TITLE: The effects of biological sex on estimates of persistent inward currents in the human lower limb

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Running title (70 characters): Sex differences in estimates of PICs

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ABSTRACT (250 words)

Non-invasive recordings of motor unit (MU) spike trains help us understand how neural drive is modulated by various motor commands and/or physiological conditions. The majority of study participants in human and non-human animal physiology studies are male, and it is assumed mechanisms uncovered in these studies are shared between males and females. However, sex differences in neurological impairment and physical performance warrant the study of sex as a biological variable in human physiology and performance. To begin addressing this gap in the study of biophysical properties of human motoneurons, we quantified MU discharge rates and estimates of persistent inward current (PIC) magnitude in both sexes by quantifying ΔF. We decomposed MU spike trains from the tibialis anterior (TA), medial gastrocnemius (MG), and soleus (SOL) using high-density surface electromyography and blind source separation algorithms. Ten participants of each sex performed slow triangular (10s up and down) isometric contractions to a peak of 30% of their maximum voluntary contraction. We then used linear mixed effects models to determine if peak discharge rate and ΔF were predicted by the fixed effects of sex, muscle, and their interaction. Despite similar peak discharge rates across all muscles, ΔF was larger ($\chi^2_{(1)} = 6.26$, $p = 0.012$) in females (4.73 [0.242 pps]) than males (3.81 [0.240 pps]). These findings suggest that neuromodulatory drive and/or biophysical properties of motoneurons differ between the sexes and may contribute to differences in MU discharge patterns.

Keywords (3-5): Hysteresis, motor unit, sex differences, motoneuron

45 **KEY POINTS**

46 *- Sex differences have been revealed in motor unit studies as inclusion of female participants*
47 *becomes more common, however, mechanisms for these differences are not yet clear.*

48 *- Levels of sex hormones differ in concentration between females and males, and these hormones*
49 *are known to affect monoaminergic signaling throughout the central nervous system.*

50 *- Estimates of persistent inward currents (i.e., ΔF) were observed to be greater in females than*
51 *males in the tibialis anterior, medial gastrocnemius, and soleus muscles.*

52 *- This suggests that neuromodulatory drive, monoaminergic signaling, or descending control may*
53 *differ between the sexes, likely due to differences in sex hormone concentrations.*

INTRODUCTION

Historically, studies in physiology, and in particular neurophysiology, have excluded female participants. Woitowich and colleagues (2020) recently surveyed the literature and showed that females currently represent only 15% of both non-human subjects and human participants in biological studies. This has resulted in an extensive understanding of the neural control of movement in males, but a poor understanding of what mechanisms underlie differences in motor control observed in females (Jenz & Pearcey, 2022).

No matter the biological sex, all movement is achieved through the activation of motoneurons and the muscle fibers they innervate, together known as motor units (MUs; Heckman & Enoka, 2012). Under normal conditions, motoneurons have a one-to-one spike ratio with each fiber they innervate, and therefore the discharge patterns of motoneurons and their MU are the same (Johnson *et al.*, 2017; Thompson *et al.*, 2018). This creates a unique opportunity to study the central nervous system, as motoneurons are the only neuron that can be recorded non-invasively in humans. Thus, the non-invasive assessment of MU behavior can reveal important information on how the cortex, spinal cord, and individual neurons communicate and whether sexual dimorphisms exist within these neural commands.

A contemporary understanding of the physiology underlying motor control suggests that motoneurons receive a complex combination of excitatory, inhibitory, and neuromodulatory input (Heckman et al. 2009). This neuromodulatory input involves two important monoamines (among others), serotonin (5HT) and norepinephrine (NE), that are released via long descending projections from the brainstem nuclei (raphe nucleus and locus coeruleus, respectively) onto motoneurons (Montague *et al.*, 2013). Release of 5HT and NE facilitates voltage-sensitive persistent inward currents (PICs; Hounsgaard *et al.*, 1988; Heckman *et al.*, 2005), and is thought to contribute to the gain control required for humans to perform an impressive variety of movements, from long term postural control during waking for hours, to precise targeted movements like reaching to grasp a pint in a crowded pub (Heckman *et al.*, 2008).

PICs modulate motoneuron discharge in a non-linear fashion by creating a strong initial acceleration and subsequent discharge rate attenuation during linear increases in synaptic input (Binder *et al.*, 2020). The most robust hallmark of PICs in humans, however, manifests as discharge rate hysteresis between the recruitment and de-recruitment of MUs (Khurram *et al.*, 2022). Typically, PICs are estimated in humans using a paired MU analysis technique to quantify discharge rate hysteresis (Gorassini *et al.*, 1998, 2002*a*, 2002*b*). Although many labs have utilized

this technique with MU spike trains obtained while participants perform voluntary triangular shaped contractions, the role of descending monoaminergic (i.e., 5HT and NE) input from the brainstem is broadly understudied in the motor system and has not been compared between the sexes.

In recent years, studies that have included an adequate number of females have demonstrated sex differences in several MU discharge properties, but the results vary across different tasks and conditions. The consensus is that MU discharge (English & Widmer, 2003; Harwood *et al.*, 2014; Peng *et al.*, 2018; Parra *et al.*, 2020; Inglis & Gabriel, 2020; Nishikawa *et al.*, 2022) and neuromuscular fatigue (Hunter & Enoka, 2001; Celichowski & Drzymała, 2006; Ansdell *et al.*, 2019; Inglis & Gabriel, 2021; Augsburger *et al.*, 2022) differ between the sexes (Lulic-Kuryllo & Inglis, 2022). Recent data suggest that females use different recruitment strategies than males at relatively low efforts due to smaller MU size and higher discharge rates (Guo *et al.*, 2022) but the specific mechanisms underlying these differences remain unknown.

