166)=3.717$, $p=0.013$, Fig 3F). This decrease in spikes at higher current injections in older males seems to indicate a tendency to enter depolarization block sooner than females or younger males.

DA neurons from males 18 months and older, but not females, show a longer rebound spike delay

DA neurons are characterized by I_H, which can be measured in current clamp as a large sag during hyperpolarizing current injections (Neuhoff *et al.*, 2002; Dufour *et al.*, 2014). Moreover, I_H has been shown to interact with I_A, the rapidly inactivating type A potassium channel current, and modulate the rebound spiking properties in dopamine neurons (Amendola *et al.*, 2012). We next assessed I_H and rebound firing properties by delivering a hyperpolarizing current injection for 1 second, bringing the potential at the end of the sag to approximately –80 mV (-83.73±1.007 across all cells). Sag and rebound spiking was recorded from animals at 4 months (n=13 from 4 males; n=15 from 3 females), 12 months (n=13 from 5 males; n=12 from 5 females), 18 months (n=17 from 4 males; n=17 from 3 females) and 24-30 months (n=16 from 8 males; n=21 from 7 females). No change was detected in sag amplitude across ages and between sexes, suggesting no change in I_H. This agrees with previous findings of no change in I_H current with age (Branch & Beckstead, 2014). However, there was a robust age-sex interaction detected for rebound spike delay ($F(3,116)=5.177$, $p=0.002$). At ages 18 months and older, males show a 483.2% greater average than females of the same age for rebound spike delay. As with the CV_{ISI}, a similar proportion of cells from males ages 18 months and older showed rebound spike delays less than 504 ms, the average for 4 months, plus 1 standard deviation. 35.29% of total cells from the 18 month old group and 50% of the cells from the 24-30 month age group were in the range of the 4 month old males. At 18 months, those healthy cells were recorded from all the animals of the age group and at 24-30 months those cells came from 62.5% of the animals. Analyzing each of those animals at 24-30 months, on average 75.0±10.1% of their cells were in the range of the 4 month males. This further suggests that at 18 months there is a division in the population of the cells, while at the oldest ages this is further confounded by a division in the population of the animals.

Males show loss of midbrain DA neurons with age, while females do not

Based on the electrophysiological findings, at least 60% of DA neurons recorded from males ages 18 months and older showed robust deficits in pacemaking and rebound spiking, while females showed very few deficits. In order to more accurately interpret the findings from our electrophysiological data, we next needed to address how the population of DA neurons in the region was affected by aging. A semi-quantitative analysis of TH positive neurons in the midbrain was performed in mice at 4 months of age (n=4, males; n=5 females) and 27 months of age (n=4, males; n=4 females). At -3.28 mm from bregma, an age-sex interaction was detected ($F(1,12)=7.307$, $p=0.019$), where males showed a 28.82% decrease in the number of dopamine neurons with age and females did not change. In fact, females showed 31.36% fewer dopamine neurons at 4 months compared with males.

The substantia nigra from young females show higher expression for neuroprotective traits than young males

In order to narrow down underlying causes to the changes seen in firing properties and action potential waveform, RNA seq analysis was performed on dissected substantia nigra regions dissected from 4 month old mice (n=5, males; n=5 females) and 27 month old mice (n=5, males; n=4, females). The expression of ion channels that participate in the DA neuron action potential and pacemaking were analyzed. Compared with 4 month males, 4 month old females were found to have higher expression of BK channels, calcium activated “big” conductance potassium channels (6A, *Kcnma1;* t-test, $p=0.0139$; 142.01±5.4%) and SK channels, calcium activated “small” potassium channels (6B, *Kcnn2* t-test, $p=0.059$; 129.62±3.0%). Indeed, a higher level of BK channels could explain the narrower half width and faster decay kinetics seen in young females compared with young males (Kimm *et al.*, 2015). It must be considered as well that the higher levels of BK and SK channels in younger females could be an underestimate, since the 4 month old females have approximately 70% of the dopamine neurons in the substantia nigra as males. Both BK and SK channels have been identified as potentially neuroprotective (Contet *et al.*, 2016; Honrath *et al.*, 2018).

To further support this finding, young females also have higher L-type calcium channel expression (6F, *Cacna1c;* t-test: $p=0.034$; 124.47±5.49%), which is likely the source of calcium required for the BK channel activation (Vandael *et al.*, 2010). In addition, HCN channels, mediating the I_H current and have been shown to be mechanistically linked with BK channels (Contet et al., 2016), are also higher in younger females than males (127.83±3.98%) and decrease with age only in females (67.02±10.41%) (6E, *HCN1*: $F(1,15)=4.012$, $p=0.064$). Also, calmodulin, a calcium

binding protein associated with the SK channel complex (Griffith *et al.*, 2016), was higher in young females than males and decreased slightly with age (6C, sex-age interaction: *Calm1*, F(1,15)=4.992, p=0.041; 6D, *Calm2*, F(1,15)=4.643, p=0.048), while males did not change with age. Both BK and SK channels decreased with age in females (6A, BK channels: t-test, p=0.08; 74.9±13.07%; 6B, SK channels: t-test, p=0.009; 75.35±7.25%), while males did not show any clear change.

