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Research Article

Electromyography of spontaneous neuromuscular release of ACh: variations according to sex and age

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ABSTRACT

Neuromuscular transmission plays a crucial role in muscle function, with differences in neurotransmitter release potentially influenced by sex and aging. While hormonal effects on neurotransmission have been studied, their impact on acetylcholine (ACh) release at the neuromuscular junction remains largely unexplored. Understanding these differences could provide insights into age-related muscle function decline and sex-specific variations in neuromuscular disorders. This study aims to investigate sex- and age-related differences in spontaneous ACh release at the neuromuscular junction in mammals, focusing on variations in electromyographic (EMG) activity. Experiments were conducted on young (2 months) and old (15 months) Swiss male and female mice. EMG recordings were taken from the gastrocnemius muscle, analyzing the percentage of sites with endplate noise, noise frequency, and spike frequency. Estrous cycle stages in female mice were identified to account for hormonal fluctuations. Males exhibited a higher percentage of sites with endplate noise than females. Old females had the highest frequency of endplate noise, while old males had the largest amplitude. Spike frequency was higher in females and increased with age. These results suggest sex and age differences in neuromuscular activity, potentially influenced by hormonal and autonomic regulation. In conclusion, neuromuscular transmission differs between sexes and across aging. Estrogens may modulate cholinergic receptor function, and autonomic nervous system activity likely contributes to observed differences. These findings highlight distinct aging patterns in neuromuscular function between males and females.

Introduction

In healthy skeletal muscle synapses, only two types of spontaneous electrical events are observed (Fatt and Katz, 1951). Endplate potentials (EPPs) arise from the coordinated release of multiple ACh-filled synaptic vesicles and can trigger action potentials in the muscle fiber. In contrast, miniature endplate potentials (MEPPs) result from the exocytosis of a single synaptic vesicle; they occur roughly once per second and do not reach the threshold required to trigger an action potential.

Motor neurons can spontaneously release vesicles filled with synaptic vesicles, generating miniature end-plate potentials (MEPPs). These events maintain continuous communication between the nerve and muscle, influencing muscle membrane potential and calcium signaling (Katz and Miledi, 1969) and helping to preserve acetylcholine receptor (AChR) clustering and postsynaptic structure (Sanes and Lichtman, 1999). Furthermore, continuous spontaneous neurotransmission transmits essential trophic signals from the nerve to the muscle. Denervation or suppression of spontaneous secretion can lead to muscle atrophy and altered expression of contractile proteins (Slater, 2017). Finally, it has been established that spontaneous release is part of a feedback loop that allows the neuromuscular junction and muscle fiber to adjust receptor density and excitability to maintain stable function, a process known as homeostatic plasticity (Turrigiano and Nelson, 2004). Spontaneous ACh release constitutes an important element of neuromuscular function, justifying the study of its age- and sex-related variability.

The spontaneous neuromuscular release of ACh is modulated by multiple factors. Membrane cholesterol limits spontaneous vesicle fusion at the neuromuscular junction. When cholesterol is depleted,

Abbreviations: ACh, acetylcholine; ANS, autonomic nervous system; EPPs, endplate potentials; EMG, electromyographic activity.; MEPPs, miniature endplate potentials; NMJ, neuromuscular junction; NMDA, N-methyl-D-aspartate; PKA, protein kinase A; PKC, protein kinase C.

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spontaneous release increases due to greater presynaptic Ca^{2+} influx (Krivoi and Petrov, 2019; Petrov et al., 2014). The presynaptic K^{+} conductance also regulates this process: reduced conductance (depolarization) enhances, while increased conductance (hyperpolarization) suppresses release (De Lorenzo et al., 2004). Additionally, protein kinase activity—particularly PKC—promotes spontaneous ACh secretion by phosphorylating presynaptic proteins (Bukharaeva et al., 2022; Obis et al., 2015).

