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ORIGINAL PAPER

Analysis of Motor Function in Amyloid Precursor-Like Protein 2 Knockout Mice: The Effects of Ageing and Sex

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Abstract

The amyloid precursor protein (APP) is a member of a conserved gene family that includes the amyloid precursor-like proteins 1 (APLP1) and 2 (APLP2). APP and APLP2 share a high degree of similarity, and have overlapping patterns of spatial and temporal expression in the central and peripheral tissues, in particular at the neuromuscular junction. APP-family knockout (KO) studies have helped elucidate aspects of function and functional redundancy amongst the APP-family members. In the present study, we investigated motor performance of APLP2-KO mice and the effect sex differences and age-related changes have on motor performance. APLP2-KO and WT (on C57Bl6 background) littermates control mice from 8 (young adulthood) to 48 weeks (middle age) were investigated. Analysis of motor neuron and muscle morphology showed APLP2-KO females but not males, had less age-related motor function impairments. We observed age and sex differences in both motor neuron number and muscle fiber size distribution for APLP2-KO mice compared to WT (C57Bl6). These alterations in the motor neuron number and muscle fiber distribution pattern may explain why female APLP2-KO mice have far better motor function behaviour during ageing.

Keywords Amyloid precursor protein · Amyloid precursor-like protein · Knockout · Motor neurons · Ageing · Sex differences

Abbreviations

AD	Alzheimer's disease
APP	Amyloid precursor protein
APLP1	Amyloid precursor-like protein 1
APLP2	Amyloid precursor-like protein 2
EDL	Extensor digitorium longus
GA	Gastrocnemius
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PLA	Plantaris
ROI	Regions of interest
SOL	Soleus
TA	Tibialis anterior
WT	Wildtype

Introduction

The amyloid precursor protein (APP) is best known for its association with the pathophysiology of Alzheimer's disease (AD). APP belongs to a gene family that includes the amyloid precursor-like proteins 1 (APLP1) 1 and 2 (APLP2) [1, 2]. Most research on APP has centred on its role in the pathophysiology of AD, making the normal physiological function of APP, and its family members a relatively understudied area. The APP-family has been attributed with a broad range of actions in both the neuronal and non-neuronal systems, including regulation of cellular and neuronal differentiation, metal homeostasis, nuclear signalling and glucose metabolism [3–6].

Gene knockout studies have provided useful insights into the roles of the APP-family. All three members are important in synaptic formation, maintenance and plasticity in vivo [7–12]. The APLP2-KO mouse exhibit normal body weight and size, with comparable forelimb grip strength, balance, nociceptive reflexes, and normal cognition [13]. In contrast, the APP-KO mouse exhibit reduced body weight (15–20%), reduced grip strength, impaired locomotor activity and increased susceptibility to brain injury [14–17] compared

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to a control mouse. While single gene deletion knockout mice of the APP-family are viable, the double knockout and triple knockouts can lead to perinatal lethality. While the APP/APLP1 double knockout mouse survives to adulthood, the APP/APLP2 or APLP1/APLP2 double knockout mouse and the triple knockout APP/APLP1/APLP2 mouse are not viable and die at birth [3, 11, 13, 14, 18, 19]. Based on these combination knockout mouse models, it is apparent that APLP2 is an essential member of the APP gene family and has a vital role for postnatal survival and its lack of expression cannot be compensated for by either APP or APLP1 expression. These observations highlight both functional redundancy between the APP-family members, but also clear functional differences.

Ageing is associated with progressive changes in the brain and its associated sensory, motor, cognitive functions and body mass composition [20–24]. In the present study, we investigated if sex and age differences from 8 weeks (young adulthood) to 48 weeks (middle age) of age could affect motor performance of APLP2-KO compared to WT (C57Bl6) mouse. We found that the APLP2-KO female mouse has less age-related motor impairments. We also observed age and sex differences in motor neuron and muscle fiber size distribution in the APLP2-KO mouse. The changes in the motor neuron and muscle fiber distribution pattern may explain the improvement of motor function observed in female APLP2-KO mouse during ageing.

Materials and Methods

Animals

The APLP2-KO mouse [13] are null mutants generated by deleting their promotor and first exon. These mice were backcrossed greater than 14 times on to the same C57BL6/J background mouse resulting in greater than 99.99% background purity. Genotyping was determined by the polymerase chain reaction using primers as described by von Koch et al. [13]. The C57Bl6/J mouse were sourced from the Animal Resources Centre (Western Australia, Australia) and were used as wild type (WT) control in this study. For the rotarod experiments, the WT control (on C57BL6 background) are littermates control with sex and age matched to the APLP2-KO mouse. All mouse experiments in this study complied with the National Health and Medical Research Council code for the care and use of animals for scientific purposes and they were approved by the University of Melbourne Animal Ethics Committee (Project Number: 1413304). Mice housing cages contained standard bedding supplemented with tissue paper and cardboard tubes and/or plastic containers for environmental enrichment with access to feed and water ad libitum and housed on a reversed 12/12 h light/dark cycle and maintained at a temperature of 22 ± 2 °C. The experimenter was blinded to the mouse's age and sex until completion of the behavioural testing.

Rotarod

Before each experiment, all mice were habituated to the rotarod instrument six times before the training period. The habituation trials were conducted on two consecutive days, three times per day. Each trial involved placing a mouse on the rotating rod set at a constant speed of 10 rpm for 300 s to allow the mouse to explore the apparatus freely. Mice that repeatedly fall off were placed back onto the rotarod until they can stay on the rod for the remainder of the 300 s. Following habituation, mice were trained on the rotarod for three consecutive days with the rotation speed initially set to 4 rpm and increasing to 40 rpm over 180 s. On testing day, mice were transported to the testing room at least 30 min prior to testing and weighed before being placed on the rotarod with the same training speed parameters. Mice that were still on the rod after 180 s were recorded as having no detectable locomotor deficit. Mice that could not continue on the rotarod for the 180 s, fell on to a padded base. The fall latency was recorded by the experimenter and is defined as the physical fall off the rod or slipping if the hindlimbs and only the front paws were still grasping on the rod. Rotarod performances were performed 2 days per week, with three trials on each day of testing with intervals of 10 min of rest between each trial and the times for each test recorded.