In non-human animal models, sex differences in MU physiology have been identified at the cellular level. For example, male rats have more fast-fatigable type MUs in the gastrocnemius muscle, while female rats have more fatigue-resistant MUs (Celichowski & Drzymała, 2006), with male rats having a greater number of MUs than females (Celichowski & Drzymała-Celichowska, 2007). Most relevant to the current investigation, Kirkwood and colleagues (Kirkwood *et al.*, 2002; Ford & Kirkwood, 2006) have shown that plateau potentials, which are dependent on Ca^{2+} PICs and are not typically observed in anesthetized preparations, have been observed only in anesthetized ***estrous*** female cats suggesting a strong role of sex hormones in the modulation of biophysical motoneuronal properties.

Beginning at development, and throughout the lifespan, females and males differ primarily in terms of their levels and cycles of sex hormones. Following puberty, the hormones estradiol (one of the three endogenous estrogens) and progesterone fluctuate across the female menstrual cycle, and peak at levels much higher than males during specific days (Sisk & Foster, 2004). Estradiol has been shown to affect 5HT signaling throughout the central nervous system (Michopoulos *et al.*, 2011; Bethea & Reddy, 2012; Barth *et al.*, 2015). Therefore, to understand the effect of biological sex on motoneuronal properties in humans, we measured MU behavior during slow isometric ramps contractions to the same relative intensity and compared them between females and males. We hypothesized that the higher level of estradiol in females would enhance PICs, which would manifest as greater peak discharge rates and more discharge rate hysteresis – an indication of greater PIC magnitudes.

METHODS

Participants and ethical approval

Young and healthy females (n = 10; 25.5 ± 4.8 years) and males (n = 10; 27.2 ± 5.8 years) were recruited for this study. Participants had no history of muscular or neurological impairment and provided written informed consent, approved by the Institutional Review Board of Northwestern University (STU00216360).

Experimental Apparatus

***Torque**:* During each session, participants were seated in a Biodex chair (Biodex Medical Systems, Shirley, NY) and their right foot was placed in a footplate attachment with an ankle angle of 95 degrees, a hip flexion angle of 100 degrees, and knee fully extended. The footplate was attached to a Systems 2 Dyanomometer (Biodex Medical Systems, Shirley, NY) and Velcro straps were wrapped around the foot to secure it to the plate and prevent movement. Torque was sampled at 2048 Hz and smoothed offline with a 10 Hz low-pass filter (fifth-order Butterworth filter).

High-density surface electromyography (HDsEMG): Before electrode placement, excess hair was removed and the skin overlying the muscle of interest was lightly abraded. High-density surface EMG (HDsEMG) electrodes (64 electrodes, 13x5, 8mm I.E.D., GR08MM1305, OT Bioelettronica, Inc., Turin, IT) were placed over the tibialis anterior (TA), and the medial gastrocnemius (MG) and soleus (SOL), which are dorsiflexor and plantarflexor muscles of the ankle, respectively. A reference electrode strap was placed around the right ankle, overlying the medial and lateral malleolus (Figure 1). HDsEMG data was recorded in single differential mode, sampled at 2048 Hz, amplified x150, and band-pass filtered at 10-500Hz using a Quattrocento signal amplifier (OT Bioelettronica, Inc., Turin, IT). EMG and torque were temporally synced with a 1-second TTL pulse transmitted to the Quattrocento at the onset of each trial.