Electromyographically, action potentials are recorded as spikes, while MEPPs appear as endplate noise (Kimura, 2013). Endplate noise has been associated with the development of myofascial trigger points (Margalef et al., 2019; Gerwin, 2023). These regions within skeletal strap muscles are thought to be responsible for myofascial pain syndrome (Gerwin, 2023), a regional musculoskeletal pain condition that affects approximately 65 % of women and 37 % of men (Bodine, 2023). This highlights the importance of understanding potential sex-related differences in neuromuscular neurotransmission.

A possible cause of sex differences is the variation in sexual hormonal levels and fluctuations observed in females. In the mouse model (including rats and mice) commonly used for neurotransmission studies, the estrous cycle lasts approximately four days. On the first day (proestrus), serum estradiol levels rise rapidly, with ovulation occurring at the end of the day. Progesterone levels increase briefly before dropping sharply during estrus. The subsequent two days, metestrus and diestrus, are characterized by much lower serum hormone levels (reviewed by Scharfman and MacLusky, 2014). During the proestrus and estrus phases, female hormones exert the greatest influence on biological processes. In contrast, in the male murine model, hormonal influence remains constant.

Surprisingly, sex differences in neurotransmitter release remain largely unexplored. While the effects of female hormones on neurotransmission have been more extensively studied, their influence on neurotransmitter release specifically is less well understood. For instance, Pongrac and colleagues (Pongrac et al., 2004) observed an increase in newly synthesized ACh after administering estrogens to cultures of cholinergic neurons from the basal forebrain. In a zebrafish model, the number of neuromuscular synaptic vesicles was found to potentially correlate with estrogen levels (Houser et al., 2011).

The study of receptors and their interaction with female hormones is better documented. For example, estrogens have been shown to modulate postsynaptic ACh receptors in zebrafish (Houser et al., 2011). Estradiol reduces both the number and affinity of muscarinic receptors on cholinergic neurons in the cortex and hippocampus of ovariectomized rats following estrogen administration (van Huizen et al., 1994). In this ovariectomized rat model, the number of postsynaptic muscarinic receptors in the hippocampus also changes (Cardoso et al., 2010). Estrogens have been strongly associated with 5-HT1A receptors (Paredes et al., 2019) and μ-opioid receptors (Guajardo and Valentino, 2021). Estradiol is also closely linked to N-methyl-D-aspartate (NMDA) and AMPA/kainate receptors (Weaver et al., 1997; Gu et al., 1999). Despite these evidences, sex differences in neuromuscular neurotransmission remain largely unexplored.

Protein kinases also play a role in the exocytosis of neuromuscular synaptic vesicles (Santafé et al., 2014; 2015). In the hypothalamus and hippocampus, estrogens have been shown to rapidly activate several protein kinase pathways, including protein kinase C (PKC) and protein kinase A (PKA). These pathways modulate signal transduction, protein phosphorylation, and ion channel activity (Roepke et al., 2011).

In recent decades, the Western population has been aging (de Meijer et al., 2013). In humans, aging induces several muscular changes that are more pronounced in women (Zhang et al., 2024). Several studies have examined age-related changes in basal mEPP frequency using isolated muscle preparations (*ex vivo*). Decreases have been reported in the *extensor digitorum longus*, *soleus*, and *extensor digitorum communis* muscles, but not in the gastrocnemius or diaphragm of male mice (Banker et al., 1983). In contrast, age-related increases in spontaneous event frequency have been observed in the *gluteus maximus muscle* of male mice (Fahim, 1997). More recently, a study found no significant changes in mEPP frequency in the *epitrochleoanconeus* muscle of aged male mice (Li et al., 2025). Moreover, the regulation of neuromuscular function in the *extensor digitorum longus* muscle by autoreceptors and neurotrophins has been shown to be altered in older rats compared with younger ones (Balanyà-Segura et al., 2024). The impact of aging on neuromuscular neurotransmission between sexes remains notably unexplored.

There are no published clinical data detailing differences in spontaneous acetylcholine (ACh) release at the neuromuscular junction observed by electromyography (EMG) between young and elderly patients, or between women and men, in healthy humans. Then, the primary objective of this study is to provide an initial exploration of the differences between sexes in neuromuscular ACh release in mammals, considering both young and old animals.

