The effect of age and exercise on the proprioceptive and vestibular system

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1 Summary

Aging is a physiological process associated with decreased mental abilities but also declining muscle function, posture and balance (Camicioli, Panzer, & Kaye, 1997; Charlier, Mertens, Lefevre, & Thomis, 2015; Lord & Ward, 1994; Murman, 2015). Consequently, every third elderly person above 60 falls at least once per year, greatly affecting quality of life and independence (Fuller, 2000). Body posture, gait and balance can be improved by physical activity (Cadore, Rodríguez-Mañas, Sinclair, & Izquierdo, 2013; Gauchard, Gangloff, Jeandel, & Perrin, 2003; King et al., 2002; Lelard & Ahmaidi, 2015; Ruffieux, Mouthon, Keller, Walchli, & Taube, 2017). However, it is not at all understood why and how exercise improves balance function because the neuropathological mechanisms underlying age-associated balance disorders are not well understood.

Two main sensory system complement each other in guiding proper balance: The vestibular system, residing in the inner ear, monitors gravitational forces while the proprioceptive system tracks the velocity and force of the muscle movement. Motor neurons, responsible for initiating muscle contractions, receive monosynaptic feedback from both sensory system. Thus, direct and functional vestibular and proprioceptive feedback to motor neurons are indispensable for proper balance (Angelaki & Cullen, 2008; Proske & Gandevia, 2012). It is already well understood that the neuromuscular system experiences age-associated changes affecting muscle mass and force and that exercise is beneficial to counteract aging (Pearson et al., 2002; Valdez et al., 2010; Verdijk et al., 2009; White et al., 2016). However, a comprehensive overview about synaptic connectivity between motor neurons and vestibular and proprioceptive system

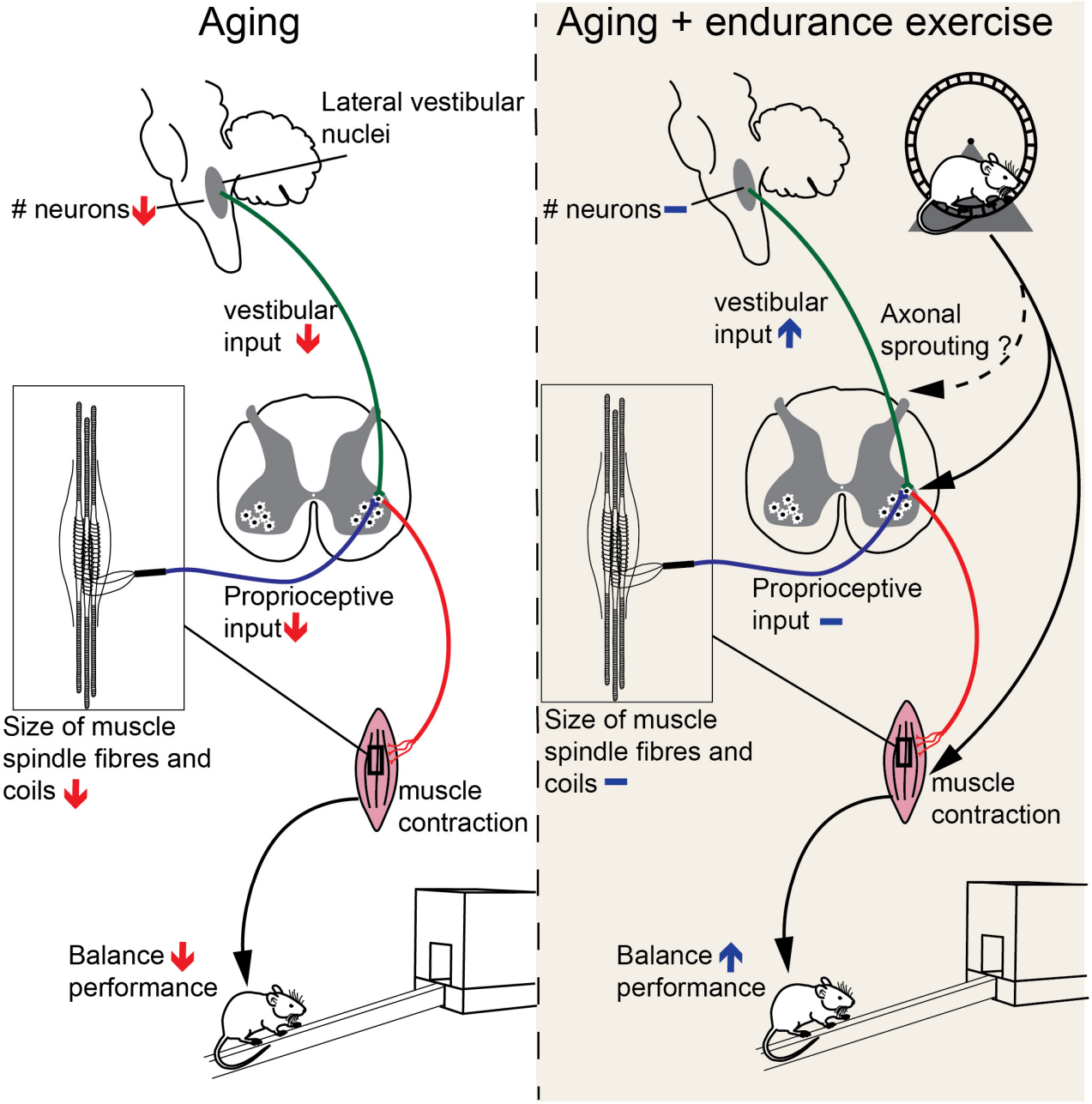
in aging is so far lacking. Furthermore, the effects of exercise on vestibular and proprioceptive spinal circuits in aging are also unknown.

Here, we used anterograde and retrograde neuronal and synaptic tracing approaches combined with balance and gait phenotypic assessment of aged exercised mice to relate declining balance to alterations in the morphology and synaptic networks of the proprioceptive and vestibular system. To that end, mice of different ages were trained for 6 to 12 weeks on treadmill and running wheels.

In **Fig. 1** the main findings of the study are graphically summarized.

As expected from epidemiological studies done in humans, balance and gait of aged exercised mice was superior to aged sedentary mice, for some parameters statistically indistinguishable from 7-month-old control mice. These results show that exercise done late in life is sufficient to substantially improve balance and gait in aged mice. Interestingly, loss of balance with age was accompanied by morphological changes on the level of muscle spindles concomitant with decreased proprioceptive input to motor neurons. However, we did not observe any improvement in muscle spindle morphology or proprioceptive input to motor neurons with training, showing that exercise likely does not modulate the proprioceptive system. Since also the vestibular system is crucial for maintaining balance, we next asked if the improvements in gait and balance in response to exercise could be mediated by the vestibular system. Interestingly, vestibular input to motor neurons in aged mice was substantially decreased even more than the proprioceptive input suggesting that decreased vestibular signalling with age could be the

Figure 1: Summary of the main findings



main driver for age-associated loss of balance. Strikingly, vestibular input to motor neurons in aged exercised mice was significantly higher than in aged sedentary mice, strongly indicating that balance improvement in response to exercise is due to increased vestibular input to motor neurons.

The mechanism of increased vestibular synapses on the level of motor neurons due to exercise is unknown, but could involve neurotrophic-factor-induced axonal sprouting.

Interestingly, exercise improves recovery from spinal cord injury by promoting axonal sprouting and synapse formation and elevates neurotrophic factors, able to induce synapses formation and axonal sprouting, in the spinal cord (English, Wilhelm, & Ward, 2014; Gomez-Pinilla, Ying, Opazo, Roy, & Edgerton, 2001; Houle & Cote, 2013; Molteni, Zheng, Ying, Gomez-Pinilla, & Twiss, 2004; Sakuma & Yamaguchi, 2011).