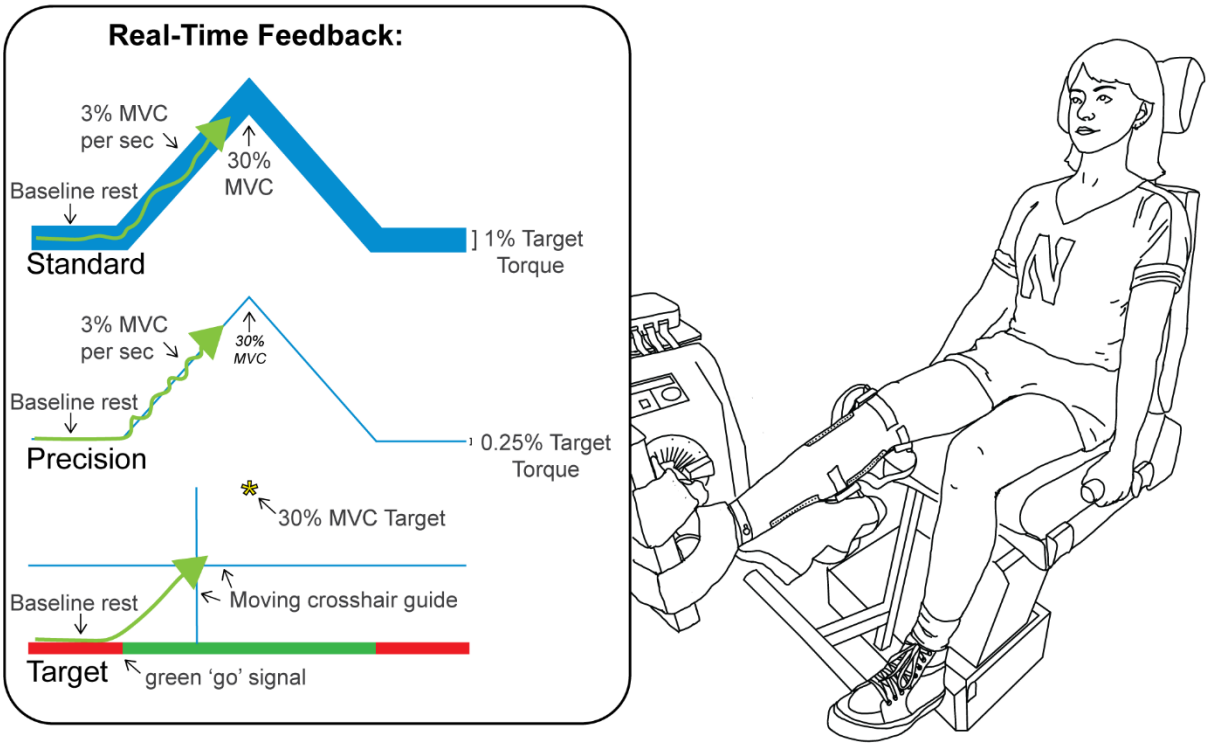

Figure 1. *Experimental setup and visual feedback conditions are shown above. Participants were seated with their right ankle secured in a device to measure isometric joint torque, with HDsEMG placed on their tibialis anterior and soleus muscles. During the experiment real-time visual feedback was provided on a monitor in front of participants, with three different types of feedback, to cue triangular ramp contractions.*

Experimental Protocol

Before beginning data collection, real-time HDsEMG recordings were visually examined to ensure a high signal-to-noise ratio. First, participants were asked to perform maximum voluntary contractions (MVCs) with their plantarflexors and dorsiflexors. Three MVC trials in each direction were collected. If the last trial had the highest peak torque, additional trials were added as needed. Verbal encouragement was given to participants during MVC trials to ensure maximal performance, and participants were given 2 minutes of rest in between trials to prevent fatigue. The maximum torque achieved was used for subsequent normalization of all trials.

The remaining trials consisted of a single triangular-shaped isometric dorsiflexion or plantarflexion torque ramp, with visual feedback to guide participants. For all ramps, participants increased torque (3% MVC/s) to 30% MVC over 10 seconds and then decreased at the same

rate to 0% MVC. Throughout the experiment, a large monitor was positioned in front of the participants to provide real-time feedback of torque performance.

***Visual feedback*:** This dataset was obtained from a larger ongoing project, and therefore participants performed contractions with three different types of visual feedback, all of which had the same goal of a 10 s linear ascending phase to a peak of 30% MVC and then a 10 s linear descending phase to rest. During all three of these conditions, participants received real-time torque feedback on the screen in front of them, which was comprised of either: 1) a standard guideline with a width of 10% of target torque (i.e., 3% MVC); 2) a precise guideline with a width of 2.5% of target torque (i.e., 0.75% MVC); or 3) cross hairs to provide feedback about the amplitude and timing of torque generation, along with cues about when to start and stop contracting and a peak target (see Figure 2). At a minimum, participants completed 4 trials while receiving each of the three types of feedback in each direction (i.e., plantarflexion and dorsiflexion), totaling to 12 ramps for both dorsiflexion and plantarflexion. Trials were discarded if they did not exhibit a smooth increase and decrease of torque (i.e., substantial deviations from the intended target profile), and the trial was then repeated.

Data Analysis

MU Decomposition: After data collection, EMG signals were manually inspected. Channels with substantial artifact, noise, or analog to digital saturation were removed (on average 1-3 channels per array). Individual MU spike trains were decomposed using a convolutive blind-source separation algorithm with a silhouette threshold of 0.87 (Negro et al., 2016).

After decomposition, MU spike trains were manually edited by a trained technician who was blind to the participant's sex. This inspection used a custom-made graphical user interface in MATLAB to correct minor errors identified by the decomposition algorithm to improve MU spike trains using well-validated local re-optimization methods used in recent studies (Hug et al, 2021; Boccia et al., 2019; Afsharipour et al., 2020; Del Vecchio et al., 2020; Hassan et al., 2020; Martinez-Valdes et al., 2020). Instantaneous discharge rates of each MU were determined by computing the inverse of the interspike interval of each MU spike train and smoothed using support vector regression (Beauchamp *et al.*, 2022) with custom-written MATLAB scripts. Within these scripts, the initial, peak, and final discharge rates were extracted from the smoothed spike trains. Ascending duration was calculated as the time that a MU exhibited sustained discharge before peak torque, and descending duration as the time a MU exhibited sustained discharge from peak torque to derecruitment. Ascending-descending phase duration ratio was calculated

as the difference in ascending duration and descending duration, divided by the total duration of discharge where a more negative value indicates more duration on the descending portion of the contraction, and thus more sustained discharge on the descending portion of the torque ramp.