Materials and methods

Animals

The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. The mice were cared for in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, following the European Community's Council Directive (2010/63/EU) and the Spanish Royal Decree 53/2013 for the humane treatment of laboratory animals. The Animal Research Committee of the Universitat Rovira i Virgili (Reference number: 11337) reviewed and approved all experiments on animals. The experiments were performed on young adult Swiss male and female mice (6 weeks, Charles River, L'Arbresle, France): 33 young males, 28 young females, 30 old males, 14 old females. Mice were habituated to the facility for at least 1 week prior to studies and were housed in groups of four, with sawdust bedding and ad libitum access to water and food throughout. The animals' room temperature was maintained at 22 ± 2 °C, a relative humidity of 50 ± 10 %, and a 12 h light/dark automatic light cycle. Experiments have been carried out with young (2 months) and old (15 months) animals, males and females. Older adult animals (15 months) have been evaluated since in female mice, reproductive function is lost around 15 months.

Estrous cycle analysis

Vaginal exfoliative cytology was examined under a light microscope using a 10 × objective lens (Byers et al., 2012). The stage of the estrous cycle was determined based on the morphology of cells observed in the smear, categorizing them into one of the four possible stages of the cycle: proestrus, estrus, metestrus, or diestrus. In our study, 49.8 % of the females were in the proestrus phase, 16.8 % in estrus, 17.7 % in metestrus, and 16.7 % in diestrus. In this study, females in the proestrus stage were selected.

Electromyography (EMG) recordings

Needle EMG recordings were always obtained from an anesthetized animals at a controlled room temperature of 22–25.8 °C. The gastrocnemius muscle was chosen for this study due to its easy accessibility and suitability for analysis.

Recordings were made using an electromyography (Medelec-Synergy; Oxford Instrument Co., Surrey, UK) with a monopolar EMG needle (Length, 25 mm; Diameter, 0.36 mm; Natus Manufacturing Limited). Rodent skin is unusually tough; therefore, to perform electromyographic recordings, the skin is incised to expose the area of interest. This allows for more accurate needle insertion. The mouse gastrocnemius muscle has a thickness of less than 1 mm, making it impossible to record at multiple muscle depths. The muscle was divided into twelve sections to

ensure comprehensive coverage and to prevent recording the same endplate noise twice. A new needle insertion is performed at each recording point. After each insertion, a 2-second pause is allowed for insertional activity to cease. The needle is then slowly rotated to 90 every second to search for spontaneous activity. Each recording lasts 5 s. No more than one spontaneous recording is obtained per insertion point. If no spontaneous electromyographic activity is detected, a recording is still made. The values of number of places with endplate noise are expressed as "percentage". This is defined as: [number of places with endplate noise / 12] X 100. For each muscle, the number of places with endplate noise (maximum twelve) and the frequency of noise (number of events per second) were recorded. To analyze the amplitude, the Synergy program was accessed, where the horizontal cursors were selected to adjust them to the amplitude of the record. The μV shown by the cursors were acquired. The experimental unit was the number of places with endplate noise and are expressed as a percentage (number of areas with endplate noise respect to total). The number of places presenting spikes and the number of events per minute (frequency) in each place were also counted.

Fig. 1 shows examples of prototype electromyographic recordings. A few seconds after inserting the recording needle, an almost flat trace is obtained when the recording site is outside the neuromuscular junction zone (Fig. 1.A). When the needle is placed within a motor endplate region, endplate noise is recorded, which may be either small or large (Fig. 1.B and 1.C, respectively). Spikes can also be recorded in the neuromuscular junction area, allowing simultaneous detection of endplate noise (Fig. 1.D). However, spikes are more commonly recorded outside the endplate region (Fig. 1.E).

All animals were anesthetized with 2 % tribromoethanol (0.15 ml/10 g of body weight, i.p.) for EMG recordings and immediately sacrificed by exsanguination while deeply anesthetized.