Therefore, we propose that exercise elevates synaptic vestibular input to motor neurons by releasing neurotrophic factors promoting axonal sprouting and synapse formation in the spinal cord, which ameliorates loss of balance in aged mice.

2 Abbreviations

AAV-Syn-tag, AAV-synaptophysin-GFP-tag

ALS, amyotrophic lateral sclerosis

BDNF, brain-derived neurotrophic factor

BPPV, benign paroxysmal positional vertigo

BrdU, bromodeoxyuridine

CMT, Charcot-Marie-Tooth

CNTF, ciliary neurotrophic factor

CSA, cross-sectional are

DRG, dorsal root ganglia

E rB2, epidermal growth factor

EDL, extensor digitorum longus

Egr3, Early growth response 3

EPSP, excitatory postsynaptic potentials

GDNF, glial cell-derived neurotrophic factor

GS, gastrocnemius

LVe, lateral vestibular nuclei

MHC, myosin heavy chain

NeuN, neuronal Marker

NFH, neurofilament H

Ngr1, neuregulin 1

NMJ, neuromuscular junction

NT, neurotrophin

PGC-1α, peroxisome proliferator-activated receptor-γ coactivator

PV, parvalbumin

Runx3, Runt-related genes 3

SMA, spinal muscular atrophy

TA, tibialis anterior (TA)

Trk, tyrosine kinase receptor

VAChT, vesicular acetylcholine transporter

Ve, vestibular

vGlut, vesicular glutamate transporter

3 Introduction

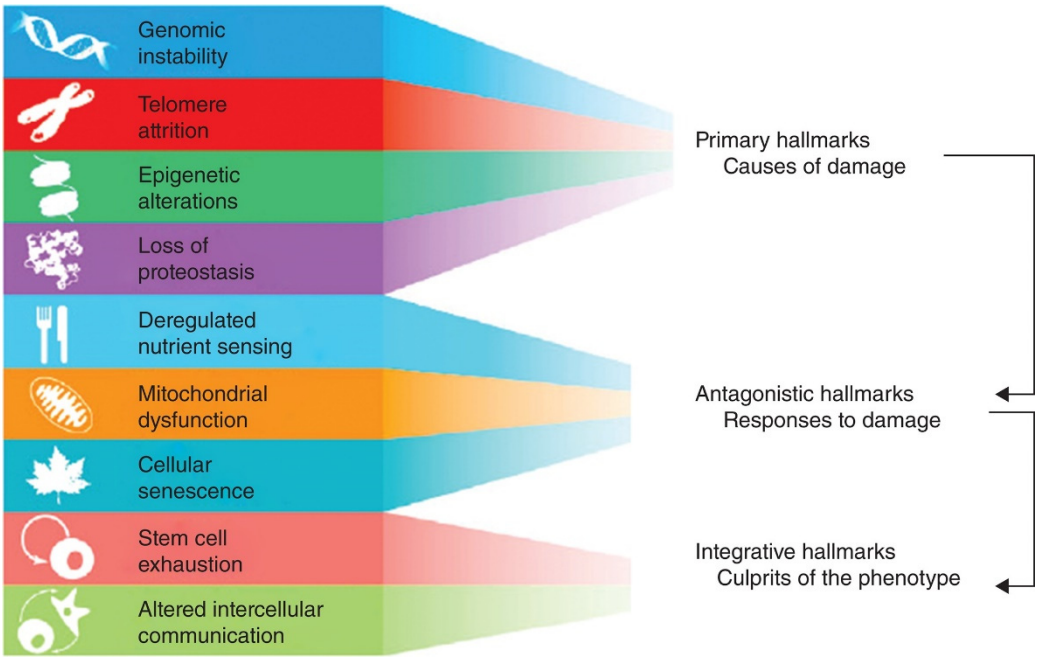
3.1 Hallmarks of aging

Aging can be defined as a gradual degeneration of function, which increases mortality after maturation. As such, many do not consider it as a diseased state but as natural process (Callahan, Topinkova, & Aging, 1998; Hayflick, 2007). However, old age increases the vulnerability to other diseases such as Alzheimer's and Parkinson's (Burns & Zaudig, 2002; Collier, Kanaan, & Kordower, 2011). Considering that the age population above 60 is the fastest growing population (World population aging 2015, United Nations), studying age-related disease will become vitally important.

3.1.1 Molecular mechanisms of aging

Why does the cell age? This question has so far not been fully answered. Nevertheless, **Fig. 2** summarizes the most prominent factors involved in the aetiology of aging. On a

Fig. 2: The molecular mechanisms of aging



(Aunan, Watson, Hagland, & Soreide, 2016)

cellular level, the aging cell accumulates damage on the DNA and epigenetic alterations

occur more frequently, leading, among other factors, to increased DNA instability. In addition, telomere length shortening has also been linked to decreased life span in yeast (Pusceddu et al., 2015; Xie et al., 2015). With increasing age, also mitochondrial DNA becomes more unstable, leading to damaged mitochondria and increased release of reactive oxygen species, exacerbating DNA and protein instability. Ultimately, the accumulated damage on DNA, proteins and membrane lipids leads to the loss of the cell's function and to a senescent state of the cell in which it can no longer divided. Stem cell exhaustion and altered intercellular communication in turn lead to a loss of regenerative capacity of organs further exacerbating loss of function (Aunan et al., 2016). Cause and consequence in the molecular aging mechanism remains enigmatic. Moreover, it is not understood why some organs are more susceptible to aging than others are.