Tissue Collection

Mice were killed with an intraperitoneal injection of anaesthetic drugs, xylazine (16 mg/kg body weight) and ketamine (120 mg/kg body weight) together in neutral saline solution. The depth of anaesthesia was evaluated by pinching the toes and looking for withdrawal reflex and once unconscious. An incision was made on both sides of the diaphragm to open the sternum and to expose the heart in the chest cavity. The aorta was identified and cut then a 23G butterfly needle inserted into the left ventricle of the heart. This needle was connected to a 30 ml syringe filled with ice cold saline solution supplemented with 20 U/ml of heparin. Using gentle pressure, cold saline solution was perfused through the mouse for 2–3 min at a flow rate of 5 ml/min. Proper perfusion is indicated by the liver becoming pale. Following perfusion, tissues were removed for analysis. *Spinal cord sections* The spinal cord lumbar region was fixed by immersing the tissue in freshly prepared 4% paraformaldehyde and incubating overnight at 4 °C and cryo-protected with 30% sucrose for at least 24 h. Lumbar regions were embedded in Tissue-Tek O.C.T compound (Sakura FineTek), and snap frozen with liquid nitrogen in isopentane bath. Each mould

contains lumbar regions from 5 to 6 animals of the same phenotype. Lumbar tissues were sectioned serially on a cryostat (Leica) at 20 µm thickness. More than six sections were collected for each slide with each section at 200 µm apart. *Muscle histology* Hind limb skeletal muscles collected included the gastrocnemius (GA), tibialis anterior (TA), plantaris (PLA), soleus (SOL), and extensor digitorum longus (EDL). The muscles were immersed in Optimal Cutting Temperature (OCT) medium (Tissue-Tek; Sakura Finetek, USA) and laid flat on the end of a syringe and snap frozen with dry ice/liquid nitrogen in isopentane bath. This procedure was used to optimally preserve tissue morphology for subsequent ATPase staining and immunofluorescence. Muscles were stored at − 80 °C and then warmed to − 20 °C for sectioning using a cryostat (Leica, Germany). All sections were collected on to poly-lysine coated slides (Thermo Fisher, Australia), air dried at room temperature for at least 1 h and stored at − 80 °C until staining. The gastrocnemius muscle was sectioned transversely using with 10 µm thick sections cut through the mid-belly region to best observe muscle fiber morphology. Tibialis anterior muscle was sectioned longitudinally at 10 µm thick. Serial sections of the muscle were cut with each section placed 30 µm apart on the slide.

Nissl Staining of Spinal Cord Sections

The spinal cord frozen sections were allowed to air dry to room temperature for 1 h then circled with a hydrophobic barrier PAP pen (VWR, Australia). Sections were washed with Phosphate Buffered Saline (PBS) for 5 min to remove OCT compound. Prior to staining the tissue sections would undergo a "defatting step" (to remove the fat content in the tissue and reduce background staining levels) where the slides were soaked in 1:1 (v:v) alcohol/chloroform solution overnight at room temperature. Sections were rehydrated by bathing in 100% and 95% alcohol for 2 min each then placed in distilled water. Tissue sections were stained by incubating in 0.1% cresyl violet solution (0.1 g cresyl violet acetate, 100 ml distilled water, 0.3 ml glacial acetic acid) for 1 h in a 37 °C oven (to improve tissue penetration and enhance evenness in tissue staining). Sections were checked under the microscope for the intensity of the staining and subjected to longer incubations if required. The slides were rinsed in distilled water for 3 s and dehydrated in 70%, 90% and 100% alcohol for 30 s each. The slides were cleared in xylene solution two times (5 min each) and mounted in Safety mounting medium (Trajan, Grale). The spinal cord sections were imaged using a digital slide scanner Panoramic SCAN II (3Dhistech, Hungary) with Carl Zeiss Plan Apochromat 20×/NA 0.8 (Zeiss, Germany). The slides were viewed using Case Viewer software (ver 2.2, 3Dhistech, Hungary).

Neuron Counting Analysis of Spinal Cord Sections

Nissl stained spinal cord sections were imported into Image J/Fiji software (ver. 1.52e, NIH) for semi-quantitative analysis of motor neuron numbers. For each spinal cord section, both the left and right ventral horn regions were selected as regions of interest (ROIs). Neurons were analysed using the manual thresholding command, followed by cell segmentation and particle analysis with neurons having a soma diameter of less than 10 µm excluded from further analysis. A total of seven spinal cord sections were analysed for each animal, and each cut section was at least 200 µm apart.

ATPase Staining for Muscle Tissue

The fiber types of the GA muscle from the mouse hind limb were analysed histochemically by staining for myosin ATPase activity. The frozen transverse muscle sections were allowed to air dry at room temperature for 1 h before circling the tissue sample with PAP pen. Slides were fixed in 4% paraformaldehyde solution for 1 h then washed with PBS three times. Muscle slides were then incubated in Myosin ATPase activity buffer at pH 4.3. Slides were pre-incubated in 0.1M acetate buffer at pH 4.3 for 10 min, then washed in distilled water then placed in sodium barbital solution and incubated at 37 °C for 30 min. Slides were brought to room temperature and washed in distilled water three times, incubated in fresh 1% $CaCl_2$ for 10 min then in 2% $CoCl_2$ for 10 min. Slides were washed thoroughly in distilled water and allowed to develop for 15 s in freshly prepared 1% ammonium sulfide solution in a fume cupboard. Slides were washed again in distilled water and dehydrated through 70%, 90% and 100% (2 times) alcohol solutions (2 min each), cleared in xylene two times (5 min each) and mounted in Safety mounting medium (Trajan, Grale).

Muscle Fiber Typing Analysis

Slow twitch and fast twitch fiber composition can be distinguished based on their reactivity with myofibrillar enzymes ATPase activity (pH 4.3), with slow twitch fibers staining dark brown and fast twitch muscle staining light beige. Type I (slow twitch) and type II (fast twitch) fibers constitute the majority of the fibers in skeletal muscles. Stained muscle sections were imaged using a Zeiss Axioscope 2 light microscope through a × 10 objective and images were acquired using Axiocam 503 color camera with Zenpro software 2011 (Zeiss, Germany). Acquired images were exported in tif file format and imported into Image J/Fiji software (ver. 1.52e, NIH) for further analysis. Total fiber numbers and the composition of each fiber type group were counted manually using the cell counter function. For each muscle fiber type, the cross-sectional area of each muscle fiber cell was

measured using ROI function. This was repeated for greater than 150 muscle cells per animal.

Statistical Analysis

All data are expressed as mean ± SEM with p values of 0.05 or less considered as significant. Statistical significance was assessed using a one-way analysis of variance with Bonferroni's post-hoc test to compare the different genotypes within the same sex groups. A two-way analysis of variance was used when assessing genotypes and sex, followed by Tukey's post-hoc tests. All statistical analyses were performed using GraphPad Prism software (Ver. 7, San Diego, CA, USA).

Results

APLP2 Deficiency in Female Mice Rescues Age-Dependent Motor Function Decline

To investigate motor function in mice over time (from 8 to 48 weeks of age), male and female mice from both WT and APLP2-KO mice were tested on an accelerating rotarod set at a speed of 4–40 rpm over 180 s longitudinally. Comparison of latency to fall at 12 weeks and 48 weeks, we found performances significantly decreased at 48 weeks for both male ($p = 0.0013$) and female ($p = 0.0014$) WT mice but there was no difference between the sexes (Fig. 1a). Similarly, a significant reduction in rotarod performances were observed in both male ($p = 0.0254$) and female ($p = 0.0023$) APLP2-KO mice at 48 weeks of age when compared to 12 weeks. However, we found that female APLP2-KO mice performed significantly better on the rotarod as they aged compared to male APLP2-KO mice and this was statistically different ($p = 0.0170$) at the 44 week time point (Fig. 1b). Moreover, these female APLP2-KO mice also performed significantly better compared to the age and sex matched WT mice ($p = 0.0440$) from 39 to 45 weeks of age (Fig. 1c). In contrast, the male APLP2-KO mice displayed a similar decline in rotarod performance as the age and sex matched WT mice (Fig. 1d).