Statistical procedure

Data were analyzed using SPSS version 21.0 (SPSS, Inc., Chicago, IL, USA). Values are expressed as mean ± SD, considering the 95 % CI. We used a one-way analysis of variance (ANOVA) to evaluate differences between groups and the Bonferroni test. Differences were considered significant at $P < 0.05$.

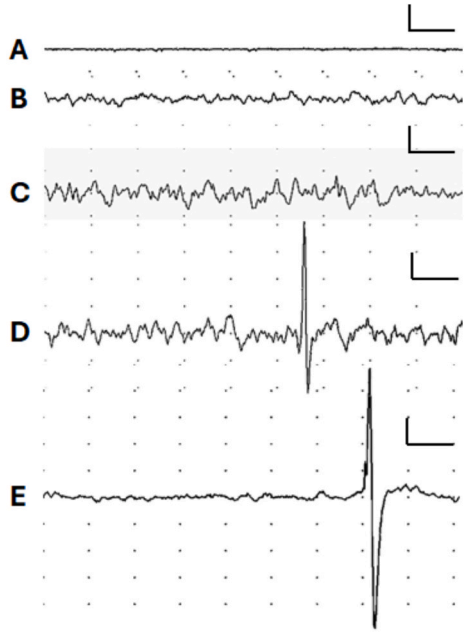

Fig. 1. Representative electromyography traces. (A) Trace recorded outside the endplate zone (baseline noise). (B) Trace within the endplate zone showing low-amplitude endplate noise. (C) Trace within the endplate zone showing high-amplitude endplate noise. (D) Spike associated with endplate noise (within the endplate zone). (E) Spike without endplate noise (recorded away from the endplate zone). Scale bars: Horizontal, 10 ms. Vertical, 10 μV.

Results

Endplate noise

From each animal, 12 areas of the gastrocnemius muscle were recorded and the % of areas that presented plate noise was calculated. When plate noise increases, it is easier to record it and the number of sites where it can be recorded increases. As can be seen in Fig. 2.A, male animals presented more places with endplate noise than female animals in both young and old (young male respect to young female: P =

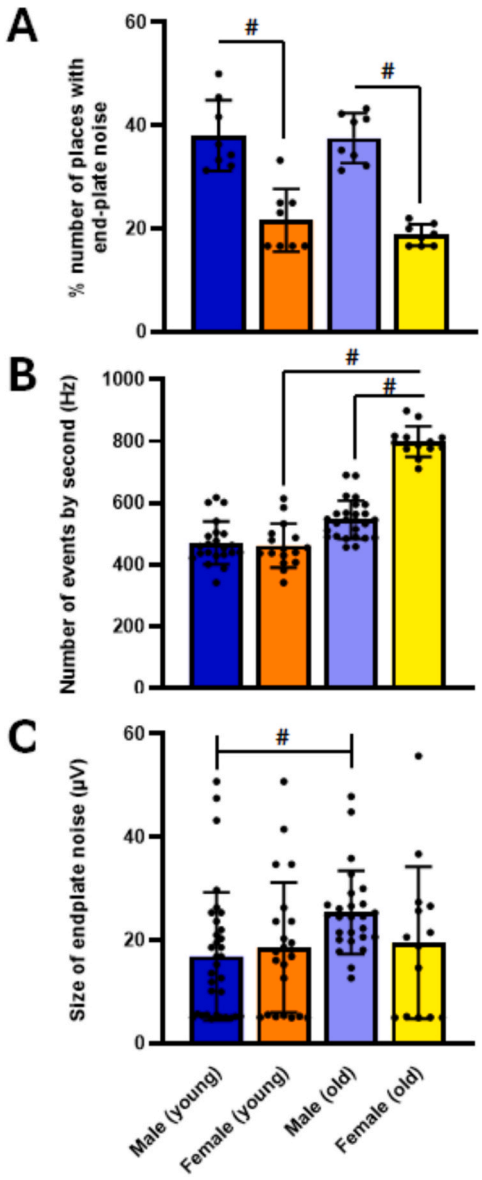

Fig. 2. Endplate noise. A, % number of places presenting plaque noise (n = 96 places tested from 4 animals (8 legs) in each experimental group). B, number of events per minute (frequency) in the places presenting endplate noise (n of places with endplate noise = 33 in young males, 28 in young females, 30 in old males, 14 in old females). C, endplate noise size (amplitude) (number of determinations: 33 for young males, 28 for young females, 30 for old males and 14 for old females). # $P < 0.05$.