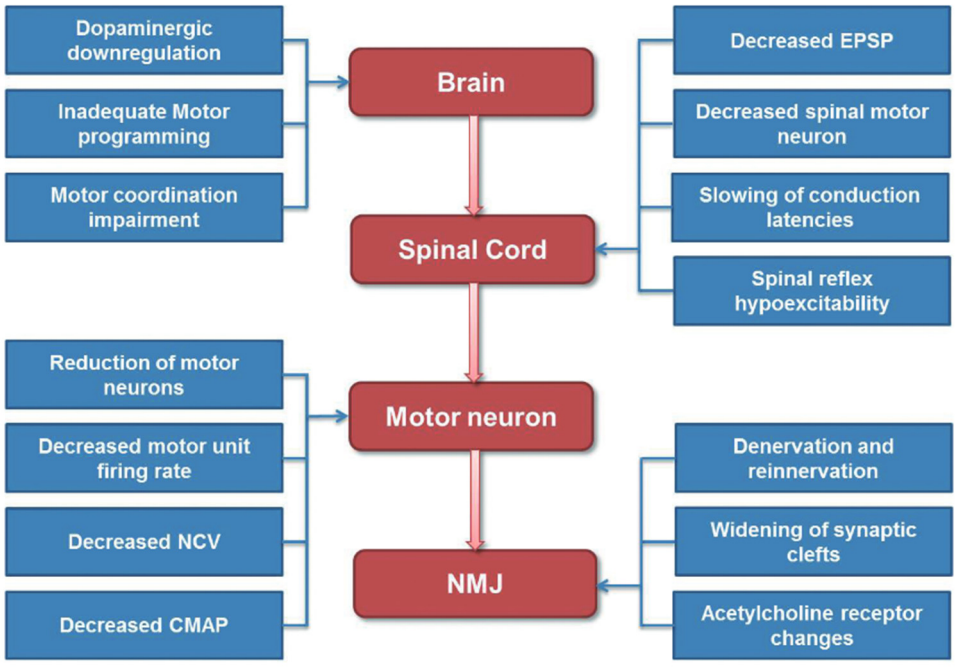
3.1.2 Aging of the nervous system

Overall, the whole nervous system experiences deterioration of neurons, glia and synaptic remodelling, impacting on important physiological functions. However, it seems that not all brain regions age at a same rate (Coleman & Flood, 1987). As we age, the brain volume decreases by 5% every decade (Svennerholm, Bostrom, & Jungbjer, 1997) and further drops after the age of 70 years of age (Scahill et al., 2003). Concomitant with decreased brain volume, cognitive functions, memory and learning ability decline (Li & Lindenberger, 2002; Murman, 2015; Peters, 2006). Indeed, decreased dopamine levels in the aging brain have been implicated in decreased mental and motor abilities (Nora D. Volkow et al., 1998). **Fig. 3** summarizes age-associated functional deteriorations in regions within the CNS and the neuromuscular system. In addition to declining brain volume, aging leads to a loss of motor neurons in the spinal cord (Cruz-Sanchez, Moral, Tolosa, de Belleroche, & Rossi, 1998; Tomlinson & Irving, 1977) and to a decrease in

the amplitude of excitatory postsynaptic potentials (EPSP) in spinal motor neurons in aging humans (Eisen, Entezari-Taher, & Stewart, 1996). Correlating, the number of myelinated fibres in the corticospinal tract have been shown to progressively decrease with age (Terao, Sobue, Hashizume, Shimada, & Mitsuma, 1994), further indicating a loss in conduction velocity. However, studies done in rodents suggest that the loss of motor neurons is comparatively small compared to the observed changes on the synaptic distribution on the dendritic tree. While no loss of motor neurons or size of motor neurons was observed in aged mice and monkeys, vesicular glutamate transporter (vGlut) 1 and vesicular acetylcholine transporter (VAChT) synapses on motor neurons were decreased (Maxwell et al., 2018).

Moreover, motor units also experience age-associated remodelling (Roos, Rice, &

Fig. 3: Aging of the nervous system



(Kwon & Yoon, 2017)

Vandervoort, 1997). While the overall number of motor units decrease with age, the size of individual motor units increase with age (S. M. Ling, Conwit, Ferrucci, & Metter,

2009). Increased motor unit size could represent a compensatory response in order to cope with loss of force in aging muscle (see Chapter 3.1.3 Sarcopenia). Aging is also associated with a decreased motor and sensory conduction velocity (Dorfman & Bosley, 1979), which is probably linked to the loss of myelination in motor and sensory axons (Verdu, Ceballos, Vilches, & Navarro, 2000).

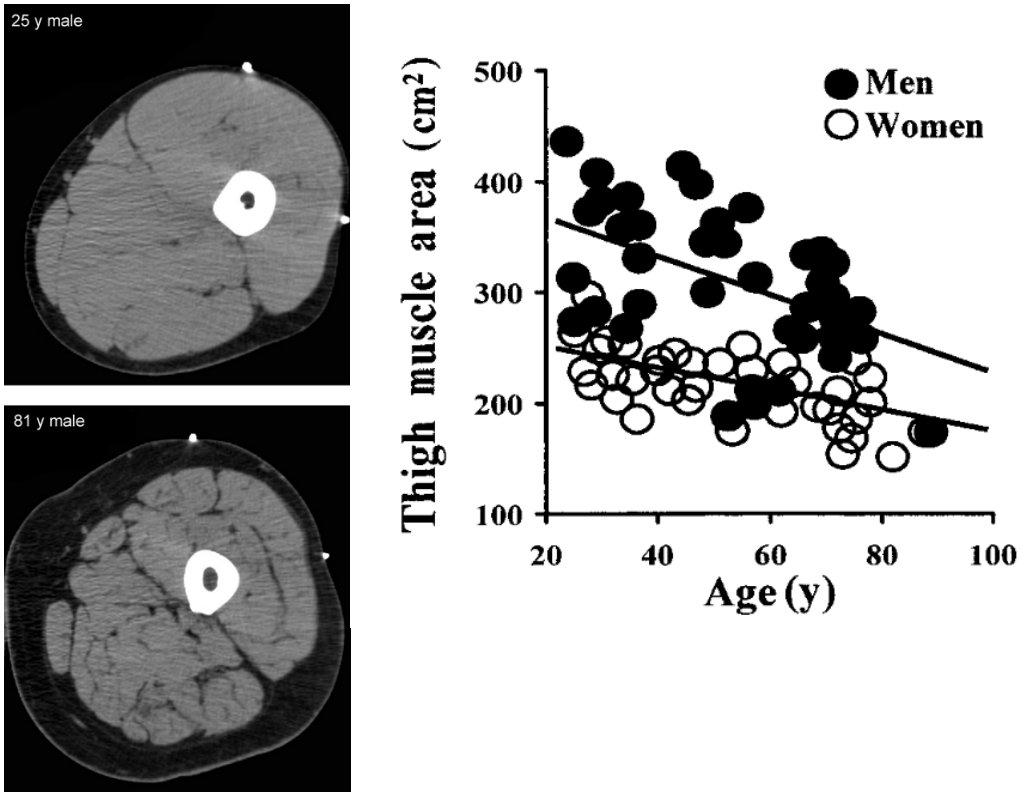
In addition to altered conduction velocity, studies done in animal models indicate that the neuromuscular junction (NMJ), the synapses between motor neuron endplate and α-motor neuron, experiences morphological changes with age, including increased presynaptic branching, postsynaptic NMJ fragmentation, a reduction in the coupling of the presynaptic vesicles and postsynaptic receptors and denervation and subsequent reinnervation (Valdez et al., 2010). Thus, it is believed that the aging NMJ becomes less stable with increasing age, which leads to impaired neuromuscular transmission (Hunter, Pereira, & Keenan, 2016; Jang & Van Remmen, 2011). However, questions have been raised if the aging rodent NMJ is an adequate model to represent the human NMJ. A recent report showed that the human NMJ is substantially smaller and more fragmented than the mouse NMJ. Furthermore, as opposed to the mouse NMJ the human one did not show any morphological remodelling with age (Jones et al., 2017).

Nevertheless, morphological changes on the level of the neuromuscular system, such as remodelling of the motor unit size, and decreased conduction velocity represent a common aging mechanism between rodent and humans. These changes with age are most probable tightly linked to the development of sarcopenia.

3.1.3 Sarcopenia

Sarcopenia was defined as the age-related loss of muscle mass when it has been first coined by Irwin H. Rosenberg 20 years ago (Rosenberg, 1997). Since then the term sarcopenia has been expanded to also refer to the combined loss of muscle mass and function (Cooper et al., 2012). As illustrated in **Fig. 4**, elderly humans progressively loose muscle thigh area (Janssen, Heymsfield, Wang, & Ross, 2000) and muscle mass (Charlier et al., 2015) with age. However, the loss of muscle function is suggested to be greater than the actual loss of muscle mass (Narici & Maffulli, 2010; Young, Stokes, & Crowe, 1985), highlighting that deterioration of muscle mass and function do not seem to be proportional. The prevalence of sarcopenia has been reported to be 13-24% in persons

Fig. 4: Loss of muscle mass with age



Adapted from (Koopman & van Loon, 2009) and (Nair, 2005)

between 60 to 70 years of age and in people aged over 80 years, the prevalence increases to over 50% (Baumgartner et al., 1998). However, considering that the age group above

60 is the fastest growing population (World population aging 2015, United Nations) these numbers probably are increasing.