α-Motor Neurons are Decreased in 12 Week Old APLP2-KO Mice but Unchanged in Female APLP2-KO at 48 Weeks of Age

To understand if the reduction in motor performance with ageing correlates with neuronal degeneration, we quantitated Nissl stained neurons in the lumbar spinal cord of WT and APLP2-KO mice at 12 and 48 weeks of age for both sexes (Fig. 2a). Alpha neurons in the ventral horn of the spinal cord can be identified by their large soma size (with cell body diameter > 20 µm) [25, 26]. The α motor neurons have large cell bodies and they innervate the extrafusal muscle fibers, the bulk of the skeletal muscle tissue and are responsible for generating tension via muscle contraction [27, 28]. The number of α motor neurons in the ventral horn of the spinal cord was lower for both sexes of APLP2-KO (female = 19.7 ± 1.0, male = 27.6 ± 2.2; $p = 0.0010$) mice compared to WT mice (female = 34.9 ± 2.1, male = 42.7 ± 1.4) at 12 weeks of age

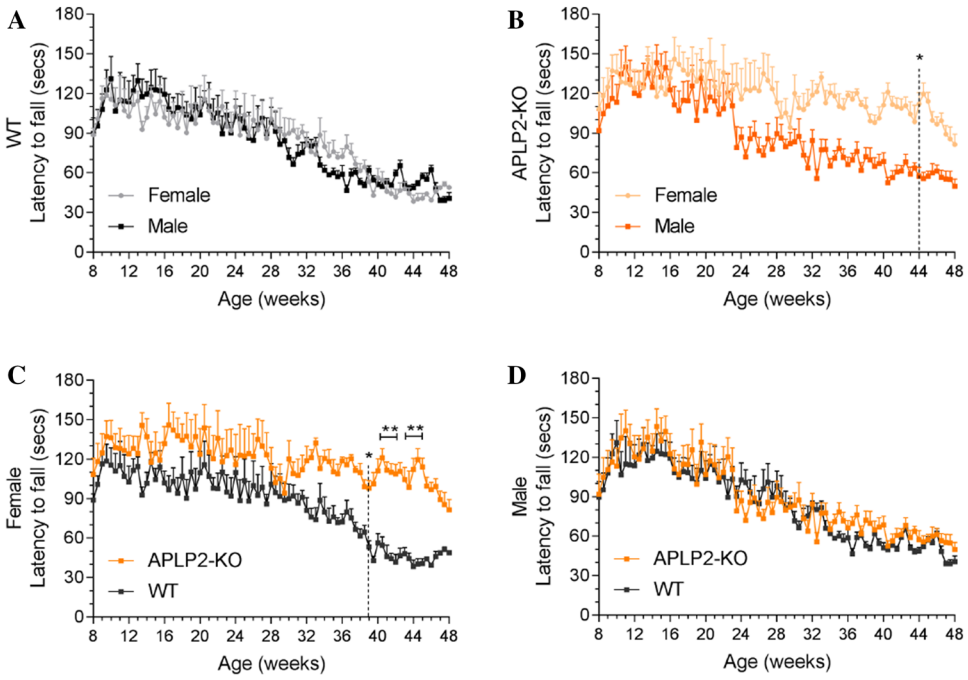

Fig. 1 Female APLP2-KO mice performed better than their female WT littermate controls, and male APLP2-KO mice on the rotarod as they age. Rotarod performance of WT littermate control mice and APLP2-KO mice beginning at 8 weeks of age **a** WT and **b** APLP2-KO. Genotype comparison of rotarod performance of **c** female and **d** male mice. Data presented as mean ± SEM. Statistical comparison was performed using a two-way repeated measure *ANOVA* and Tukey's posthoc test, *$p < 0.05$. $N = 6$