Estimating Persistent Inward Currents (PICs): Effects of PICs on MU discharge patterns can be estimated by quantifying the amount of onset-offset hysteresis (i.e., ΔFrequency, ΔF) of a higher-threshold (test) MU with respect to the discharge rate of a lower-threshold (reporter) MU (Gorassini *et al.*, 1998, 2002*a*, 2002*b*). Rather than providing ΔF values for each test-reporter unit pair, we calculated 'unit-wise' values, which gives each test unit one ΔF value based on the average values obtained from multiple reporter units (Figure 3). Criteria for inclusion of ΔF values from MU pairs were that 1) the test MU was recruited at least 1 second after the reporter unit to ensure full activation of the PIC (Bennett *et al.*, 2001; Powers *et al.*, 2008; Hassan *et al.*, 2020), test unit-reporter unit pair exhibited rate-rate correlations of $r^2 > 0.7$ to ensure MU pairs received common synaptic input (Gorassini *et al.*, 2004; Udina *et al.*, 2010; Stephenson & Maluf, 2011; Vandenberk & Kalmar, 2014), and 3) the reporter unit modulated its discharge rate by at least 0.5 pps while the test unit was active (Stephenson & Maluf, 2011).

Statistical Procedures

All statistical analyses were performed using R Statistical Software (v4.1.0; R Core Team 2021). To determine if variables of interest were predicted by the fixed effects of sex, muscle, and their interactions we used linear mixed effects models with covariates of recruitment threshold and the type of visual feedback, and a random intercept and muscle slope for participant (lmer R package; v1.1.27.1; Bates et al., 2015). To determine significance, we applied Satterthwaite's method for degrees of freedom (lmerTest R package; v3.1.3; Kuznetsova et al., 2017). Both individual participant means and group estimated marginal means (male and female) were computed for each variable (emmeans R package, v1.8.0, Lenth et al., 2022). Effect size (Cohen's d) was calculated to determine the standardized magnitude of the effect of sex from the estimated marginal means of the male and female data from the model (emmeans R package, v1.8;0, Lenth et al. 2022). All data were visualized in R (ggplot R package, v3.3.6, Wickham et al., 2022).

RESULTS

In this study, we characterized sex differences in MU discharge properties during isometric triangular shaped contractions to 30% of MVC. For females, we decomposed a total of 2,126 MUs (TA: 933, MG: 691, SOL: 502), and for males a total of 3,935 MUs (TA: 1651, MG: 1484, SOL: 800).

A greater number of low threshold MUs are identified in males

Plotting decomposed MUs by recruitment threshold revealed that a greater number of low threshold MUs were decomposed in males than females (Figure 2). Thus, sex was a significant predictor of recruitment torque ($\chi^2_{(1)} = 13.56$, $p = 0.00023$), and was higher in females across all three muscles (Figure 2). To account for this in all subsequent analyses, we included a covariate of recruitment torque in all models.

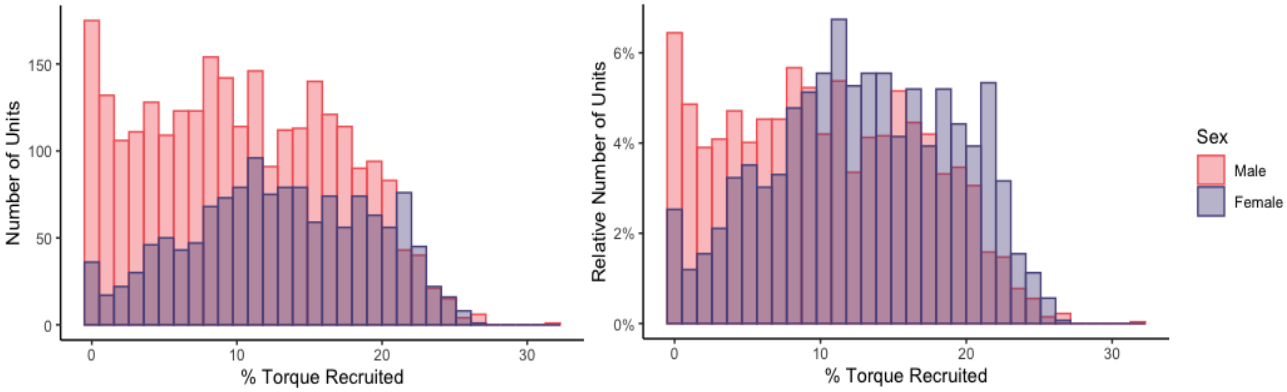

Figure 2. *Decomposed MUs by recruitment threshold for all participants. Female units are shown in purple, and male units in pink. For each figure, the x-axis shows the percentage of MVC the units were recruited at in 1% bins. On the left, the y-axis represents the total number of units recruited. On the right, the y-axis represents the percentage of total units recruited at each torque level.*

Estimates of persistent inward currents are greater in females

Across all muscles, ΔF was larger ($\chi^2_{(1)} = 6.26$, $p = 0.012$) in females (4.73 [0.242 pps]) than males (3.81 [0.240 pps]) with an effect size of d = 0.653. Figure 3 shows an example trial from one male and one female, with individual ΔF values provided for the average of all test units and individually for each MU. In Figure 4, within participant means (diamonds) and individual ΔF values for each test unit along with distributions across all three muscles are shown. No significant

interaction between sex and muscle was identified ($\chi^2_{(2)} = 1.79$, $p = 0.409$) , and therefore ΔF was 0.92 pps higher in females based on estimated marginal means.