In addition to decreased size, the aging muscle experiences changes on a morphological, molecular, and functional level. Overall, there is a loss of muscle fibres and decreased fibre size as well as a fibre type grouping (Lexell, Henriksson-Larsen, Winblad, & Sjostrom, 1983) with a preferential loss of fast-twitch type II fibres in rodents (Caccia, Harris, & Johnson, 1979) and humans (Lexell, 1995), leading to decreased force and oxidative capacity (M. R. Deschenes, 2004). The underlying changes in metabolism are many fold. They include the deregulation of muscle proteostasis and nutrient sensing as well as lower ATP synthesis in the mitochondria (Ghosh et al., 2011; Short et al., 2005). In particular, a reduced mitochondrial enzyme activity (Rooyackers, Adey, Ades, & Nair, 1996) as well as increased release of reactive oxygen species (Bejma & Ji, 1999) exacerbate loss of muscle function (Fulle et al., 2004). Moreover, the loss of satellite cells proliferation and activation and thus the loss of regenerative potential lead to a net loss of muscle mass (Conboy & Rando, 2005). It is still unclear if the loss of muscle mass is the cause or consequence of altered NMJ morphology, motor unit size and the denervation of NMJs. However, some studies done in rodents detected early neurological changes before the onset of muscle mass loss (Tamaki, Hirata, & Uchiyama, 2014; Valdez et al., 2010). Therefore, these findings would suggest that denervation and subsequent reinnervation could lead to the depletion of satellite cells and decreased regenerative capacity resulting in a net loss of muscle mass. Conversely, reduced mitochondrial activity and increased release of ROS could further exacerbate muscle damage.

3.1.4 Exercise as a therapeutic intervention

The ability of the muscle to contract is at the core of every movement. Skeletal muscle fragility due to aging impinges greatly on the quality of life in affected people and on health life span (Sayer et al., 2008). Therefore, it is crucial to maintain healthy muscle function throughout life.

One powerful way to maintain and extend health life span represents physical activity (Harridge & Lazarus, 2017). It was reported that resistance exercise could slow down the rate of strength decay in men (Pearson et al., 2002) and women (Caserotti, Aagaard, Larsen, & Puggaard, 2008). In addition, 12 weeks of resistance training improved muscle mass and increased satellite cell activation in elderly males (Verdijk et al., 2009).

On the other hand, 8 weeks endurance training improved ATP synthesis rate and increased mitochondrial enzyme activity in elderly people (Ghosh et al., 2011). Old rats with life-long access to running wheels exhibited higher muscle fibre cross-sectional are (CSA) and decreased amount of infiltrated connective tissue in-between muscle fibres than sedentary aged-matched controls. Consistently, in mice, 8 weeks of running wheel access ameliorated the age-associated fibre type shift (Graber, Ferguson-Stegall, Liu, & Thompson, 2015). In addition, one month of running wheel access in old mice improved NMJ morphology compared to sedentary mice (Valdez et al., 2010). Strikingly, endurance exercise did not only prevent NMJ degeneration but it also partially restored alterations on the level of the NMJ that had already occurred (Valdez et al., 2010). These studies highlight the high potential for endurance and resistance exercise in ameliorating sarcopenia.

However, aside from its effect on the muscle, exercise also acts in a systemic way. It is known that the contracting muscle can release myokines, such as irisin, and the neurotrophin brain-derived neurotrophic factor (BDNF) (see 3.1.5 Neurotrophic factors and aging) exerting many different effects (Schnyder & Handschin, 2015). Irisin has been shown to lead to adipose tissue browning and increased thermogenesis (Bostrom et al., 2012). Furthermore, BDNF levels are increased in the hippocampus after two days of running wheels access (Oliff, Berchtold, Isackson, & Cotman, 1998). Strikingly, long-term running wheel access in aged mice increased neurogenesis in the hippocampus and improved memory. Concomitantly BDNF was higher in aged exercised mice than sedentary mice, suggesting that higher BDNF level due to exercise improved memory function (van Praag, Shubert, Zhao, & Gage, 2005).

In sum, exercise is a powerful treatment strategy to counteract aging. However, it is highly depending on the patient's compliance. Especially elderly people tend to exercise less (Heath & Stuart, 2002; Hughes, Salmon, Galvin, Casey, & Clifford, 2018) exacerbating progression of the aging process (Booth, Laye, & Roberts, 2011; Dogra & Stathokostas, 2012). Thus, there is a need to study the effect of exercise on the aging body to implement targeted pharmacological interventions.

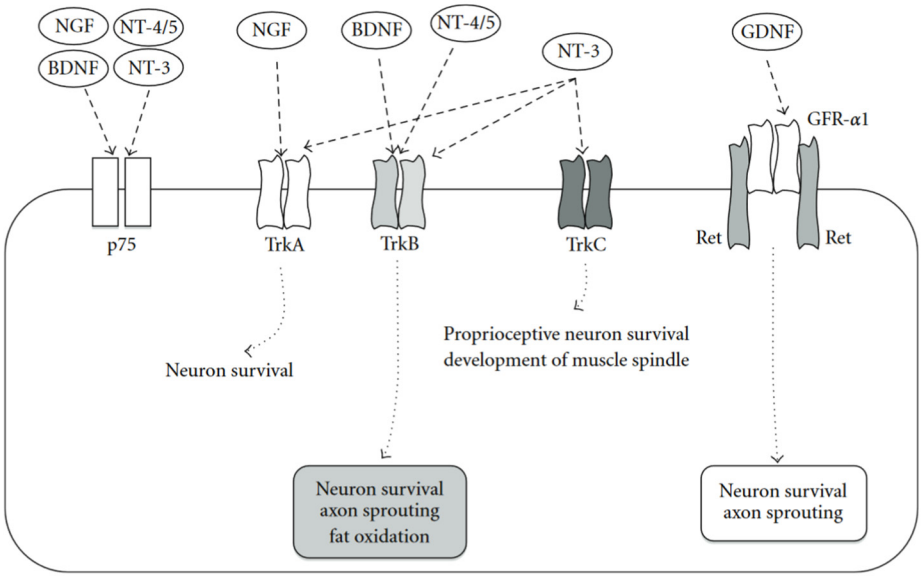
3.1.5 Neurotrophic factors and aging

The family of neurotrophins include BDNF, neurotrophin (NT) 3 and 4. They are needed for neuronal development and survival, as well as synaptic plasticity and promote neuronal survival by binding to tyrosine kinase receptor (Trk) A, B and C (Huang & Reichardt, 2001). In addition, there are other neurotrophic factors, like glia-derived growth factor (GDNF) and ciliary neurotrophic factor (CNTF), which promote axonal survival (Sakuma & Yamaguchi, 2011). **Fig. 5** shows and overview of the different

neurotrophic factor ligands and their receptors. Because these factors are crucial for neuronal and axonal development as well as for neuronal survival, it is not surprising that neurotrophic signalling in the neuromuscular system is deregulated in old age. In aged rodents, BDNF, NT3 and NT4 are downregulated (Y. Ming, Bergman, Edstrom, & Ulfhake, 1999b), while GDNF is upregulated in muscle (Y. Ming, Bergman, Edstrom, & Ulfhake, 1999a). Consistently, levels of TrkA and C are also lower. However, in motor neurons, NT3, NT4 and GDNF are upregulated while BDNF and CNTF are downregulated. Moreover, in the sciatic nerve BDNF, NT3, NT4, GDNF and CNTF are increased in aged animals compared to young (Guillet, Auguste, Mayo, Kreher, & Gascan, 1999; Ulfhake et al., 2000). Consistent with these findings, whole body knock out of NT4 and reduced levels of TrkB in mice lead to a fragmented and instable NMJ reminiscent of the aging NMJ (Kulakowski, Parker, & Personius, 2011). These findings suggest that deregulated neurotrophic factors likely contribute the progression of sarcopenia. However, it is unclear if altered neurotrophic signalling with age is the cause or consequence of motor axon denervation and reinnervation. Interestingly, increased levels of BDNF (Cuppini et al., 2007), NT4 (Funakoshi et al., 1995) and GDNF (McCullough, Peplinski, Kinnell, & Spitsbergen, 2011) in the muscle have been suggested to be promoted by electrical activity and exercise. Moreover, neurotrophic factors have been implicated in regeneration of muscle fibres (Sakuma & Yamaguchi, 2011), suggesting that exercise could ameliorate sarcopenia by enhancing neurotrophic factor signalling.