0.00102, t = 5.08; old male respect to old female: P < 0.0001, t = 6.41). Old females were the group with the highest average number of events recorded in each area (frequency; Fig. 2.B) clearly above old males (Old females respect to old males: P < 0.0001, t = -12.739, n = 14 places with endplate noise in old females and 30 places with endplate noise in old males) and young females (old females respect to young females: P < 0.0001, t = -14.447, n = 14 places with endplate noise in old females and 28 places with endplate noise in young females). The largest size of the endplate noise events was in the group of old males (Fig. 2.C; old male respect to young male: P = 0.0218, t = -3.034, n = 30 places with endplate noise in old males and 33 places with endplate noise in young males). See an example of large end plate noise in Fig. 1.C.

Spontaneous spikes

The number of areas with spikes has been obtained in a similar way to plate noise. The % of areas with spikes was similar between males and young females (Fig. 3.A; young males respect to young females: P = 1.0, t = 1.194, n = 96 places tested in males and females). However, among old animals, males had more places with spikes than females (Fig. 3.A; Old males respect to old females: P < 0.0001, t = 6.417, n = 96 places tested in males and females). On the other hand, old males will have more places with spikes than young ones (Fig. 3.A; Old males respect to young males: P < 0.0001, t = -8.923, n = 96 places tested in young and old). The number of spikes per minute (frequency) was always higher in female animals than in males and always higher in old animals than in young ones (Fig. 3.B:young females respect to young males: P = 0.000156, t = -4.33; old females respect to old males: P = < 0.000225, t = -4.26; old males respect to young males: P = 0.0227, t = -2.95; old females respect to young females: P = 0.00104, t = -3.84; number of places with spikes = 62 in young males, 68 in young females, 65 in old males and 72 in old females).

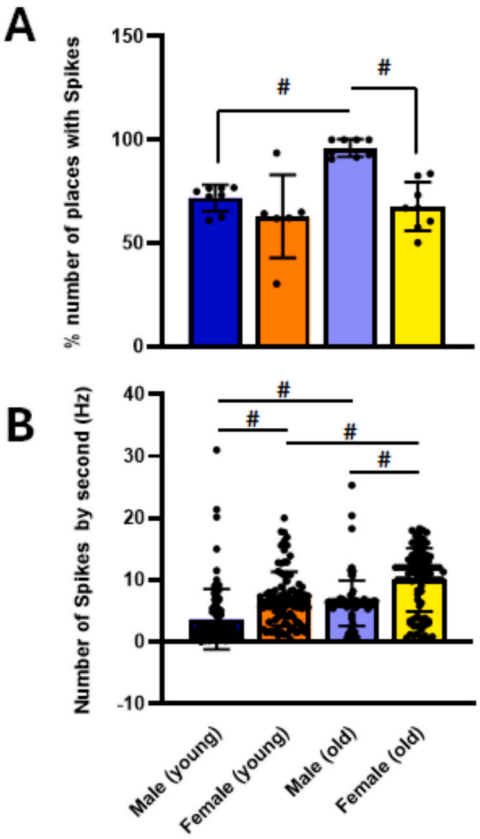

Fig. 3. Spikes. A, % of the number of areas with spikes (n = 96 areas explored from 4 animals (8 legs) in each experimental group). B, number of spikes per minute (frequency) in the areas with spikes (n of areas with spikes = 62 in young males, 68 in young females, 65 in old males, 72 in old females). #, P < 0.05.