No changes were found in the expression of any other ion channel in males with age, but also the action potential waveform showed very limited changes when comparing between 4 months and 24-30 months. Despite this, an age-sex interaction was detected for *PINK1* (6G, F(1,15)=4.862, p=0.043), where expression only increased in males with age (138.35%) and did not change in females. *PINK1* encodes PTEN induced putative kinase 1, which accumulates on damaged mitochondria and activates parkin, the E3 ubiquitin ligase, which facilitates mitochondrial degradation (Srivastava, 2017). Notably, mutations in *PINK1* and parkin have been identified in autosomal recessive forms of early onset Parkinson's Disease, as damaged mitochondria accumulate and lead to degeneration of dopamine neurons. Parkin expression was also found to increase with age (6H, F(1,15)=10.086, p=0.006), in both males and females. However, the increase in males with age (182.83%) was more than females (123.20%). These suggest that dopamine neurons from may be under greater mitochondrial stress than females, which may contribute to the overall functioning and health of the cells.

Males and females show slight declines in spontaneous locomotor activity with age

The final question was to determine if these changes and deficits seen with age (impaired firing activity, loss of DA neurons) translate into any meaningful behavioral phenotype. Spontaneous locomotor activity was assessed in animals across ages 4 months (n=15, males; n=13, females), 12 months (n=11, males; n=8, females), 18 months (n=10, males; n=8, females), and 24-30 months (n=7, males; n=8, females). Distance traveled and average velocity both decreased by 18 months of age in males and females (distance: F(3,72)=5.771, p=0.001; velocity: F(3,72)=20.413, p<0.001). Females overall showed higher average velocities than males (F(1,72)=4.835, p=0.031), especially noticeable in the youngest age group. It has been previously shown that young female mice have higher basal locomotor activity than males (Caldarone *et al.*, 2008; Van Swearingen *et al.*, 2013). It has also been reported consistently that parkinsonian motor impairments appear only after at least a 50% of dopamine neurons in the substantia nigra are lost (Cheng *et al.*, 2010). Given that the overall changes in locomotor activity were very minor

in these mice across age, it is likely that the alterations in neuronal properties represent more of a vulnerability to insults, rather than any active disease process.

Discussion

To our knowledge, this is the first in-depth report of dopamine firing parameters across age in both male and female mice. Overall, dopamine neuron parameters from females remained fairly stable across the lifespan, with few changes in firing properties and no decrease in dopamine cell number with age. However, dopamine neurons from males began to show signs of physiological decrements in firing activity by 18 months of age and a loss of dopamine neuron number. Indeed, we qualitatively noted that most animals at 18 months had a population of both “healthy” cells and “poor” cells, while at 24-30 months animals tended to have either a majority of healthy cells or a majority of poor cells. There were slight decreases in open field locomotor behavior with age, but nothing that reached the level of impairment observed in single neurons. Data from both human Parkinson’s patients and animal models of the disease indicate that a loss of at least 50% of dopamine neurons is needed to see a change in locomotor activity (Cheng *et al.*, 2010). It is likely therefore that the decrease in locomotor activity is unrelated with the loss of functionality of the dopamine neurons. Together, we believe these results represent vulnerability in the dopamine cells from males, more so than from females, to insults that could trigger the degeneration of dopamine neurons seen in Parkinson’s disease.

Even at the youngest ages tested (4 months), from mice in young adulthood, there were observable differences between dopamine neuron from males and females. Females overall had 30% fewer dopamine neurons in the midbrain than males (previously observed, see Murray et al., 2003), their action potentials were much larger with faster kinetics, and they exhibited higher mRNA expression for many ion channels important in the dopaminergic action potential. In addition, females had higher locomotor activity than males, which may be possibly related to the differences in the dopaminergic systems from males. Such a higher level of basal locomotor activity in females has also been observed previously (Caldarone *et al.*, 2008; Van Swearingen *et al.*, 2013).