In sum, the central, peripheral and the neuromuscular nervous system show signs of age-related degeneration that translate to a functional decline. Exercise can ameliorate loss of muscle strength and can improve oxidative capacity. Moreover, neuromuscular activity could release neurotrophic factors that have been suggested to be beneficial to counteract age-associated degeneration of muscle fibres and NMJ integrity.

Fig. 5: Neurotrophic factors and their receptors



(Sakuma & Yamaguchi, 2011)

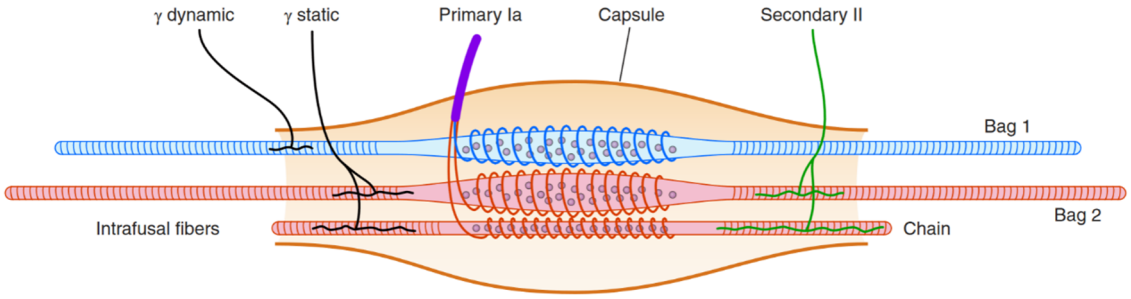
3.2 The proprioceptive system

Aside from a loss in muscle function, elderly humans also experience a declining sense of balance and proprioception (Lei & Wang, 2018; Lord & Ward, 1994). Proprioception is the inherent sense of the relative position of one's own limbs and their location in space. Thus, without visual cues, our senses are able to estimate the location of limbs in time and space and at what angle they are positioned (Kandel Eric R., 2012 Principles of Neural Science). Decreased proprioception in the elderly population is reflected in the very high incidence of falls in this age group (Fuller, 2000; Rubenstein, 2006). Statistically, more than 10 % of elderly people older than 75 years will fall within the next 6 months (Franse et al., 2017). Therefore, it is important to study the age-associated changes in the sensory systems, and their impact on decreased balance. In order to guide proper balance, sensory information from vision, touch, proprioception as well as vestibular system are integrated (see 3.3 The vestibular system) (Bacsi & Colebatch, 2005; Sturnieks, George, & Lord, 2008).

3.2.1 The proprioceptive receptors

The term proprioception has first been coined by Charles Sherrington, the same ingenious biologist who also defined the term synapse (Sherrington, 1907). The proprioceptive system consist of the muscle spindle receptor, located in the muscle, the Golgi tendon

Fig. 6: the structure of the muscle spindle



(Proske & Gandevia, 2012).

Fig. 7: Axon Classification, Axon Diameter, Receptor Types, and Function

Sensory and Motor Fibers[b]	Sensory Fibers[c]	Diameter (nm)	End Organ/Receptor	Function
A-alpha	Ia	10–20	M: extrafusal fibers	Muscle contraction
			S: nuclear bag and chain intrasfusal fibers	Detect changes in the length and velocity of muscle stretch
	Ib	10–20	S: GTO	Detect muscle tension
			S: GTO ligament receptors	Detect tension in ligaments
A-beta	II	4–12	S: nuclear bag 2 and chain fibers	Detect changes in length of muscle stretch
			S: Meissner's corpuscle (skin)	Vibration and discriminative touch
			S: pacinian corpuscle (skin)	Vibration and discriminative touch
			S: Merkel disk (skin)	Pressure on the skin
			S: Ruffini's endings (skin)	Skin stretch
			S: Ruffini's joint receptor	Extremes of range of motion and more to passive than active motion
			S: pacinian joint receptor	Joint range of motion
A-gamma		2–8	M: dynamic-nuclear bag 1 fibers	Muscle spindle alignment
			M: static-nuclear bag 2 and chain fibers	Muscle spindle alignment
A-delta	III	1–5	S: free nerve endings (skin and joints)	Crude touch, pain, temperature
C	IV	<1	S: free nerve endings (skin and joints)	Detect pain, temperature

[a] M=motor branch, S=sensory branch, GTO=golgi tendon organs.
[b] Erlanger J, Gasser HS. *Electrical Signs of Nervous Activity*. Philadelphia, Pa: University of Pennsylvania Press; 1937.
[c] Lloyd D. Neuro patterns controlling transmission of ipsilateral hindlimb reflexes in cat. *J Neurophysiol*. 1943;6:293–315.

(Shaffer and Harrison 2007)

organs within the tendon, as well as specialized joint and skin receptors. The muscle spindle is composed of an encapsulated structure containing specialized muscle spindle fibres, also termed intrafusal fibres. Intrafusal fibres run in parallel to and in-between extrafusal fibres (Proske & Gandevia, 2012). However, intrafusal fibres are about ten times smaller than extrafusal muscle fibres and express other myosin heavy chain (MHC) isoforms such as slow tonic and α cardiac-like MHC (Soukup, Pedrosa-Domellof, & Thornell, 1995). There are three types of intrafusal fibres, which were classified according to their appearance and function. Nuclear bag_1 fibres are innervated by dynamic γ motor axons, while nuclear bag_2 fibres and nuclear chain fibres are innervated by static γ motor axons as well as by Type II secondary sensory axons terminating on muscle spindle poles.

The central region of the muscle spindle, nuclear chain fibres and nuclear bag_2 fibres are innervated by proprioceptive sensory neurons, also termed primary Ia endings. The peripheral projecting sensory axon is wrapped in a regular manner around intrafusal fibres and forms intrafusal fibres sensory coils **(Fig. 6)** (Proske & Gandevia, 2012). γ-motor axons form cholinergic synapses at the pole of intrafusal fibres (Zhang, Wesolowski, Karakatsani, Witzemann, & Kroger, 2014). On the other hand, both the centrally projecting and peripheral projecting branch of the Ia sensory axon form vGlut1 positive synapses. Thus, vGlut1 positive synapses in contact with motor neurons is used as a marker for proprioceptive synapses (Basaldella, Takeoka, Sigrist, & Arber, 2015; de Nooij, Doobar, & Jessell, 2013; Wu et al., 2004)