Discussion

Spontaneous acetylcholine (ACh) release at the neuromuscular junction (NMJ) shows clear age- and sex-dependent differences in vivo. Using needle EMG as an extracellular readout of spontaneous synaptic activity, we found that males present a higher proportion of sites with endplate noise than females at both ages, whereas old females exhibit the highest endplate-noise frequency. In contrast, endplate-noise amplitude is selectively increased in old males. Together, these results indicate that the aging trajectory of spontaneous NMJ transmission is not uniform between sexes.

End plate noise

Kimura links plate noise and miniature endplate potentials as "endplate noise represents the extracellular recording of miniature endplate potentials (MEPPs), which are non-propagating depolarizations caused by the spontaneous release of acetylcholine (ACh)" (Kimura, 2013). The higher percentage of endplate-noise sites in males suggests a greater spatial prevalence of spontaneous ACh release along the gastrocnemius NMJ region. This aligns with the idea that spontaneous release is sensitive to presynaptic homeostatic state and can differ across biological variables such as sex. Importantly, the sex divergence becomes more evident with aging: old females display a marked increase in event frequency, while males do not.

Tsentsevitsky et al. (2024) evaluated mice of both sexes (3- and 12-month-old) using extracellular recording and found that spontaneous and evoked exocytosis remained unaltered. In contrast, the present study reveals sex-specific differences in the number of events per minute: older females exhibit increased spontaneous release, while males show no change. Similarly, Tsentsevitsky et al. (2024) reported decreased MEPP amplitude in older mice. However, our study demonstrates divergent endplate noise size behavior by sex: it increases in older males but remains unmodified in older females. Discrepancies with Tsentsevitsky et al.'s findings may stem from age-range differences (2–15 months here vs. 3–12 months in their study), though this seems unlikely. Alternatively, the blending of male and female data in their study may have diluted age-related differences between young and old mice.

The results obtained suggest that these modulators exhibit sensitivity to both sex and age. In male animals, Banker et al. reported a decrease in the number of MEPPs with aging (Banker et al., 1983). This finding coincides with the present study, since the number of areas that presented endplate noise was lower in older animals (both males and females). However, Banker et al. classified old mice as those aged 29 to 33 months, whereas in the present study, animals were considered old at 15 months. This suggests that extreme senescence may not be a determining factor and that these variations occur much earlier, as observed in our study.

Skeletal muscle, independent of the neuromuscular junction, has been extensively studied concerning sex and age. For instance, Hill et al. (2020) conducted a comprehensive analysis of age-, sex-, and muscle-specific changes, demonstrating that contractile muscle function is highly complex and non-uniform, depending on factors such as age, sex, mode of muscle activation, and the specific muscle studied. Additionally, estrogen regulates key pathways involved in skeletal muscle function, energy metabolism, calcium signaling, and protein trafficking, all of which are essential for maintaining normal muscle function.

Electromyographic recordings of endplate noise revealed significant differences based on sex and age. Specifically, endplate noise was more frequently observed in males than in females, regardless of age. Moreover, older females exhibited a higher number of endplate noise events

per minute compared to younger females. Mechanistically, the selective increase in frequency in old females is compatible with hormonal modulation of presynaptic function. In the central nervous system, estrogens have been shown to modulate major neurotransmitter systems by influencing neurotransmitter production, release, or turnover, as well as through direct interactions with neurotransmitter receptors (Manev and Pericic, 1987; McDermott et al., 1994; Malyala et al., 2005). Estrogens influence cholinergic signalling and have been linked to changes in synaptic vesicle availability and cholinergic tone in different models (Newhouse and Dumas, 2015; Russell et al., 2019). Thus, fluctuations or long-term age-related shifts in estrogenic milieu may bias spontaneous vesicle fusion probability in females (Newhouse and Dumas, 2015; Russell et al., 2019). In males, the increase in endplate-noise amplitude with aging suggests a different adaptation, possibly involving altered quantal size, postsynaptic responsiveness, or presynaptic–postsynaptic matching (for discussion of age- and sex-related neuromuscular changes, see Russell et al., 2019). Future work directly measuring MEPPs and vesicle parameters at single NMJs will be needed to pinpoint the substrate of these sex-specific trajectories (as suggested by current evidence on estrogen–cholinergic interactions and neuromuscular aging; Newhouse and Dumas, 2015; Russell et al., 2019).