This work builds on our previous study in male C57Bl/6 mice, where cell-attached recordings from two age groups showed decreases in firing rate and pacemaking fidelity with age (Branch and Beckstead, 2014). Here we explored that further and found that these deficits in spontaneous firing, starting at 18 months, are also accompanied by a propensity to enter depolarization block

and very long delays in rebound spiking. An analysis of the the action potential waveform across the ages showed, surprisingly, that males show a biphasic change throughout their life in spike height and kinetics. Specifically, this was an increase in spike height and kinetics by 12 months, followed by a progressive decrease. There were very few differences in the action potential waveform between the youngest and oldest age groups, which was supported by a lack of change in expression of ion channels at those ages. Dopaminergic firing activity is highly dependent on oscillations of calcium, of which control is lost through age and mitochondrial stress (Surmeier, 2007). One interpretation of these data could be that from 4 to 12 months, calcium levels are rising in the cells, due to a loss of control over the homeostasis, and more calcium is available to activate BK and SK channels which leads to larger spikes with faster kinetics. Over time however, the amount of intracellular calcium increases, leading to a disruption in function of many ion channels, which produces the deficits in firing activity. An indication of stress in the cells from the old males was the elevated levels of *PINK1* expression, suggesting the presence of more damaged mitochondria. In fact, L-type calcium channel blockers are being tested as a therapy for Parkinson's disease, to prevent intracellular calcium increases that are toxic for the cells (Ilijic et al., 2011).

Aging of dopamine neuron firing parameters in females has not been previously studied. However, there is evidence from animal models of Parkinson's that cells from females are slightly more resistant than cells from males to neurotoxic lesions (Gillies et al., 2004). This suggests the disposition to cellular damage could be fundamentally different. The only changes observed with age in the dopamine neuron firing activity in females were slight decreases in spike height and kinetics of the action potential waveform, which was further supporter by decreases in gene expression of ion channels. In most cases, these changes brought the old females down to the ranges of parameters and expression levels in young males, but was not to disrupt firing. It has been suggested that neuroprotective effects of sex hormones, including estrogen and progesterone, account for the dopamine cell resistance in lesion studies (Siddiqui et al., 2016; Gillies et al., 2014). However, while mice do not formally enter menopause like humans, they do show very low plasma levels of hormones by 18 months of age (Lu et al., 1979). It is possible that the hormonal presence earlier in life exerts protective effects that can last through aging, or the resilience of female cells could be due to other mechanisms that have yet to be identified.