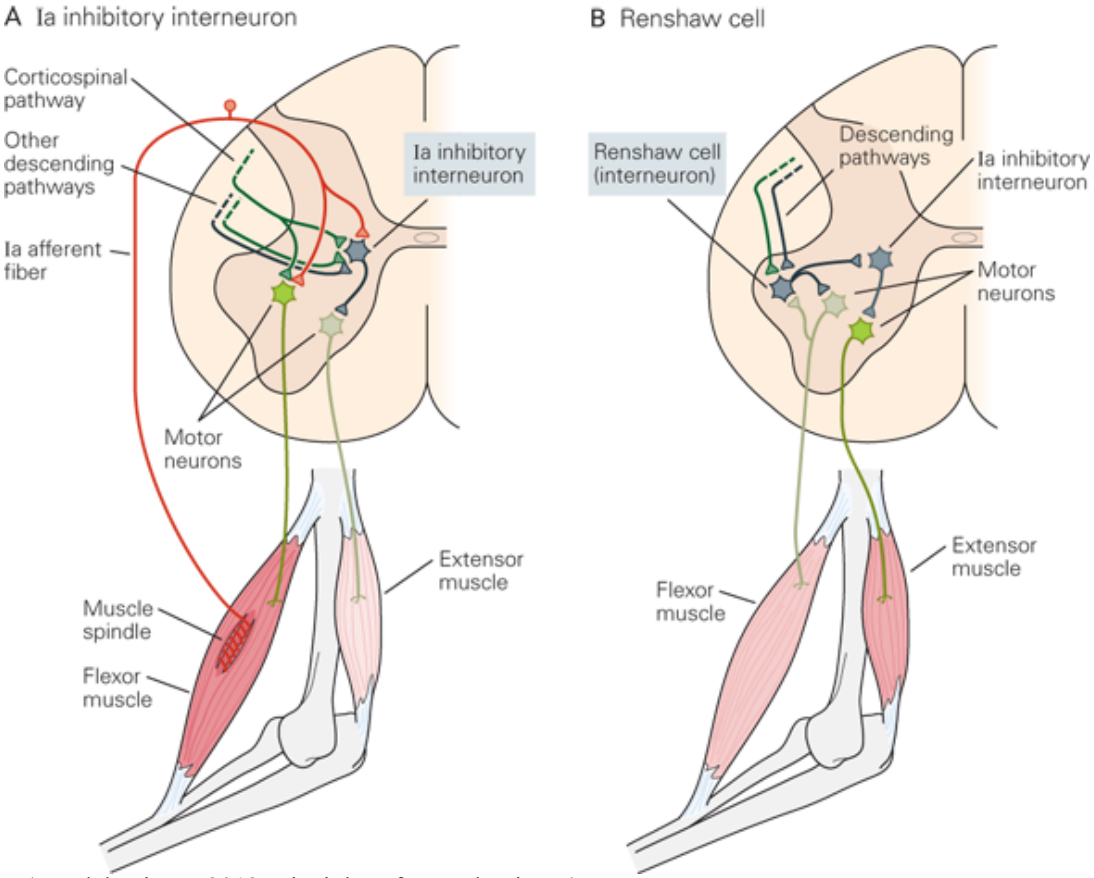
The Golgi tendon organ in the myo-tendinous junction is also encapsulated and innervated by primary type Ib sensory axons (Jami, 1992). **Fig. 7** shows an overview of the different sensory axons and their classification and functions. While the Golgi tendon organ, monitoring muscle tension, fire when the muscle is contracted and relaxed, the muscle spindle receptor is mainly responsive to stretch. Thus, muscle spindle monitor muscle length over time while the Golgi tendon organ sense the strength of the muscle contraction (Shaffer & Harrison, 2007). How this mechanotransduction is achieved is still not completely understood. It is believed that the stretch or the contraction of the muscle activates mechano-sensitive ion channels in the sensory axons of type Ia and Ib endings, leading to the generation of an action potential that is propagated directly to motor neurons (see 3.2.2 The proprioceptive spinal circuits) (Bewick & Banks, 2015; Bewick, Reid, Richardson, & Banks, 2005). In line with this theory, the principal component of the mechanotransduction channel in proprioception has been proposed to be Pizeo2, a nonselective cation channel. Mice deficient in Piezo2 specifically in proprioceptive

sensory neurons displayed abnormal movements and ataxia. Concomitantly, stretch-induced firing of proprioceptive sensory neurons in muscle nerve recordings was markedly reduced in Piezo2 KO mice, suggesting that Piezo2 mitigates mechanotransduction in proprioceptors (Woo et al., 2015).

3.2.2 The proprioceptive spinal circuits

Sensory neurons have no dendrites but one bifurcating axons, one projecting to the periphery, in this case the proprioceptors, and one projecting centrally to connect to other neurons, such as motor neurons in this example. The sensory neuron cell bodies are

Fig. 8: The spinal circuits involved in proprioception



(Kandel Eric R., 2012 Principles of Neural Science)

located in dorsal root ganglia (DRG) that are part of the spinal nerve. The main tasks of the proprioceptive sensory is to relay sensory information from the muscle directly to motor neurons, ensuring that adequate muscle tone and length is maintained at all times.

The most prominent example of proprioceptive reflexes is the stretch reflex, which is activated when the muscle is stretched, leading to firing of the muscle spindle (Kandel Eric R., 2012 Principles of Neural Science). Type Ia sensory axons project directly to motor neurons of the homonymous muscle but also to type Ia inhibitory interneurons, located near to motor neurons. Inhibitory Ia interneurons connect to motor neurons of the antagonistic muscle, thereby inhibiting motor neuron firing (**Fig. 8)** (Windhorst, 2007). Thus, activity of the muscle spindle in response to muscle stretch leads to contraction of the same muscle while the antagonistic muscle is relaxed, enabling targeted muscle activation (Windhorst, 2007). The most famous example of the stretch reflex activity is the knee jerk reaction. Tapping with a hammer on the patella tendon stretches the quadriceps muscle, activating muscle spindle firing. Consequently, motor neurons of the quadriceps muscle fire, leading to contraction of the quadriceps muscle and extension of the leg (Kandel Eric R., 2012 Principles of Neural Science).

When the muscle contracts, sensory information from the Golgi tendon organ is processed via type Ib interneurons (Jami, 1992). At the same time as α- motor neurons, γ-motor neurons are also activated, leading to contraction of intrafusal fibres, a process that is termed α-γ-coactivation (Macefield & Knellwolf, 2018; Vallbo, 1971). The main function of γ-motor neurons, as far as is known, is to contract intrafusal fibres to ensure that they do not slacken. Therefore, γ-motor neurons regulate the sensitivity or the gain of muscle spindle firing and are important to modulate spinal circuits (Dimitriou, 2014; Macefield & Knellwolf, 2018). γ-motor neurons are considerably smaller than α-motor neurons (Moschovakis, Burke, & Fyffe, 1991). However, compared to the wealth of knowledge available about α-motor neuron, little is known about γ-motor neurons, partly because of the lack of specific markers (Kanning, Kaplan, & Henderson, 2010). Nevertheless, it has

been suggested that these two motor neuron subtypes could be transcriptionally distinguished. It has been found that γ-motor neurons express the transcription factor Err3 and are negative for neuronal marker (NeuN) (Friese et al., 2009). Knowing about transcriptional identity of γ-motor neurons will enable us to specifically ablate this neuronal population to find out more about the function of γ-motor neurons.

To add another layer of complexity to spinal circuits, Renshaw cells, inhibitory interneurons, project to Ia inhibitory interneurons. In turn, Renshaw cells receive input from, type II sensory neuron, from muscle spindles, descending pathways from the corticospinal tract, as well as excitatory input from homonymous α-motor neurons (Rosales & Dressler, 2010; Windhorst, 2007). Moreover, other higher-level brain regions, such as the vestibular system, also project to interneurons (Murray, Croce, Belton, Akay, & Jessell, 2018). Therefore, the proprioceptive spinal circuits are not only needed for involuntary reflex actions but also for targeted movements, motor coordination and balance (Akay, Tourtellotte, Arber, & Jessell, 2014; Macefield & Knellwolf, 2018; Windhorst, 2007).