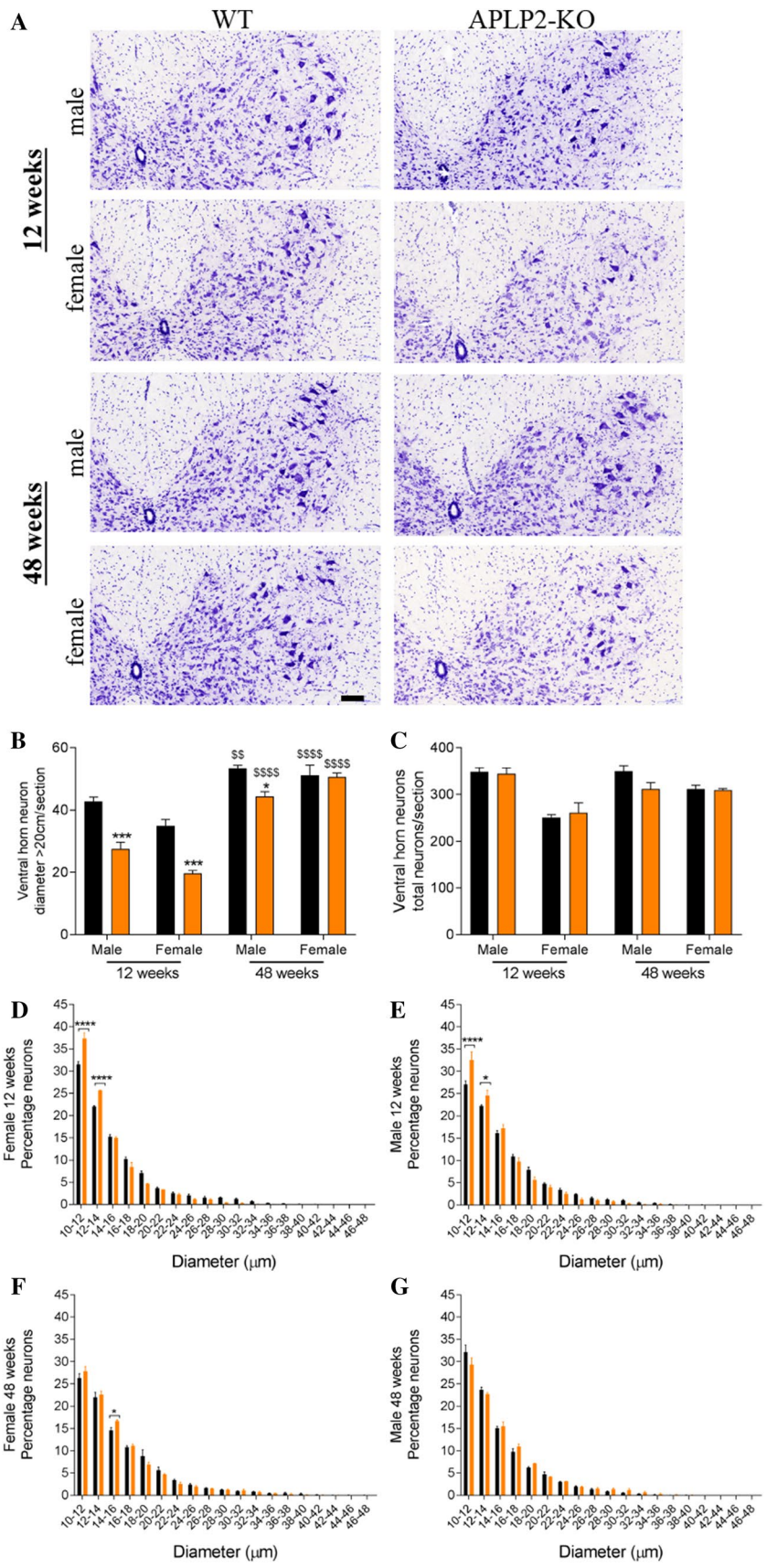

Fig. 2 Reduction in ventral horn neurons in APLP2-KO mice. The ventral horn lumbar spinal cord (L4–L5) were **a** sectioned and stained with cresyl violet for WT (black) and APLP2-KO (orange) mice at 12 and 48 weeks of age. **b** Stereological motor neuron counts with a diameter greater than 20 µm per section and **c** the total number of neurons per section of the ventral horn spinal cord. Frequency distribution of neuron calibre of both sex (female and male) and genotype (WT and APLP2-KO mouse) at **d, e** 12 weeks and **f, g** 48 weeks of age. Data represented as mean ± SEM. Statistical comparison was performed using two-way ANOVA with Bonferroni's posthoc test, genotype comparison (WT vs. APLP2-KO) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; age comparison (12 vs. 48 weeks) $^{\$\$}p < 0.01$, $^{\$\$\$\$}p < 0.0001$. N = 3–4. Scale bar = 100 µm

(Fig. 2b). At 48 weeks of age, the number of these larger motor neurons in male APLP2-KO (44.3 ± 1.5, p = 0.0141) were significantly reduced compared to WT mice (53.3 ± 1.0) but no statistical differences were found when comparing the female APLP2-KO (50.6 ± 1.3, p = 0.99) to WT (51.1 ± 3.3) mice at the same age (Fig. 2b).

Motor neurons in the spinal cord with diameter ≥ 10 µm were examined only. The total number of neurons were quantified in the anterior horn of the spinal cord were not significantly different between the male APLP2-KO and WT at both 12 and 48 weeks of age (Fig. 2c). To further quantify the changes in motor neuron soma size, we next examined the size distribution of ventral horn motor neurons by binning neuron soma diameter and expressing it as a percentage of the total number of neurons. We observed there was a significantly higher proportion of the smaller neurons (10–12 µm and 12–14 µm ranges) in the APLP2-KO female (Fig. 2d) and male (Fig. 2e) compared to WT mice at 12 weeks of age. This may explain the lower number of large neurons observed in APLP2-KO at 12 weeks. At the 48 week mice cohort, the percentage of the 14–16 µm diameter neurons were significantly higher in the female APLP2-KO compared to the WT mice (Fig. 2f) but no differences were observed in the distribution of the different neuronal sizes for males between APLP2-KO and WT (Fig. 2G).

Muscle Fiber Distribution is Altered in APLP2-KO Mice

To examine whether the changes in spinal cord motor neuron soma size for the APLP2-KO mouse causes alterations of muscle fiber caliber composition, the ATPase muscle staining technique was used to examine muscle fibre morphology. Slow twitch and fast twitch fiber composition can be distinguished based on their reactivity with myofibrillar enzymes ATPase activity at pH 4.3, with slow twitch fibers (Type 1) staining a dark brown colour while the fast twitch muscle fiber (Type II) stain is a beige or light brown in colour (Fig. 3a). Type I and II fibers represent the majority of the muscle fiber types present in skeletal muscles [29]. Histological analysis of the cross sectional gastrocnemius muscle showed no gross morphological differences in the pattern of fiber distribution between the WT and APLP2-KO groups and this was confirmed following analysis of fiber distribution (Fig. 3b). At 48 weeks of age, the female APLP2-KO mouse had a significantly higher proportion of type I fibers compared to both the age matched

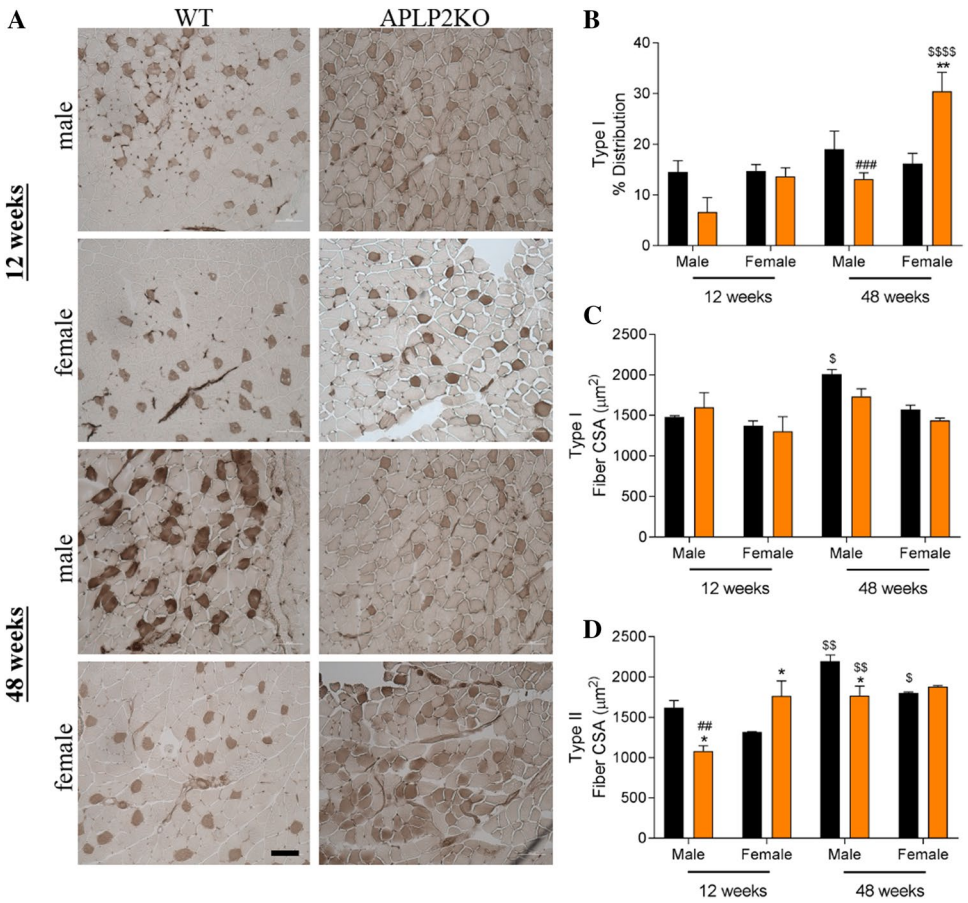

Fig. 3 Analysis of the gastrocnemius muscle from 12 to 48 weeks old APLP2-KO mice. **a** Myosin ATPase staining (pH 4.3) of representative cross-sectional gastrocnemius muscles isolated from male and female WT (black) and APLP2-KO (orange) mice at 12 and 48 weeks of age. Percentage distribution of **b** type I muscle fiber types and cross sectional area of **c** type I and **d** type II muscle fiber types are shown. Data represented as mean ± SEM. Statistical comparison was performed using a two-way ANOVA with Bonferroni's post-hoc test, genotype comparison $^{*}p < 0.05$, $^{**}p < 0.01$; sex comparison $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; age comparison $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$\$}p < 0.0001$. N = 3–4. Scale bar = 100 µm