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Figures and Legends

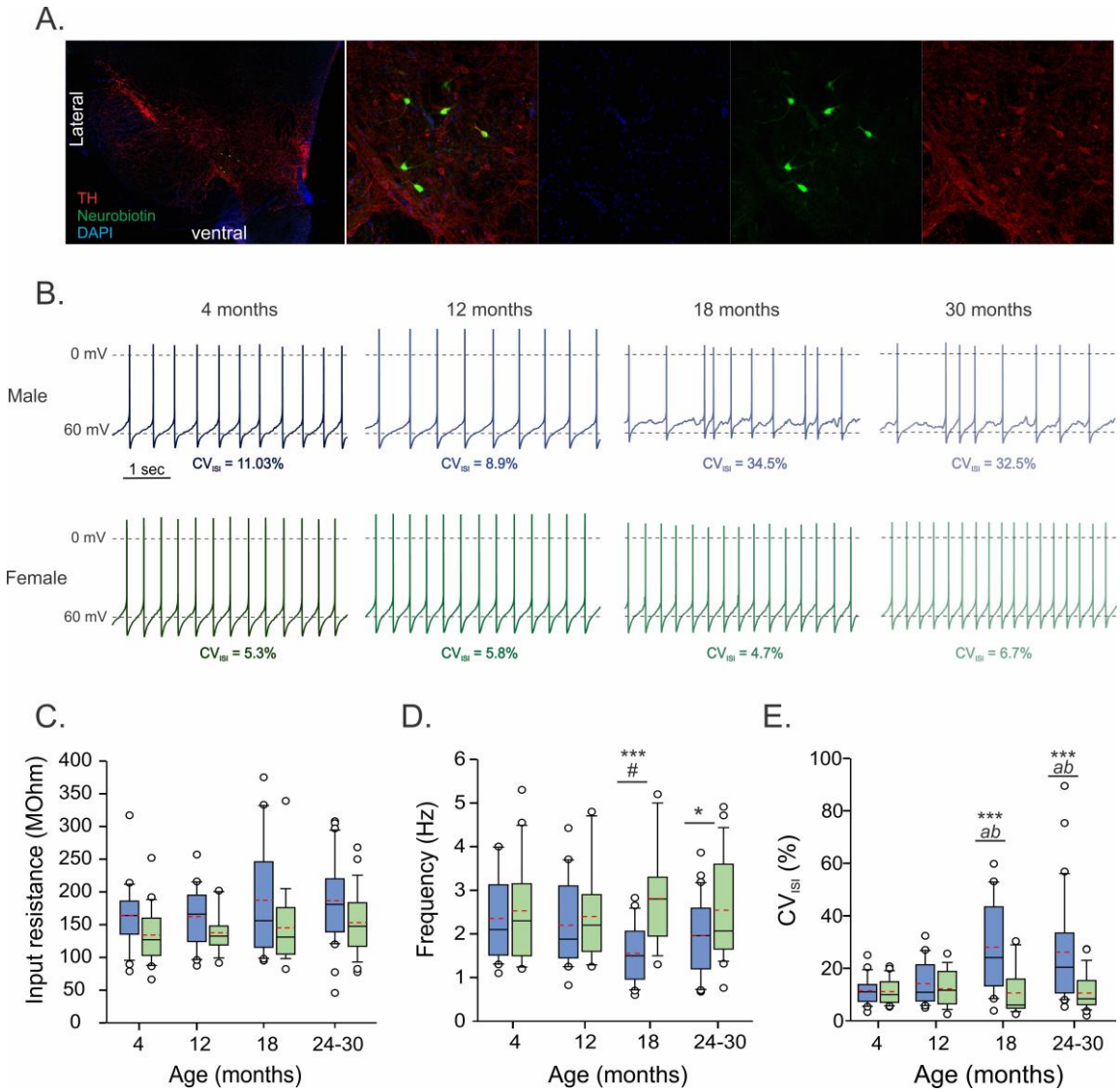

Figure 1. Evolution of spontaneous firing activity across ages. A. Images from a recorded slice immunolabeled for tyrosine hydroxylase (TH, a marker for DA neurons) and neurobiotin (a tracer added to the patch pipette). Far left, image of the midbrain dopamine region from the whole slice (a bisected coronal section). Images to the right show zoom in of labeling for TH (red), neurobiotin (green), DAPI counterstain (blue). B. Representative recordings of spontaneous firing in current clamp from cells at 4, 12, 18, and 30 months from males (top, blue) and females (bottom, green). Box and whisker plots show input resistance (C), firing rate (D), and coefficient of variation for the interspike interval (CV_{ISI}, E). Red dashed lines indicate means. # - 18 month males differ from 4 month males, p=0.054. Males differ from females, *p<0.05, ***p<0.001; *a* - 18 or 24-30 month males differ from 4 month males, p<0.001; *b* - 18 or 24-30 month males differ from 12 month males p≤0.005