3.2.3 Development of the muscle spindle

Like extrafusal fibres, intrafusal fibres originate from primary and secondary myotubes. During development, some myotubes are contacted by proprioceptive sensory neurons, which upregulates transcription factors promoting muscle spindle development (Walro & Kucera, 1999). For intrafusal fibre development, innervation by the sensory neuron is absolutely necessary but innervation from γ-motor neurons seems to be dispensable (Kucera & Walro, 1992). Interestingly, intrafusal innervation of γ-motor axons has been shown to be dependent on GDNF signalling. Decreased amount of GDNF lead to fewer muscle spindles that were innervated by γ-motor axons, while GDNF overexpression lead

to more γ-motor axons innervation (Gould, Yonemura, Oppenheim, Ohmori, & Enomoto, 2008; Whitehead, Keller-Peck, Kucera, & Tourtellotte, 2005).

Furthermore, type Ia sensory axons in contact with myotubes release neuregulin 1 (Ngr1), which upregulates transcription factor programs promoting muscle spindle morphogenesis, such as the transcriptional regulator early growth response 3 (Egr3) (Hippenmeyer et al., 2002). Consistently, this process has been shown to be dependent on functional epidermal growth factor 2 (E rB2) receptor signalling in myotubes (Leu et al., 2003).

Moreover, mice deficient for NT3 show abnormal movements because they lack muscle spindles, showing that NT3 is needed for muscle spindle development (P. Ernfors, Lee, Kucera, & Jaenisch, 1994). Indeed, overexpressing NT3 in skeletal muscle promoted the formation of more muscle spindles. Consistently, re-expressing NT3 in developing skeletal muscle of mice lacking NT3 was sufficient to restore muscle spindle formation (Wright, Zhou, Kucera, & Snider, 1997), suggesting that muscle derived NT3 is necessary for muscle spindle development. However, it has been suggested, that intrafusal fibres derived NT3 might not be important for initial muscle spindle formation. Instead, it was proposed that intrafusal fibre-derived NT3 was needed to regulate the strength of muscle spindle connections with motor neurons (Gorokhova, Gaillard, & Gascon, 2009; Shneider, Mentis, Schustak, & O'Donovan, 2009).

In addition to its function for muscle spindle development, NT3 signalling has also been implicated in the muscle spindle fibre repair process. Intramuscular supplementation or muscle specific overexpression of NT3 protected the muscle spindle from crush injury induced degeneration (Taylor, Holdeman, Weltmer, Ryals, & Wright, 2005; Wright,

Williams, McDonald, Carlsten, & Taylor, 2002). Consistently, NT3 transcripts levels in intrafusal fibres decreased when the muscle was denervated. Subsequent re-innervation of the muscle spindles by type Ia sensory axons restored NT3 levels (Copray & Brouwer, 1997). These experiments suggest that NT3 also has an important role in muscle spindle repair. This begs the question, if NT3 is also needed for muscle spindle maintenance. So far, this question has not been addressed (Gorokhova et al., 2009)

Mice deficient in skeletal muscle Egr3 exhibited balance difficulties. Muscle spindle from these mice showed that they had normal type Ia innervation but their muscle spindles were non-functional, indicating that Egr3 is dispensable for Ia axon guidance but is needed for muscle spindle maturation (Oliveira Fernandes & Tourtellotte, 2015).

How the proprioceptive sensory neurons finds its target myotubes and how this is regulated is still not completely understood. It has been suggested that signals from the developing limb mesenchyme and a specific expression pattern in the DRG guide proprioceptive sensory axons to the limbs to contact myotubes (Poliak, Norovich, Yamagata, Sanes, & Jessell, 2016). Moreover, proprioceptive sensory axons of mice deficient in Runt-related genes 3 (Runx3) failed to connect to muscle spindles and motor neurons, suggesting that Runx3 is also needed for proprioceptive axon guidance (Inoue et al., 2002).

In sum, intrafusal fibre development depends on Ia sensory axon innervation leading to the induction of NT3 and Nrg1 and ErB2 signalling promoting muscle spindle maturation.

3.2.4 Diseases affecting proprioceptive feedback

In neurodegenerative diseases such as spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS), motor neurons degenerate consequently leading to muscle atrophy

(Messina, 2018; Yedavalli, Patil, & Shah, 2018). However, evidence from mouse models suggest that these diseases affect not only motor neurons, but also proprioceptive sensory neurons. ALS mutant mice showed signs of degenerated Ia/II proprioceptive nerve endings in muscle spindles even before disease onset. In addition, they observed reduced amount of proprioceptive synapses on motor neurons in ALS mutant mice (Vaughan, Kemp, Hatzipetros, Vieira, & Valdez, 2015). Similarly, SMA model mice also displayed a reduced amount of proprioceptive synapses on motor neurons and a loss of proprioceptive sensory neurons (K. K. Ling, Lin, Zingg, Feng, & Ko, 2010; Mentis et al., 2011). Interestingly, muscle spindles also showed signs of muscle spindle degeneration very early in the disease progression (Mentis et al., 2011).

Another neuromuscular disease leading to muscle weakness is Charcot-Marie-Tooth (CMT), a common genetic neuropathy affecting mainly Schwann cell development and peripheral axons. These patients suffer from decreased balance and ataxia, suggesting that proprioceptive sensory neurons could also be degenerated (Antonellis, Goldfarb, & Sivakumar, 1993; Saporta, 2014). Indeed, a mouse model of CMT showed a loss of muscle spindles and a high fraction of muscle spindles lacking sensory innervation (Sleigh et al., 2017). Moreover, it has been reported that before disease onset the muscle spindle diameter and volume was reduced in CMT mutant mice (Villalon et al., 2017).

Strikingly, in rats one year after nerve injury and subsequent reinnervation, proprioceptive synapses on motor neurons were not restored even though motor innervation was re-established. Importantly, the loss of other synapse, such as vGlut2 positive synapses, were restored after reinnervation (F. J. Alvarez et al., 2011), indicating that the selective loss of proprioceptive vGlut1^{+} synapses is likely responsible for the diminished stretch reflex in nerve-injured rats (Bullinger, Nardelli, Pinter, Alvarez, &

Cope, 2011). Taken together, these studies suggest that proprioceptive sensory neurons could be very vulnerable to disease and injury.