female WT control ($p = 0.0022$) and male APLP2-KO ($p = 0.0004$) mice and compared to the younger 12 weeks old female APLP2-KO ($p < 0.0001$) (Fig. 3b). When we compared the cross-sectional area (CSA) of both type I and II muscle fibers across the mice groups, we found a significant increase in fiber CSA values in both the Type I and II fibers for the WT male groups at 48 weeks compared to the younger 12 week old group. Similarly, male APLP2-KO also showed a significant increase in Type II CSA at 48 weeks compared to 12 week group. Interestingly, when compared to the WT controls we found a significantly lower CSA values for Type II fibers in the male ($p = 0.0106$) APLP2-KO but this was significantly higher in the female ($p = 0.0261$) group at 12 weeks of age. The significantly lower CSA persists in the male APLP2-KO mice ($p = 0.0360$) but unchanged for female group at 48 weeks of age. However, only WT female mice showed a decrease ($p = 0.0347$) in Type II CSA at 48 weeks. Comparison of sex showed a significant increase in CSA in only in female APLP2-KO ($p = 0.0001$) mice at 3 months. We next investigated whether the muscle fiber atrophy observed in the male APLP2-KO or muscle hypertrophy observed in the female APLP2-KO mice at 12 weeks could result in a change in the muscle mass. Therefore, the tissue wet weight of different leg muscles including the soleus, plantaris, gastrocnemius, tibialis anterior, extensor digitorium longus were determined and corrected for mouse body weight. The muscle mass to body weight ratios were not statistically different between APLP2-KO and WT mice and for any muscle type or between sexes at 12 and 28 weeks of age (Fig. 4).

Discussion

We performed a longitudinal motor analysis of male and female APLP2-KO mice and examined the neuropathological features in young (12 week) and older (48 week) mice to determine if there is a correlation between motor performance, motor neuron expression in the spinal cord, and muscle fiber composition. Normal ageing is associated with progressive changes in the brain, and impairment of both sensory and motor function [21–23]. Aged C57Bl6 mice show reduced neuromuscular strength at 8–12 months of age, but no difference in motor performance in the single time point rotarod test [24]. Aged mice have impaired sensorimotor dependent behaviours and their physical activity also decreases with age [24, 30–32].

Similar to previous studies [33–35], we found no differences in the rotarod performance between female and male WT mice on a C57Bl6 background. Interestingly, while the male APLP2-KO showed a similar reduction in rotarod performance compared to the WT littermates with ageing, we observed a significantly better motor performance (less of a decline) in the APLP2-KO female cohort. While sex differences in age of onset, disease progression or disease severity have been observed [36], female rodents are often not selected for behavioural studies due to an assumed variability related to their oestrous cycle confounding the results. However, for the C57Bl/6J mouse, the locomotion activity and reflex responses remained stable across all phases the oestrous cycle [37], and the oestrous cycle plays a very minor role in mouse physical performance [38] therefore including both sexes in animal studies is

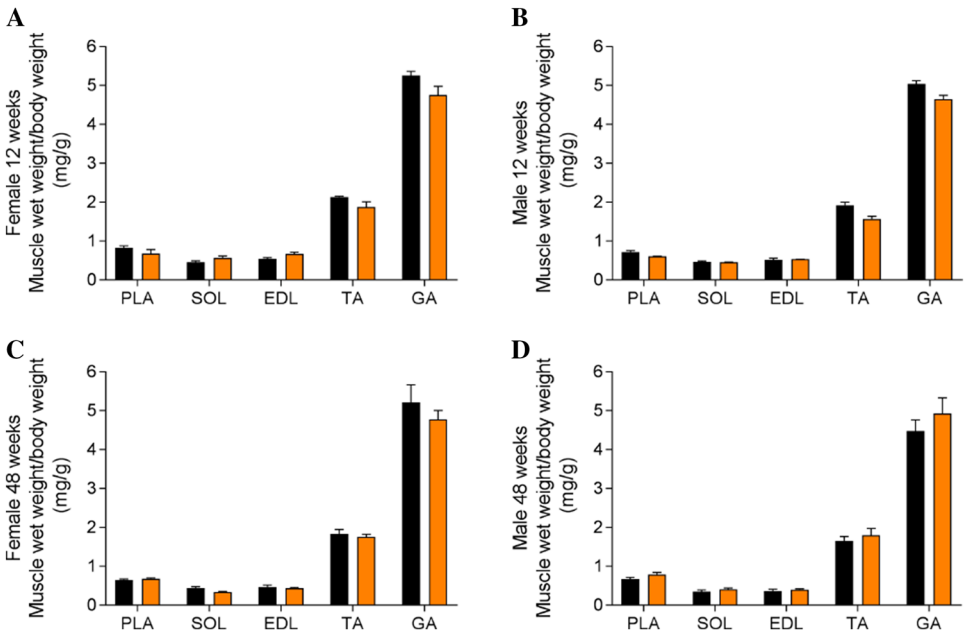

Fig. 4 Relative muscle wet weight in APLP2-KO and WT mice are similar at 12 weeks of age. The ratio of muscle wet weight (mg) relative to mouse body weight of WT (black) and APLP2-KO (orange) at **a, b** 12 and **c, d** 48 weeks of age for female and male mice groups respectively. Muscles examined included; plantaris (PLA), soleus (SOL), extensor digitorum longus (EDL), tibialis anterior (TA), and gastrocnemius (GA). Data represented as mean ± SEM. N = 3–4

important. It is noteworthy that female mice exhibit better motor and behavioural performance outcomes compared to male mice following traumatic brain injury (TBI) when using either the C57Bl6 mouse [35] and rat [39]. The basis for these differences between male and female rodents following TBI is still unknown, but in female rats, the effect of the oestrous cycle on motor performance post TBI is believed to be a minor contributor [40], and it had no effect on motor and cognitive tasks [41]. These results suggest that the oestrous cycle may play a subtle role in the physical performance of mice and therefore the molecular basis for the effect of APLP2 deletion in female sex remains to be resolved.