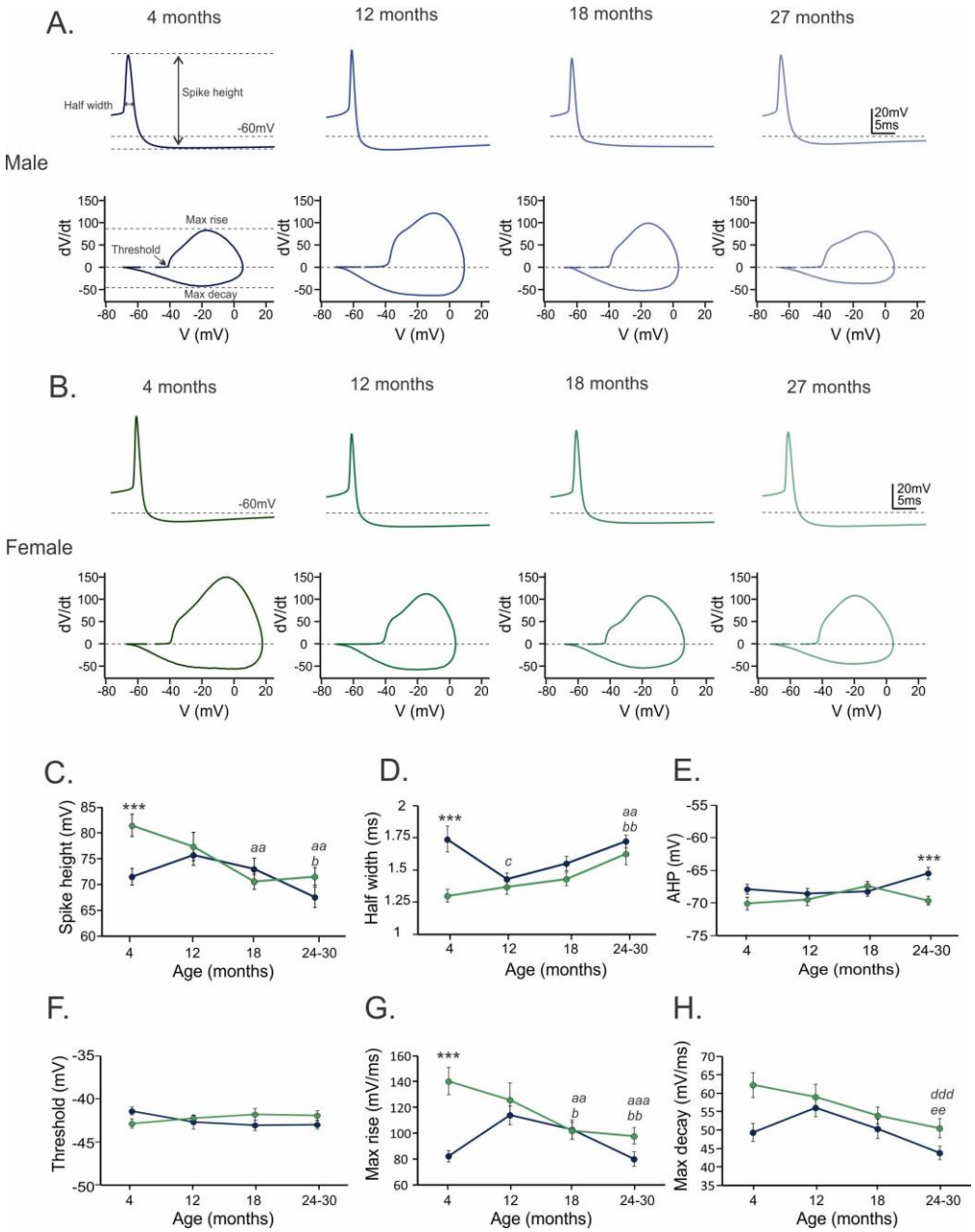

Figure 2. Differential effects of age on the action potential waveform in males and females. A. Top, representative averages of action potentials from cells in each of the 4 age groups of males (blue). Bottom, phase plane plots of the representative action potential traces. Parameters measured are indicated in the 4 month old action potential and phase plane plot on the left. B. Representative action potential averages (top) and phase plane plots (bottom) from cells in each of the 4 age groups of females (green). Males (blue) and females (green) are compared in spike height (C), half width (D), AHP (E), action potential threshold (F), maximum rise velocity (G) and maximum decay velocity (H). Bonferroni corrected t-test: ***Males and females differ, $p<0.001$; Females 18 months or 24-30 months compared with 4 months, *aa* – $p<0.005$, *aaa* – $p<0.001$; Males 24-30 months compared with 12 months, *b* – $p<0.05$, bb – $p<0.01$; Males 12 months compared with 4 months, *c* – $p<0.02$; Age group 24-30 months compared with 12 months, *ddd* – $p<0.001$; Age group 24-30 months compared with 4 months, *ee* – $p=0.003$.