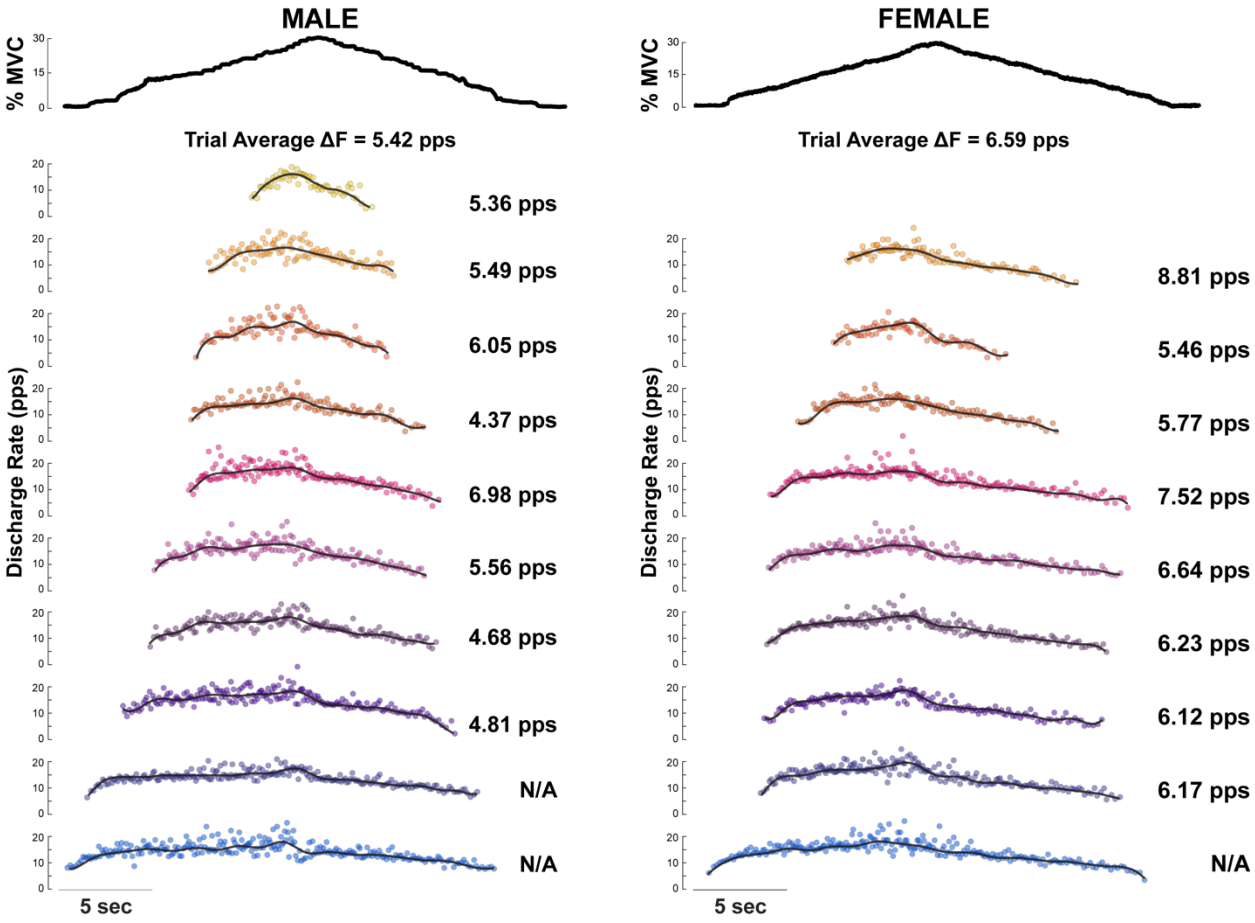

Figure 3. *An example of a dorsiflexion contraction trial from one male (left) and one female (right). Torque is plotted with a thick black line (top) and individual motor units are plotted in the bottom plots according to recruitment threshold (lower thresholds at the bottom). Each point represents the instantaneous discharge rate, and the thin black line for each unit represents the smoothed discharge rate computed using support vector regression. The test-unit mean averaged across all suitable reporter units is provided to the right of each unit. The average of all these values within the trial is provided just below the torque trace.*