At the cellular level, we found significant differences in the spinal cord neurons and in the muscle histology of the young and old APLP2-KO mice. A motor unit comprises of a single motor neuron and all the muscle fibers it innervates [42]. Skeletal muscle activities are under the control of the motor unit, triggered by signalling from a motor neuron [42]. Therefore, early motor unit loss in ageing mice could be an important driver for the deficits seen in the physical performances during ageing [43]. There are three main classes of motor neurons in the motor nucleus- alpha motor neurons, gamma motor neurons and beta motor neurons [44]. Alpha neurons in the ventral horn of the spinal cord can be identified by their large soma size (with cell body diameter > 20 µm) [25, 26]. There are conflicting reports in the literature regarding the loss of motor neuron in mice during ageing [45–47]. Studies have reported a reduction in α motor neurons in aged human and animals, and they showed that hypertrophy was observed in the remaining α neurons [48, 49]. In contrast, other studies reported no difference in the number and size of motor neurons in aged mice compared to adult mice [46, 50]. In our study, we observed a significant increase in α motor neurons in WT mice in the 48 week age group for both sexes while a significant decrease in α motor neurons was observed in the female WT group. Our result agrees with a recent study looking at α motor neurons in primates and mice which demonstrated that although motor function deteriorates with ageing, the soma of α motor neurons and innervating synaptic inputs were retained in the aged spinal cord of both species [50]. The loss of motor units in the hindlimb muscle correlated with reduced muscle contractility and was seen at a relatively early stage during the ageing process [43]. Electrophysiological studies of ageing mice demonstrated significant differences in the amplitude of the motor unit action potential in female mice during ageing suggesting that motor neurons in female mice are able to form collateral sprouts during ageing [43]. There is an increase in collateral sprouting in female rats compared to their male counterparts [51], while sarcopenia is more evident in aged male C57Bl6 mice compared to females [52]. Therefore, sex-dependent differences are an important parameter for understanding motor unit connectively during ageing.

APLP2 is ubiquitously expressed in all tissues, with higher expression levels present in neurons from the central and peripheral nervous system [53]. The APLP2 expression in pyramidal neurons from hippocampus region of the brain appear to be dispensable for normal maintenance of dendritic growth, spine formation and neuronal function in an age-independent manner [10]. This was confirmed by electrophysiological studies where field excitatory post synaptic potentials (fEPSPs) and long-term potentiation (LTP) studies showed no age related changes in the fEPSP and LTP recordings between younger (1–2 months) and older (10–12 months) aged APLP2-KO mice [10]. In the rodent brain, APLP2 protein expression was localised to the presynaptic active zone of neuronal axons and in close proximity to the synaptic vesicles suggesting that it may be an important modulator for neurotransmitter release [54, 55]. Consistent with this report, APLP2 expression in association with APP, was important for the expression of vesicular glutamate transporter 2 in synaptic transmission and plasticity [54, 56, 57]. Together, these data suggest APLP2 plays a key role in neuronal development and function and these observations highlight a functional compensation between APP and APLP2 in the neurons. Furthermore, it is known that APLP2 (as well as APP) is expressed on motor neurons and in muscle, whereas APLP1 is only expressed on the presynaptic terminal of motor neurons [11]. APLP2 and APP expression at the NMJ of skeletal muscle is regulated during development, and are important in NMJ formation and maturation [7, 8, 11, 58]. While neither APLP2-KO or APP-KO mice display obvious alterations in NMJ structure [13, 14, 59], the APP-KO/APLP2-KO double knockout mouse displayed severe alteration of NMJ morphology and impaired neurotransmission, further highlighting the compensatory roles of other APP-family members [7, 8, 11, 18, 59]. In the present study, we observed a significant reduction in α motor neurons in APLP2-KO mice at 12 weeks of age, locomotor function of APLP2-KO appears to be unaffected, possibly owing to the compensatory function of the APP family members. Interestingly, the reduction in α motor neurons was only observed in the spinal cord of male APLP2-KO and not in the 48 week female group (Fig. 2b). This data is also in agreement with the reduction in the CSA of muscle fiber type II for male APLP2-KO at 48 weeks of age, but is unchanged for the female group. The sex-related differences in motor neurons distribution and muscle fiber may explain the improvement in the motor performance decline seen in the female APLP2-KO mice during ageing. The reduction in muscle mass and the reduction of motor neurons in ageing has been associated with NMJ remodelling, which precedes muscle fiber atrophy and could be involved with initiating muscle loss [60–62]. Denervation at the NMJ is a major

contributor to the diminished motor function in older mice [45–47, 63–65]. Given the roles of APLP2 at the NMJ, it may explain the alterations in motor neuron distribution and muscle fiber size in APLP2-KO during ageing. Future studies examining the structural pattern of NMJ innervation in the muscle and electrophysiological studies examining the conduction properties of nerve axons in the spinal cord of APLP2-KO during ageing, would enhance our understanding on the role of APLP2 in motor neurons, synaptic transmission and muscle function. Also, it will be of particular interest to examine the compensatory function of the APP and APLP1 at the NMJ in the absence of APLP2 expression in mice during ageing. Further studies are needed to understand the effect of sex in the functional role of APLP2 in motor unit connectivity and locomotor function. Collectively, these data suggest that APLP2 affects muscle development and/or function.

The current study has identified a novel relationship whereby the deletion of the APLP2 gene in female mice can mitigate against age-related motor function impairments. Further studies are needed to understand the neurochemical and cellular basis for these APLP2 mediated effects on motor neurone and muscle morphology and function in females. This will include deciphering which of the different APLP2 proteolytic metabolites are mediating these effects [66]. Finally, the sex-dependent effects highlight the importance of considering both sexes in animal based studies to ensure phenotypes are not missed due to the use of only a single sex group.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

Ethical Approval All applicable international, national, and institutional guidelines for the care and use of animals were followed.