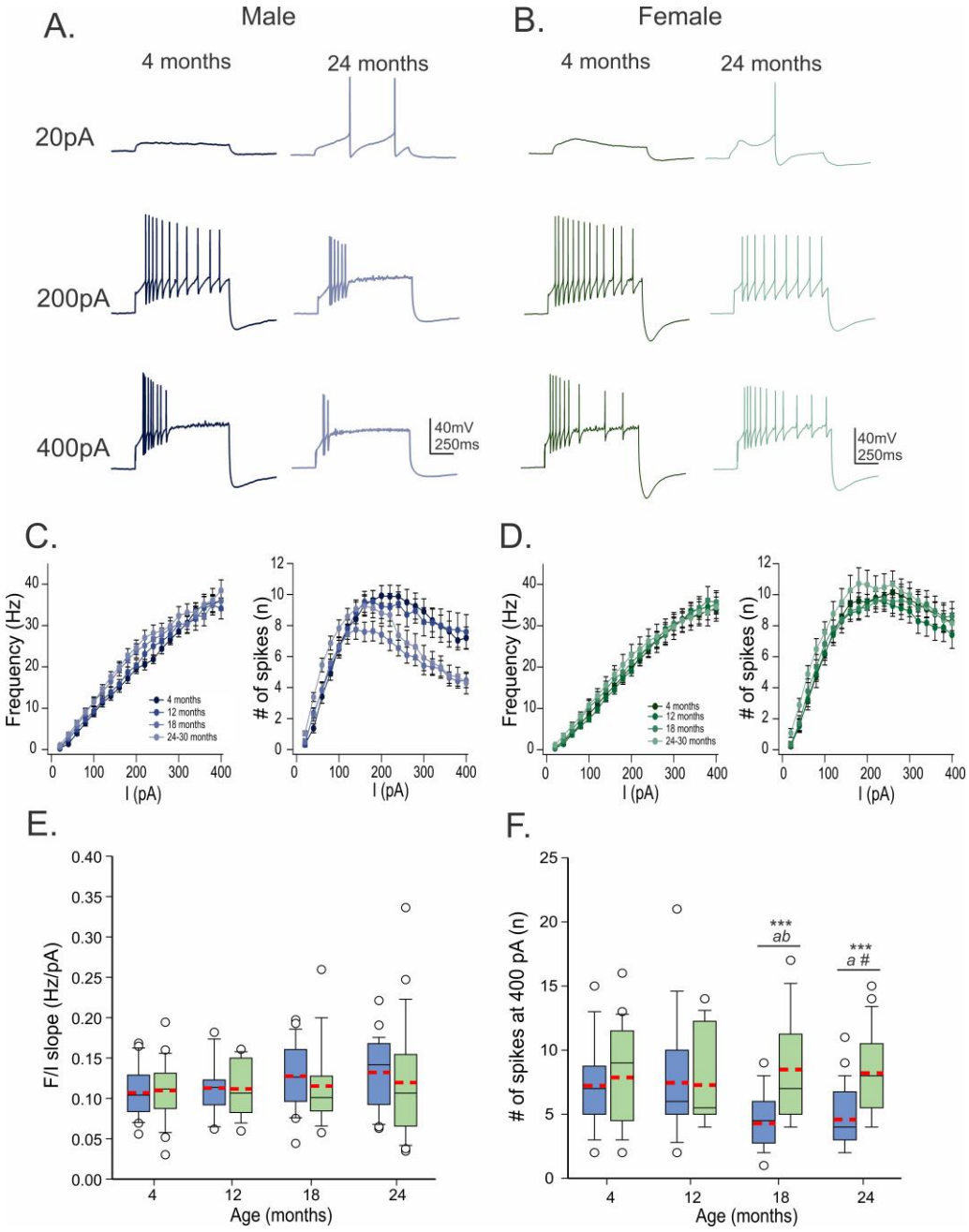

Figure 3. Membrane excitability across ages. Representative traces of current step injections at 20, 200, and 400 pA from males (A; blue; 4 months left, 24 months right) and females (B; green; 4 months left, 24 months right). F-I curves (left) (frequency x injected current) and number of spikes x injected current (right) are plotted for each age group of males (C., blue, left) and females (D., green, right). Box and whisker plots show slopes of F/I curve (E) and number of spikes at 400 pA current injection across ages in males (blue) and females (green). Red dashed lines show means. Bonferroni corrected t-test: males and females differ, ***p<0.001; *a* – 18 & 24 month males differ from 12 month males, p<0.05; *b* – 18 month males differ from 4 month males, p<0.05; # - 24 month males differ from 4 month males, p=0.057.