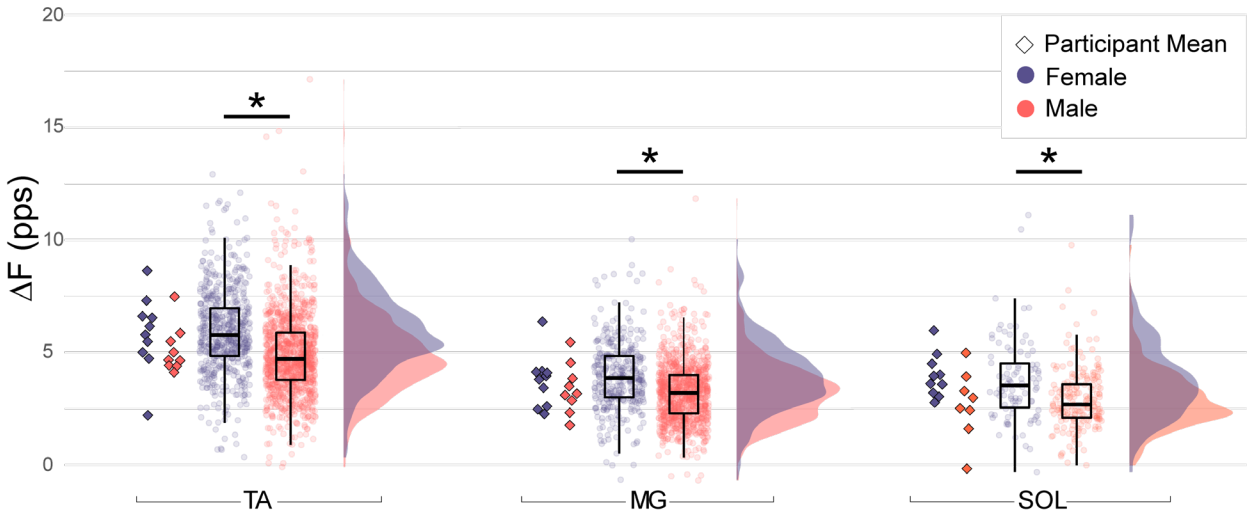

Figure 4. *Estimates of persistent inward currents (ΔF) for TA (left), MG (middle), and SOL (right) for females (purple) and males (pink). Individual participant means are shown in diamonds. ΔF measures for each unit collected across trials are shown as scatter points. Box plots represent the 25th, 50th (median) and 75th quartiles, with whiskers for minimums and maximums at 1.5 the interquartile range. Distributions of unit-wise ΔF values are shown to the right for each muscle.*

The descending phase duration of discharge, from peak torque to derecruitment, ($\chi^2_{(1)}$ = 3.316, p = 0.0686) and ascending duration, from recruitment to peak torque, ($\chi^2_{(1)}$ = 0.0342, p = 0.8532) were not significantly different between the sexes (Table 1B). Additionally, the ratio between ascending and descending durations did not differ significantly between the sexes ($\chi^2_{(1)}$ = 2.94, p = 0.086, Table 1C).

A. Ascending Phase Duration

	Female		**Male**	
	Mean (s)	*SD*	*Mean (s)*	*SD*
TA	6.09	0.52	6.11	0.52
MG	5.97	0.51	5.98	0.51
SOL	6.03	0.51	6.09	0.52

B. Descending Phase Duration

	Female		**Male**	
	Mean (s)	*SD*	*Mean (s)*	*SD*
TA	7.32	1.72	6.58	1.74
MG	5.23	1.88	4.48	1.87
SOL	5.77	1.78	5.02	1.85

C. Ascending-Descending Phase Ratio

	Female		Male	
	Mean (s)	*SD*	*Mean (s)*	*SD*
TA	-0.16	0.20	-0.02	0.20
MG	0.12	0.25	0.12	0.25
SOL	0.0369	0.22	0.0817	0.24

Table 1. *Measurements of motor unit discharge duration during the triangular ramp contractions. Estimated marginal means and standard deviations are shown for each muscle, with females on the left and males on the right. Abbreviations: TA; tibialis anterior, MG; medial gastrocnemius, SOL; soleus, s; seconds, SD; standard deviation.*

MU Discharge rates did not differ between sexes

While peak discharge rates appeared higher in females than males (see table 2A), no significant differences were detected between the two groups. Across muscles, discharge rates did not differ at recruitment ($\chi^2_{(1)} = 1.36$, $p = 0.243$), derecruitment ($\chi^2_{(1)} = 0.06$, $p = 0.805$), and maximum ($\chi^2_{(1)} = 1.15$, $p = 0.283$).

A. Discharge Rate at Recruitment

	Female		Male	
	Mean (pps)	*SD*	*Mean (pps)*	*SD*
TA	6.73	1.98	6.29	2.01
MG	5.01	1.91	4.57	1.96
SOL	4.04	1.83	3.60	1.88

B. Peak Discharge Rate

	Female		Male	
	Mean (pps)	*SD*	*Mean (pps)*	*SD*
TA	17.20	2.33	16.40	2.41
MG	13.00	2.72	12.10	2.76
SOL	11.10	2.43	10.30	2.57

C. Discharge Rate at Derecruitment

	Female		Male	
	Mean (pps)	*SD*	*Mean (pps)*	*SD*
TA	5.55	1.61	5.50	1.62

MG	4.60	1.63	4.56	1.64
SOL	3.70	1.58	3.65	1.60

Table 2. *Measurements of motor unit discharge during triangular ramp contractions. Estimated marginal means and standard deviations are shown for each muscle, with females on the left and males on the right. Abbreviations: TA; tibialis anterior, MG; medial gastrocnemius, SOL; soleus, pps; pulses per second; s; seconds, SD; standard deviation.*

DISCUSSION

This study quantified the discharge patterns of large populations of concurrently active MUs during submaximal isometric triangular shaped contractions in lower limb muscles of females and males. Although no significant differences in MU discharge rates were identified, estimates of PIC magnitude using a paired motor unit analysis technique revealed that discharge rate hysteresis (i.e., ΔF) was substantially larger in females than males. These findings suggest that descending neuromodulatory drive, monoaminergic signaling of 5HT and/or NE to motor units, and/or inhibitory commands may differ between the sexes.

Estimates of PICs are higher in females

Linear mixed effects models revealed that biological sex was a significant predictor of estimates of PIC magnitude (ΔF) across all three muscles. Indeed, ΔF was almost 1 pps higher in females than males, indicating a greater contribution of PICs to MU discharge while performing a simple isometric ramp contraction.

PICs are vital for motoneuronal function – without their amplification effects, repetitive discharge cannot be achieved (Lee & Heckman, 2001), as driving currents from descending and sensory synaptic inputs are too weak to cause repetitive MU discharge (Binder *et al.*, 2020). Therefore, identifying and understanding differences in PIC magnitude may garner insights into potential sex differences in motor command structure. Even though males in our experiment certainly displayed discharge rate hysteresis (i.e., positive ΔF values), the magnitude of this hysteresis was markedly smaller compared to females.