References

1. Wasco W, Bupp K, Magendantz M, Gusella JF, Tanzi RE, Solomon F (1992) Identification of a mouse-brain cdna that encodes a protein related to the Alzheimer disease-associated amyloid-beta-protein precursor. Proc Natl Acad Sci USA 89:10758–10762
2. Wasco W, Gurubhagavatula S, Paradis MD, Romano DM, Sisodia SS, Hyman BT, Neve RL, Tanzi RE (1993) Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid beta protein precursor. Nat Genet 5:95–100
3. Needham BE, Wlodek ME, Ciccotosto GD, Fam BC, Masters CL, Proietto J, Andrikopoulos S, Cappai R (2008) Identification of the Alzheimer's disease amyloid precursor protein (APP) and its homologue APLP2 as essential modulators of glucose and insulin homeostasis and growth. J Pathol 215:155–163
4. Nalivaeva NN, Turner AJ (2013) The amyloid precursor protein: a biochemical enigma in brain development, function and disease. FEBS Lett 587:2046–2054
5. Dawkins E, Small DH (2014) Insights into the physiological function of the β-amyloid precursor protein: beyond Alzheimer's disease. J Neurochem 129:756–769
6. Müller UC, Deller T, Korte M (2017) Not just amyloid: physiological functions of the amyloid precursor protein family. Nat Rev Neurosci 18:281
7. Yang G, Gong YD, Gong K, Jiang WL, Kwon E, Wang P, Zheng H, Zhang XF, Gan WB, Zhao NM (2005) Reduced synaptic vesicle density and active zone size in mice lacking amyloid precursor protein (APP) and APP-like protein 2. Neurosci Lett 384:66–71
8. Wang B, Yang L, Wang Z, Zheng H (2007) Amyolid precursor protein mediates presynaptic localization and activity of the high-affinity choline transporter. Proc Natl Acad Sci USA 104:14140–14145
9. Taylor CJ, Ireland DR, Ballagh I, Bourne K, Marechal NM, Turner PR, Bilkey DK, Tate WP, Abraham WC (2008) Endogenous secreted amyloid precursor protein-alpha regulates hippocampal NMDA receptor function, long-term potentiation and spatial memory. Neurobiol Dis 31:250–260
10. Midthune B, Tyan SH, Walsh JJ, Sarsoza F, Eggert S, Hof PR, Dickstein DL, Koo EH (2012) Deletion of the amyloid precursor-like protein 2 (APLP2) does not affect hippocampal neuron morphology or function. Mol Cell Neurosci 49:448–455
11. Klevanski M, Saar M, Baumkotter F, Weyer SW, Kins S, Muller UC (2014) Differential role of APP and APLPs for neuromuscular synaptic morphology and function. Mol Cell Neurosci 61:201–210
12. Weyer SW, Zagrebelsky M, Herrmann U, Hick M, Ganss L, Gobbert J, Gruber M, Altmann C, Korte M, Deller T, Muller UC (2014) Comparative analysis of single and combined APP/APLP knockouts reveals reduced spine density in APP-KO mice that is prevented by APPsalpha expression. Acta Neuropathol Commun 2:36
13. von Koch CS, Zheng H, Chen H, Trumbauer M, Thinakaran G, van der Ploeg LH, Price DL, Sisodia SS (1997) Generation of APLP2 KO mice and early postnatal lethality in APLP2/APP double KO mice. Neurobiol Aging 18:661–669
14. Zheng H, Jiang M, Trumbauer ME, Sirinathsinghji DJ, Hopkins R, Smith DW, Heavens RP, Dawson GR, Boyce S, Conner MW, Stevens KA, Slunt HH, Sisoda SS, Chen HY, Van der Ploeg LH (1995) b-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity. Cell 81:525–531
15. Senechal Y, Kelly PH, Dev KK (2008) Amyloid precursor protein knockout mice show age-dependent deficits in passive avoidance learning. Behav Brain Res 186:126–132
16. Corrigan F, Vink R, Blumbergs PC, Masters CL, Cappai R, van den Heuvel C (2012) sAPPalpha rescues deficits in amyloid precursor protein knockout mice following focal traumatic brain injury. J Neurochem 122:208–220
17. Hefter D, Kaiser M, Weyer SW, Papageorgiou IE, Both M, Kann O, Muller UC, Draguhn A (2016) Amyloid precursor protein protects neuronal network function after hypoxia via control of voltage-gated calcium channels. J Neurosci 36:8356–8371
18. Heber S, Herms J, Gajic V, Hainfellner J, Aguzzi A, Rulicke T, von Kretzschmar H, von Koch C, Sisodia S, Tremml P, Lipp HP,