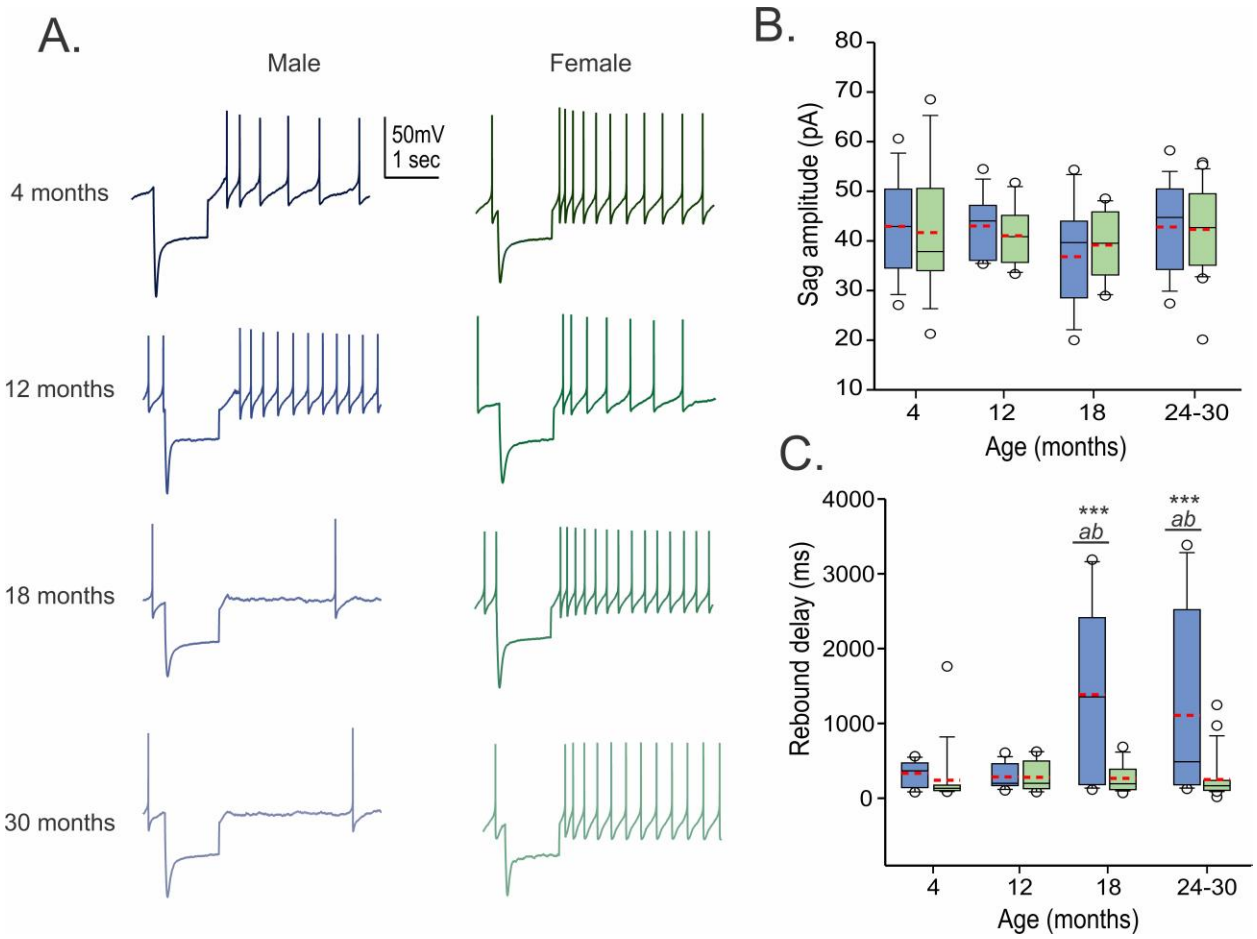

Figure 4. Sag and post inhibitory rebound spiking across ages in males and females. A. Representative traces of hyperpolarizing current injections at 4, 12, 18 & 24-30 months from males (left, blue) and females (right, green). Box and whisker plots show sag amplitude (B) across ages (males, blue; females, green) and time delay until rebound spike (C). Red dashed lines show means. Bonferroni corrected t-test: males and females differ, ***p<0.001; *a* – 18 & 24 month males differ from 12 month males, p<0.01; *b* – 18 & 24 month males differ from 4 month males, p<0.02

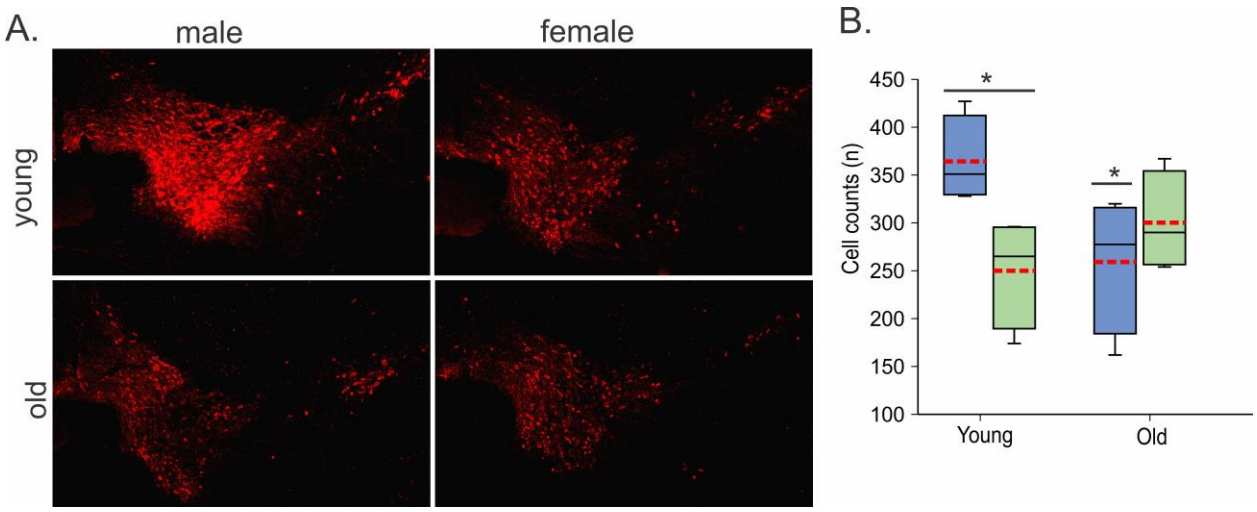

Figure 5. Semi-quantitative analysis of tyrosine hydroxylase positive neurons. A. Representative images at -3.28mm from bregma from a 4 month old male and female (top) and 27 month old male and female (below). B. Box and whisker plot showing TH positive cell counts from the same level in 4 month and 27 month old males (blue) and females (green). Red dashed lines show means. Bonferroni corrected t-test: young males differ from young females and old males differ from young males, *$p<0.05$