The factors that can influence PIC magnitude, and thus ΔF, include: 1) monoaminergic input to the motoneurons, 2) Na+ or Ca2+ channel function, and/or changes in 5HT/NE reuptake and receptor function, and 3) the amount or pattern of inhibitory input to the motoneuron. Physiologically, males and females differ most notably in their levels of sex hormones. In eumenorrheic females (premenopausal females with a regular menstrual cycle), levels of estradiol, one of the three endogenous estrogens, and progesterone fluctuate throughout the month over a menstrual cycle (Sherman & Korenman, 1975). In males, the levels of these two hormones remain low and do not fluctuate across the month. Progesterone levels in males are similar to levels of females during the early menstrual cycle (follicular phase), but after ovulation, progesterone levels rise much higher in females (Oettel & Mukhopadhyay, 2004; Tea *et al.*, 1975). Throughout the entire menstrual cycle, eumenorrheic females experience fluctuations in estradiol ten times that of males (Verdonk *et al.*, 2019). Remarkably, these two hormones have known

316 effects on neurons throughout the nervous system, in particular serotonin (Bethea *et al.*, 1998),
317 and thus, higher fluctuating levels in females may have effects on the motor system.
318
319 **The potential role of sex hormones in mediating PIC magnitude**
320 Monoamines released from the caudal raphe nucleus (i.e., 5HT) and locus coeruleus (i.e.,
321 NE) facilitate PICs, and therefore the magnitude of PICs is directly proportional to the level of
322 brainstem monoamine release (Lee & Heckman, 1998, 2000). Thus, sex differences in
323 monoaminergic release could be responsible for differences in ΔF. Most well understood are the
324 effects of estradiol on 5HT neurons in other areas of the nervous system. In nonhuman female
325 primates, estrogen receptor beta (Erβ) has been identified in the dorsal and caudal raphe nuclei
326 (Gundlah *et al.*, 2000; Bethea *et al.*, 2002). Additionally, long-term loss of estradiol in
327 ovariectomized female macaques results in a reduction in the number of 5HT neurons (Rivera &
328 Bethea, 2012). Taken together, these data suggest that females could have greater release of
329 brainstem-derived monoamines than males due to greater estradiol effects in the brainstem.
330 Based on available evidence, or a lack thereof, we must infer some effects of estradiol
331 from the supraspinal structures in order to understand the effects at the spinal level. For instance,
332 injection of estradiol in ovariectomized rats produced a significant increase in 5HT2A receptor
333 (prevalent on MN dendrites) labeling in the dorsal raphe nucleus and other brain regions (Sumner
334 & Fink, 1993). In animals with a normal estrus cycle, during spontaneous estradiol surge, 5HTA
335 receptor densities in various brain regions are markedly increased (Sumner & Fink, 1997). If
336 estradiol's effects on 5HTA receptors extend throughout the nervous system, it is logical to think
337 that higher levels of estradiol in females could increase PICs due to the pivotal role of these
338 receptors in the facilitation of dendritic PICs in motoneurons. Although studies in the reduced
339 preparation have yet to directly examine the effects of these sex hormones on 5HT neurons and
340 receptors in the periphery and motor system, the known effects of estradiol's effects on 5HT
341 signaling in the brain, in conjunction with the observed plateau potentials in the estrous and
342 anesthetized cat (Kirkwood *et al.*, 2002; Ford & Kirkwood, 2006), provide a promising explanation
343 for our observed sex differences in PIC magnitude.
344 PIC magnitude is also influenced by the amount and pattern of inhibitory input to
345 motoneurons. Both sex hormones outlined above have influences on excitatory and inhibitory
346 neuronal input, and thus this may be another possible reason for the observed sex differences.
347 In general, estradiol has a net excitatory effect on the nervous system, potentiating the activity of
348 glutamate receptors (Smith, 1994). Rats with 3-day exposure to elevated estradiol levels had
349 increased number and density of dendritic spines and glutamatergic synapse on hippocampal

neurons (Woolley & McEwen, 1992). These short-term effects of estradiol suggest that hormonal fluctuations over several days in a menstrual cycle may be significant enough to have effects on neuronal signaling. Conversely, progesterone has a net inhibitory effect, where progesterone receptors throughout the brain and spinal cord increase GABA release and decrease neuronal discharge rate (Smith, 1994; Bitran *et al.*, 1995; Melcangi *et al.*, 2014). Inhibitory inputs greatly impacts PIC magnitude (Hyngstrom *et al.*, 2008), but it is unlikely progesterone directly increases reciprocal or recurrent inhibition to MUs, as this would decrease the observed hysteresis in MUs of females. However, more inhibition of these inhibitory interneuronal pathways could manifest as greater PIC magnitude. It is also possible that other aspects of the excitatory or inhibitory circuitry are modulated with elevated levels of estradiol and progesterone differently between the sexes.

If sex hormones were in fact the cause of the sex differences, and since we did not control or measure the time of the menstrual cycle in females, we would expect greater variability in estimates of PICs across the female group due to the fluctuating levels of sex hormones in females in comparison to the relatively stable levels in males. Additional examination of values obtained from within individuals indeed suggests that there is more variability in ΔF values and discharge rates in females compared to males, and measures of variance in ΔF obtained from our models were greater in females (see figure 3). Confidence in this sentiment, however, requires further study, in which females are studied at multiple timepoints throughout their menstrual cycle, and sex hormones are quantified.