Wolfer DP, Muller U (2000) Mice with combined gene knockouts reveal essential and partially redundant functions of amyloid precursor protein family members. J Neurosci 20:7951–7963
19. Herms J, Anliker B, Heber S, Ring S, Fuhrmann M, Kretzschmar H, Sisodia S, Muller U (2004) Cortical dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family members. EMBO J 23:4106–4115
20. Barreto G, Huang TT, Giffard RG (2010) Age-related defects in sensorimotor activity, spatial learning, and memory in C57BL/6 mice. J Neurosurg Anesthesiol 22:214–219
21. Bishop NA, Lu T, Yankner BA (2010) Neural mechanisms of ageing and cognitive decline. Nature 464:529–535
22. Simen AA, Bordner KA, Martin MP, Moy LA, Barry LC (2011) Cognitive dysfunction with aging and the role of inflammation. Ther Adv Chronic Dis 2:175–195
23. Keller K, Engelhardt M (2013) Strength and muscle mass loss with aging process. Age and strength loss. Muscles Ligaments Tendons J 3:346–350
24. Shoji H, Takao K, Hattori S, Miyakawa T (2016) Age-related changes in behavior in C57BL/6J mice from young adulthood to middle age. Mol Brain 9:11
25. Lance-Jone C (1982) Motoneuron cell death in the developing lumbar spinal cord of the mouse. Brain Res 4:473–479
26. Friese A, Kaltschmidt JA, Ladle DR, Sigrist M, Jessell TM, Arber S (2009) Gamma and alpha motor neurons distinguished by expression of transcription factor Err3. Proc Natl Acad Sci USA 106:13588–13593
27. Eccles JC, Eccles RM, Iggo A, Lundberg A (1960) Electrophysiological studies on gamma motoneurones. Acta Physiol Scand 50:32–40
28. Burke RE, Strick PL, Kanda K, Kim CC, Walmsley B (1977) Anatomy of medial gastrocnemius and soleus motor nuclei in cat spinal cord. J Neurophysiol 40:667–680
29. Schiaffino S, Reggiani C (2011) Fiber types in mammalian skeletal muscles. Physiol Rev 91:1447–1531
30. Dean RL, Scozzafava J, Goas JA, Regan B, Beer B, Bartus RT (1981) Age-related differences in behavior across the life-span of the C57bl 6j mouse. Exp Aging Res 7:427–451
31. Benice TS, Rizk A, Kohama S, Pfankuch T, Raber J (2006) Sex-differences in age-related cognitive decline in C57BL/6J mice associated with increased brain microtubule-associated protein 2 and synaptophysin immunoreactivity. Neuroscience 137:413–423
32. Fahlstrom A, Yu Q, Ulfhake B (2011) Behavioral changes in aging female C57BL/6 mice. Neurobiol Aging 32:1868–1880
33. Kovacs AD, Pearce DA (2013) Location and sex specific differences in weight and motor coordination in two commonly used mouse strains. Sci Rep 3:2116
34. Ashworth A, Bardgett ME, Fowler J, Garber H, Griffith M, Curran CP (2015) Comparison of neurological function in males and females from two substrains of C57BL/6 mice. Toxics 3:1–17
35. Tucker LB, Fu AH, McCabe JT (2016) Performance of male and female C57BL/6J mice on motor and cognitive tasks commonly used in pre-clinical traumatic brain injury research. J Neurotrauma 33:880–894
36. Ober C, Loisel DA, Gilad Y (2008) Sex-specific genetic architecture of human disease. Nat Rev Genet 9:911–922
37. Meziane H, Ouagazzal AM, Aubert L, Wietrzych M, Krezel W (2007) Estrous cycle effects on behavior of C57BL/6J and BALB/cByJ female mice: implications for phenotyping strategies. Genes Brain Behav 6:192–200
38. Aguiar AS Jr, Speck AE, Amaral IM, Canas PM, Cunha RA (2018) The exercise sex gap and the impact of the estrous cycle on exercise performance in mice. Sci Rep 8:10742
39. O'Connor CA, Cernak I, Vink R (2003) Interaction between anesthesia, gender, and functional outcome task following diffuse traumatic brain injury in rats. J Neurotraum 20:533–541
40. Wagner AK, Willard LA, Kline AE, Wenger MK, Bolinger BD, Ren D, Zafonte RD, Dixon CE (2004) Evaluation of estrous cycle stage and gender on behavioral outcome after experimental traumatic brain injury. Brain Res 998:113–121
41. Monaco CM, Mattiola VV, Folweiler KA, Tay JK, Yelleswarapu NK, Curatolo LM, Matter AM, Cheng JP, Kline AE (2013) Environmental enrichment promotes robust functional and histological benefits in female rats after controlled cortical impact injury. Exp Neurol 247:410–418
42. Monti RJ, Roy RR, Edgerton VR (2001) Role of motor unit structure in defining function. Muscle Nerve 24:848–866
43. Sheth KA, Iyer CC, Wier CG, Crum AE, Bratasz A, Kolb SJ, Clark BC, Burghes AHM, Arnold WD (2018) Muscle strength and size are associated with motor unit connectivity in aged mice. Neurobiol Aging 67:128–136
44. Manuel M, Zytnicki D (2011) Alpha, beta and gamma motoneurons: functional diversity in the motor system's final pathway. J Integr Neurosci 10:243–276
45. Valdez G, Tapia JC, Kang H, Clemenson GD Jr, Gage FH, Lichtman JW, Sanes JR (2010) Attenuation of age-related changes in mouse neuromuscular synapses by caloric restriction and exercise. Proc Natl Acad Sci USA 107:14863–14868
46. Chai RJ, Vukovic J, Dunlop S, Grounds MD, Shavlakadze T (2011) Striking denervation of neuromuscular junctions without lumbar motoneuron loss in geriatric mouse muscle. PLoS ONE 6:e28090
47. Chung T, Park JS, Kim S, Montes N, Walston J, Hoke A (2017) Evidence for dying-back axonal degeneration in age-associated skeletal muscle decline. Muscle Nerve 55:894–901
48. Tomlinson BE, Irving D (1977) The numbers of limb motor neurons in the human lumbosacral cord throughout life. J Neurol Sci 34:213–219
49. Jacob JM (1998) Lumbar motor neuron size and number is affected by age in male F344 rats. Mech Ageing Dev 106:205–216
50. Maxwell N, Castro RW, Sutherland NM, Vaughan KL, Szarowicz MD, de Cabo R, Mattison JA, Valdez G (2018) α-Motor neurons are spared from aging while their synaptic inputs degenerate in monkeys and mice. Aging cell 17:e12726
51. Kovačič U, Sketelj J, Bajrović FF (2009) Sex-related differences in recovery of cutaneous nociception after end-to-side nerve repair in the rat. J Plast Reconstr Aesthet Surg 62:806–813
52. White Z, Terrill J, White RB, McMahon C, Sheard P, Grounds MD, Shavlakadze T (2016) Voluntary resistance wheel exercise from mid-life prevents sarcopenia and increases markers of mitochondrial function and autophagy in muscles of old male and female C57BL/6J mice. Skelet Muscle 6:45
53. Wasco W, Gurubhagavatula S, Paradis MD, Romano DM, Sisodia SS, Hyman BT, Neve RL, Tanzi RE (1993) Isolation and characterization of APLP2 encoding a homologue of the Alzheimer's associated amyloid b protein precursor. Nat Genet 5:95–100
54. Weyer SW, Klevanski M, Delekate A, Voikar V, Aydin D, Hick M, Filippov M, Drost N, Schaller KL, Saar M, Vogt MA, Gass P, Samanta A, Jaschke A, Korte M, Wolfer DP, Caldwell JH, Muller UC (2011) APP and APLP2 are essential at PNS and CNS synapses for transmission, spatial learning and LTP. EMBO J 30:2266–2280
55. Lassek M, Weingarten J, Einsfelder U, Brendel P, Muller U, Volknandt W (2013) Amyloid precursor proteins are constituents of the presynaptic active zone. J Neurochem 127:48–56
56. Korte M, Herrmann U, Zhang X, Draguhn A (2012) The role of APP and APLP for synaptic transmission, plasticity, and network function: lessons from genetic mouse models. Exp Brain Res 217:435–440
57. Zhang X, Herrmann U, Weyer SW, Both M, Muller UC, Korte M, Draguhn A (2013) Hippocampal network oscillations in APP/APLP2-deficient mice. PLoS ONE 8:e61198

58. Akaaboune M, Allinquant B, Farza H, Roy K, Magoul R, Fiszman M, Festoff BW, Hantai D (2000) Developmental regulation of amyloid precursor protein at the neuromuscular junction in mouse skeletal muscle. Mol Cell Neurosci 15:355–367
59. Wang P, Yang G, Mosier DR, Chang P, Zaidi T, Gong YD, Zhao NM, Dominguez B, Lee KF, Gan WB, Zheng H (2005) Defective neuromuscular synapses in mice lacking amyloid precursor protein (APP) and APP-Like protein 2. J Neurosci 25:1219–1225
60. Deschenes MR, Roby MA, Eason MK, Harris MB (2010) Remodeling of the neuromuscular junction precedes sarcopenia related alterations in myofibers. Exp Gerontol 45:389–393
61. Mantilla CB, Sieck GC (2011) Age-related remodeling of neuromuscular junctions. In: Sarcopenia age related muscle wasting and weakness. Springer, New York pp 37–54
62. Arnold A-S, Gill J, Christe M, Ruiz R, McGuirk S, St-Pierre J, Tabares L, Handschin C (2014) Morphological and functional remodelling of the neuromuscular junction by skeletal muscle PGC-1α. Nat Commun 5:3569
63. Li Y, Lee YI, Thompson WJ (2011) Changes in aging mouse neuromuscular junctions are explained by degeneration and regeneration of muscle fiber segments at the synapse. J Neurosci 31:14910–14919
64. Hepple RT, Rice CL (2016) Innervation and neuromuscular control in ageing skeletal muscle. J Physiol 594:1965–1978
65. Muller U, Cristina N, Li ZW, Wolfer DP, Lipp HP, Rulicke T, Brandner S, Aguzzi A, Weissmann C (1994) Behavioral and anatomical deficits in mice homozygous for a modified beta-amyloid precursor protein gene. Cell 79:755–765
66. Ludewig S, Korte M (2017) Novel insights into the physiological function of the APP (gene) family and its proteolytic fragments in synaptic plasticity. Front Mol Neurosci 9:161