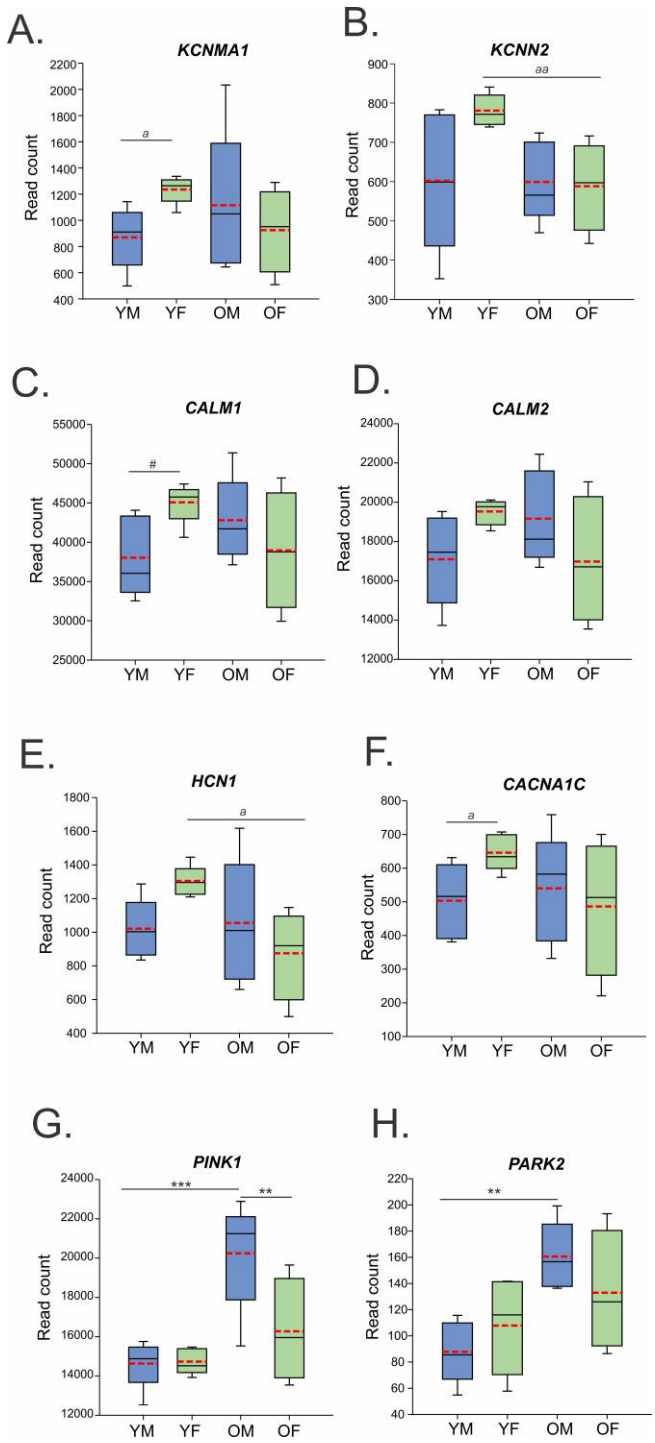

Figure 6. Gene expression changes between males (blue) and females (green) with age for BK channels (*KCNMA1*, A), SK channels (*KCNN2*, B), calmodulin 1 (*CALM1*, C), calmodulin 2 (*CALM2*, D), HCN channels (*HCN1*, E), L-type calcium channels (*CACNA1A*, F), PTEN induced putative kinase 1 (*PINK1*, G), and parkin (*PARK2*, H). T-test: a – $p<0.05$; aa – $p<0.01$. Bonferroni corrected t-test: # - $p=0.052$; **$p<0.01$; ***$p<0.001$.

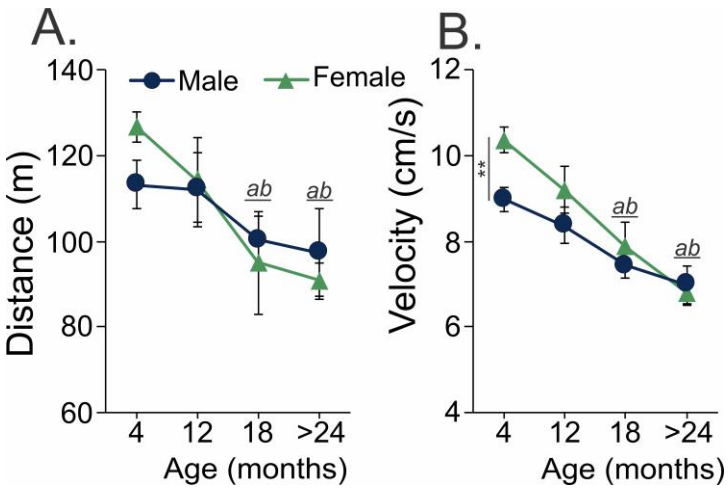

Figure 7. Assessment of locomotion across ages. A. Total distance traveled during a 30 minute session in the locomotor chambers across ages 4, 12, 18 and 24-30 months in males (blue circles) and females (green triangles). B. Average velocity of mice during the time in the locomotor chambers. Bonferroni corrected t-test: males and females differ, **$p<0.01$; a – 18 & 24 month males differ from 4 months, $p<0.02$; b – 18 & 24-30 month females differ from 4 months, $p<0.001$.

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