It is possible that the observed differences are due to an effect of anesthesia on spontaneous release that depends on sex or age. Ample experimental and bibliographic evidence demonstrates that general anesthetics can alter spontaneous (and miniature) synaptic events through presynaptic or postsynaptic mechanisms (Hao et al., 2020). Moreover, sensitivity to anesthesia and its neuronal consequences have been shown to vary with age and sex, including hormonal status (Sitdikova et al., 2014). However, no such evidence exists for tribromoethanol, and most studies refer to neurotransmission within the central nervous system rather than at the neuromuscular junction. Therefore, it seems unlikely that tribromoethanol influences the spontaneous neuromuscular release of acetylcholine (ACh) in a manner dependent on sex or age.

Collectively, substantial evidence supports the dependence of cholinergic receptors in both the central and peripheral nervous systems on estrogens (Houser et al., 2011). This study provides further evidence reinforcing this relationship at neuromuscular synapses in mammals.

Spontaneous spikes

When multiple vesicles are released simultaneously from a nerve terminal, an end-plate potential (EPP) is generated, subsequently triggering muscle fiber action potentials (MFAPs) in response (Fatt and Katz, 1951). To date, no studies have specifically evaluated EPPs in relation to age. Many years ago, Fahim (1997) elicited trains of EPPs and reported that the first EPP in a series increases with age, while subsequent EPPs are smaller compared to those observed in young animals. The spikes recorded via electromyography (EMG) correspond to action potentials propagating along muscle fibers (Kimura, 2013). Since action potentials follow the all-or-nothing principle, their amplitude is not a meaningful parameter for evaluation. Therefore, in the present study, only spike frequency—not amplitude—was assessed.

The origin of these spikes has been the subject of significant debate. It is well established that they may result from accidental stimulation of the neuromuscular junction by the recording needle, fusimotor activation, or spontaneous MFAPs (Kimura, 2013). More recently, Partanen et al. (2022) used a five-channel needle to record EMG activity from the extensor carpi radialis muscle, capturing “end-plate spikes” that do not propagate but are accompanied by motor end-plate noise. The authors conclude that end-plate spikes are potentials of fusimotor units, reflecting the activity of intrafusal muscle fibers of the muscle spindle (nuclear bag and nuclear chain fibers).

In the present study, the proportion of areas where spikes were recorded varied among groups. If spikes were propagating through extrafusal fibers, they could be detected in any muscle area, resulting in a near-100 % occurrence. Comparing the percentage of areas exhibiting non-propagating end-plate noise to those with propagating spikes reveals that spike presence is significantly high. Thus, the findings of this study support the notion that spikes do not originate from fusimotor activity, as proposed by Partanen et al. (2022), but rather align with Kimura’s findings (2013). Spike recordings also differed by sex and age. We observed a higher spike frequency in females than in males and a general increase with aging, with old males showing more spike-positive sites than old females. These findings indicate that the processes generating spikes are likewise modulated by biological sex and aging. Rather than re-defining spikes here, we interpret them as reflecting heightened excitability and/or altered neuromuscular drive in specific conditions, which may be shaped by hormonal state and age-dependent changes in peripheral or central motor control.

The study reveals significant differences in neuromuscular transmission according to sex and age, highlighting the role of estrogens and autonomic regulation in spontaneous acetylcholine (ACh) release. The results indicate that males present a greater number of sites with end-plate noise compared to females, while in old age, females exhibit a higher frequency of events at these sites. Furthermore, the amplitude of the noise is greater in aged males. These findings suggest hormonal modulation in cholinergic function and possible differences in the regulation of the autonomic nervous system.