More MUs decomposed in males than females

In the current investigation, we identified a greater number of MUs in males across all three of the sampled muscles. Recent work by our colleagues (Taylor *et al.*, 2022) also documented a higher number of MUs decomposed in the male compared to female TA muscle and our findings extend these findings to other muscles of the lower leg. Additionally, a greater proportion of lower threshold MUs were decomposed in males compared to females in the current study but methodological considerations may skew interpretations when comparing recruitment thresholds between heterogeneous groups of people (i.e., females vs males herein). For example, HDsEMG decomposition is biased toward larger and more superficial MUs (Hassan *et al.*, 2019). As shown by Guo and colleagues (Guo *et al.*, 2022), males tend to have larger MUs, which may explain why their spike times are decomposed more readily than their female counterparts. Furthermore, recent publication by Souza de Oliveira and colleagues (Oliveira *et al.*, 2022) showed that, indeed, variability in the number of decomposed MUs is predicted by the amount of subcutaneous tissue. Although not measured in this current study, differences in subcutaneous

fat between sexes may be responsible for differences in MU yield and may explain why fewer units were decomposed in females despite equal experimental conditions. These sex differences in the decomposition results are important to note, however, as they highlight what has been a barrier to female inclusion in past HDsEMG analyses.

MU discharge rates did not differ between males and females

Despite the tendency for females to have higher discharge rates overall, there were no significant sex differences in MU discharge rates. Previous studies have reported higher discharge rates in female MUs, however during these experiments participants performed isometric holds or trapezoid shaped contractions (Inglis & Gabriel, 2020; Guo *et al.*, 2022; Taylor *et al.*, 2022). In our study, discharge rates at onset, at maximum, and at offset did not differ between the sexes. The greater yield of lower threshold units in the males may have skewed the discharge rates of males to a higher level, due to the relationship of recruitment threshold and discharge rate during submaximal isometric contractions (Pearcey & Rymer, 2022), which would mitigate statistical differences between the sexes. We did, however, attempt to deal with this limitation by including a covariate of recruitment threshold in our models. It remains possible that, if participants had been asked to sustain a contraction, sex differences may have emerged as has been observed by previous studies.

Methodological considerations

The non-invasive nature of surface electromyography is advantageous in that it allows us to estimate the spike times of many MUs but since we are unable to measure from the spinal motoneurons directly, this method is unable to directly quantify the magnitude of PICs. The paried MU analysis technique to produce ΔF as a metric for PIC magnitude in humans has been well-validated, but it is possible that the mechanisms behind the observed sex differences in ΔF are not solely due to changes in monoaminergic drive. For example, differences in the patterns of inhibitory inputs, monoamine release, monoaminergic receptor activity and/or sensitivity, and/or musculoskeletal differences between the sexes could possibly account for differences in MU discharge patterns. Future studies which include a comprehensive collection of contractions, that complement the isometric triangular ramps utilized herein, and allow inferences about the biophysical properties of motoneurons or that use electrical stimulation or vibration protocols to probe excitatory and inhibitory circuits, may reveal further insight into the mechanism behind these sex differences.

An additional limitation of this study is that the menstrual cycle status of female participants was not recorded and thus could not be taken into consideration during analysis. The uncontrolled phase of the menstrual cycle in females remains a potential reason for the increased variability in female ΔF values. Previous work has shown that various aspects of motor control, including MVC (Sarwar *et al.*, 1996; Nicolay *et al.*, 2007), fatigability (Sarwar *et al.*, 1996; Ansdell *et al.*, 2019; Pereira *et al.*, 2020), and initial MU discharge rate (Tenan *et al.*, 2013) all fluctuate across the menstrual cycle. There is reason to believe that MU discharge and PIC magnitude would also be affected by fluctuating levels of sex hormones (i.e., see previous discussion on the effects of sex hormones on 5HT signaling).

Subcutaneous fat, or the distance between the muscle and the surface array, is a likely contributor to the lower MU yield in females, however we cannot confirm this because we did not perform appropriate measures for each participant. Larger distances between the electrodes and the muscle fibers in the females recruited for this study (i.e., higher average levels of subcutaneous fat) are likely, based on typical (i.e., population) anthropometrics (Deurenberg *et al.*, 1991), however future analyses that include matching subcutaneous fat thickness between the sexes will offer further insight.

Conclusion

We have revealed that estimates of PIC magnitudes are higher in lower limb muscles of females than males. This suggests female MUs receive a greater contribution from PICs than males to achieve the same relative force and discharge rate, which may be due to requirement of more persistent discharge or a consequence of more monoamine availability. Females may also use a different combination of synaptic excitation, inhibition, and neuromodulation than males to achieve the same relative force levels during triangular ramp contractions. As such, this study further highlights the necessity to include equal numbers of female participants in order to, not only, fully understand the human physiology behind motor control, but to potentially use MU analysis as biomarkers for neurological disease (Fajardo *et al.*, 2022).

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Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

Competing interests

The authors declare that they have no competing interests.

Author contributions

S.T.J., J.A.B., C.J.H., and G.E.P.P., conceptualized and designed the research; S.T.J., J.A.B., M.M.G., and G.E.P.P. performed the experiments; S.T.J. and J.A.B. analyzed the data; S.T.J., J.A.B., G.E.P.P., and C.J.H. interpreted the results of experiments; S.T.J., J.A.B., and G.E.P.P., prepared the figures; S.T.J drafted the manuscript; S.T.J., J.A.B., M.M.G., G.E.P.P., F.N., and C.J.H. revised and approved the final version of the manuscript.

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