Furthermore, it was observed that the frequency of electromyographic spikes is higher in females and increases with age. This supports the hypothesis that estrogens influence cholinergic neurotransmission, affecting both synaptic vesicle release and the function of postsynaptic receptors. Furthermore, the decrease in sympathetic activity with age may contribute to the observed changes in neuromuscular neurotransmission.

Participation of the autonomic nervous system (ANS)

The autonomic nervous system (ANS) plays a crucial role in regulating muscle tone and spindle sensitivity through indirect mechanisms, such as gamma motor neuron activation and blood flow modulation (Macefield and Knellwolf, 2018). Both the morphological and functional aspects of sympathetic innervation at skeletal neuromuscular synapses have been described (Rodrigues et al., 2019), and this innervation directly influences the spontaneous release of acetylcholine (Bosque et al., 2023). Age and sex are known to influence ANS function. For example, while no correlation exists between muscle sympathetic nerve activity and blood pressure in young men and women, a significant relationship emerges in individuals over 40 years of age (Narkiewicz et al., 2005). Additionally, Delbono et al. (2021) suggested that sympathetic regulation of neuromuscular junction transmission declines with aging.

Recent studies have identified a novel mechanism by which motor and sympathetic neurons interact at the NMJ presynapse (Khan et al., 2016; Rodrigues et al., 2019; Wang et al., 2020). Impaired neuronal crosstalk may explain age-related declines in muscle function (Rodrigues et al., 2019). More recently, D’Souza et al. (2023, 2024) employed a comprehensive range of techniques to assess motor sympathetic activity, revealing notable differences between younger (24–26 years) and older (70–71 years) men and women. Their findings indicated that young men exhibit greater increases in sympathetic action potential discharge compared to young women. Conversely, in older adults, regardless of sex, muscle sympathetic nerve activity discharges are lower during exercise than in younger individuals. In line with D’Souza et al., our study also observed differences in spike frequency between younger and older animals, as well as between sexes. Aging exerts variations in sympathetic control over the neuromuscular junction (NMJ). Thus, the expression of adrenergic receptors and functional coupling decrease with age (Wang et al., 2020). On the other hand, acute adrenergic ligands can increase the spontaneous release of acetylcholine (MEPP frequency) in fast-twitch muscles, demonstrating that the

pathway is directly targeted to the quantum release machinery (Tsentsevitsky et al., 2024). That is, aging should generally reduce the sympathetic "drive" of spontaneous release. However, in the present study, not all the evaluated parameters decreased with age. It appears that the control of the neuromuscular sympathetic system is compensated for by other factors related to sex, especially in women. These results further reinforce the strong association between ANS function and both age and sex.

Regardless of the underlying causes of these sex differences, this study provides a significant characterization of male–female variations in neurotransmission at the neuromuscular junction. Furthermore, it offers evidence of a divergent aging process at this level between males and females.

In conclusion, this study provides evidence on sex and age differences in neuromuscular transmission, highlighting the hormonal and autonomic influence on these processes. The findings could have implications for the understanding of neuromuscular pathologies and the development of sex- and age-differentiated therapeutic strategies.

Limitations of the study

As mentioned in the introduction, each muscle may respond differently to aging in terms of spontaneous ACh release. In this study, electromyographic recordings were performed only in the gastrocnemius muscle.

The EMG recording of endplate noise provides an estimate of the activity of dozens of muscle fibers simultaneously. In contrast, recordings with glass microelectrodes are more precise, as they independently capture ACh release at each neuromuscular synapse. EMG-recorded potentials may be subject to technical errors.

In females, only the proestrus stage was selected, excluding the hormonal variability associated with other phases of the estrous cycle. This approach limits the assessment of hormonal impact to a single physiological state.

Adult aged animals (15 months) were evaluated, but not geriatric animals (24 months). Therefore, the results of this study are more comparable to humans around 50 years of age rather than those around 80 years.

CRediT authorship contribution statement

R. Gutierrez: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **M. Fibla:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **E. Skorupska:** Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Conceptualization. **M.M. Santafe:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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