Similar to neuromuscular diseases, old age is associated with a loss of muscle mass and function (see 3.1.3 Sarcopenia). However, with age also proprioception decreases (Butler, Lord, Rogers, & Fitzpatrick, 2008; Lord & Ward, 1994; Sturnieks et al., 2008), suggesting that old age likely affects the morphology of the muscle spindle. Indeed, several studies show that in elderly humans the muscle spindle exhibit age-associated changes, such as a thickening of the capsule, a decrease in muscle spindle volume as well as a loss of intrafusal fibres per muscle spindle (Kararizou, Manta, Kalfakis, & Vassilopoulos, 2005; Liu, Eriksson, Thornell, & Pedrosa-Domellof, 2005; Swash & Fox, 1972). Similarly, in aged rodents muscle spindle coils were unravel and showed signs of degeneration (Kim, Suzuki, & Kanda, 2007; Vaughan, Stanley, & Valdez, 2016) In addition, increased H-reflex latency in elderly humans is consistent with morphological degeneration of muscle spindle suggesting decreased proprioceptive feedback in old age. (Kido, Tanaka, & Stein, 2004). The H-reflex is measured by electrically stimulating the neve and then to measure the EMG response of the corresponding muscle. Because sensory neurons have a lower excitability threshold than motor axons, it is possible to separate the sensory axon response (H-wave) from the motor axon response (M-wave). Therefore, H-reflex latency is used to evaluate α-motor neuron excitability and is seen as analogous to the stretch reflex (see 3.2.2 The proprioceptive spinal circuits) (Palmieri, Ingersoll, & Hoffman, 2004). Of note, by stimulating directly the sensory axons, the muscle spindle is effectively bypassed (Zehr, 2002). Thus, increased H-reflex latency with old age suggest that not only the muscles spindle degenerates but also its sensory afferent fibres. Indeed, sensory neuron conduction velocity and the number of sensory axons in elderly humans has been

shown to decrease with age (Romanovsky, Mrak, & Dobretsov, 2015; Verdu et al., 2000). Studies done in animals further confirm that the conduction velocity of single afferent discharges from muscle spindles was lower in aged rats and that there was a loss of sensory neurons in the DRGs (Kim et al., 2007). Of note, conditional satellite cell depletion in skeletal muscle of adult mice lead to changes in gait, reduced running wheel activity, and loss of balance. Interestingly, muscle spindles from these mice exhibited increased capsular thickness and intrafusal fibre atrophy (Jackson et al., 2015) reminiscent of aged muscle spindles. These unexpected findings suggest a yet unexplored role of satellite cells in the regeneration of intrafusal fibres in the aging process.

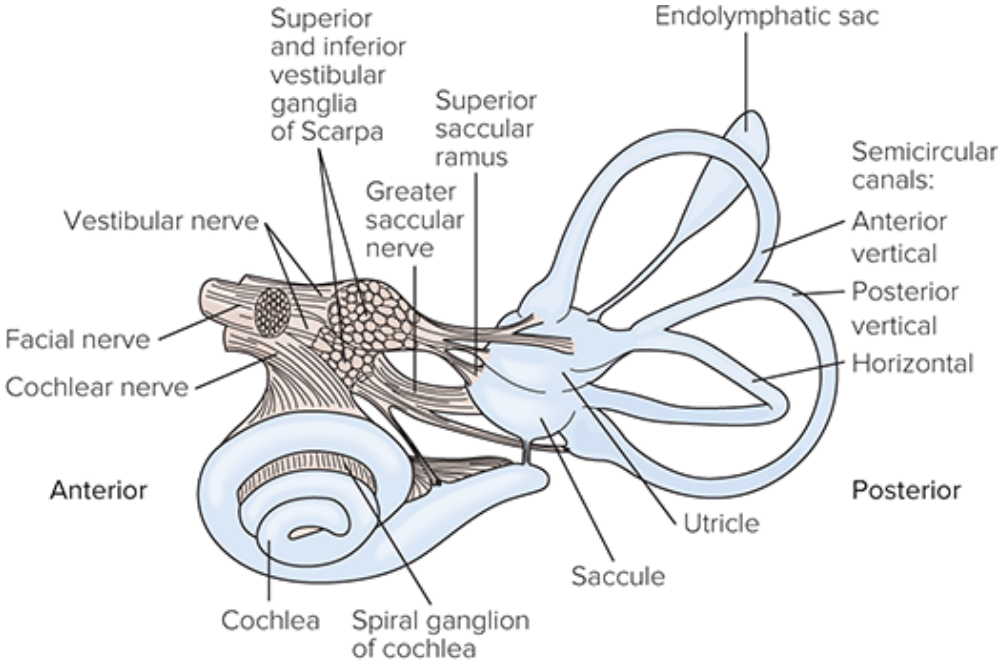
Taken together, evidence from humans and rodent studies suggest that proprioception is lower with old age because of degenerated muscle spindles and decreased conduction velocity of proprioceptive sensory neuron nerve fibres. However, the effects on age on the central synaptic spinal circuits that are involved in proprioception, balance and gait is not well understood.

3.3 The vestibular system

The vestibular system is the sensory system contributing to the sense of balance and spatial orientation necessary for body posture, gait and balance (Angelaki & Cullen, 2008). Similar to proprioception, vestibular function also decreases with age, exacerbating loss of balance in old age by increasing prevalence of vertigo and dizziness in the elderly (Eibling, 2018). Consequently, the incidence of falls in the elderly population is very high and fall-induced morbidity and mortality is increasing (Fuller, 2000), emphasizing a need to study age-related vestibular dysfunction.

3.3.1 Anatomy and function of the vestibular system

Fig. 9: Anatomy of the vestibular sensory apparatus



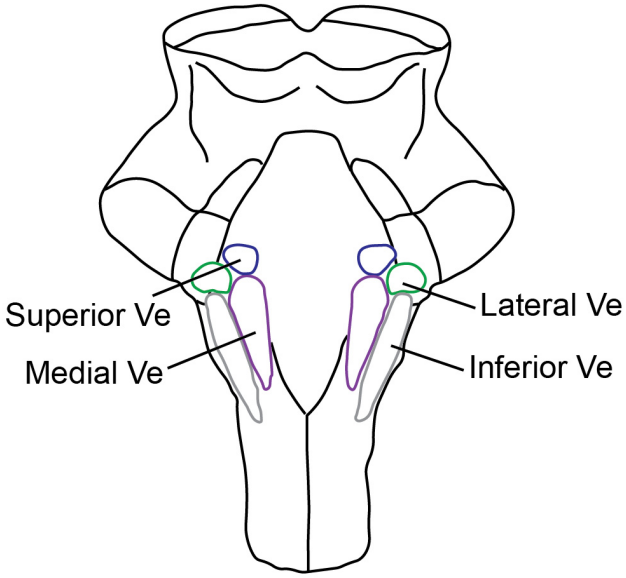
(Kandel Eric R., 2012 Principles of Neural Science)

The vestibular sensation is generated in the inner ear in the utricle, the saccule and the semicircular canals (**Fig. 9)**. The utricle and saccule together constitute the otolithic organs. They are characterized by the presence of mechanosensitive type I and type II hair cells, covered by the otolithic membrane. Distributed over the otolithic membrane,

is the free-moving otoconia, consisting of calcium carbonate. When the head experiences linear acceleration, the otoconia mass moves within the membranous labyrinth, stimulating firing of type I and II sensory hair cells. On the other hand, the semicircular canals are filled with endolymph. Similar to the function of otoconia, when the head experiences angular acceleration, by e.g. tilting the head, endolymph fluids in the semicircular canals are disturbed, which is stimulating firing of sensory hair cells. Both sensory sensation generated form otolithic organs and semicircular canals are transmitted by the vestibular nerve to the vestibular nuclei located in the brainstem. The vestibular nerve, joining the auditory nerve to from the auditory vestibular nerve, consist of vestibular sensory afferents. Sensory neurons in the vestibular ganglion project peripherally to hair cells in the inner ear, and centrally to the vestibular nuclei, transmitting mechanosensitive information about angular and linear acceleration. The vestibular nuclei in turn project to sensory hair cells in the otolithic organs (Kandel Eric R., 2012 Principles of Neural Science). The function of these efferent fibres is not well understood. However, it has been suggested that they could be involved in the discrimination of passive versus active movements (Mathews, Camp, & Murray, 2017).

The vestibular nuclear complex, located near to the IV ventricle in the brainstem, is composed of four anatomically separated nuclei: the medial, superior, lateral, and inferior vestibular nuclei (**Fig. 10)**. The medial vestibular nucleus is the largest of the four and projects to motor neurons of extraocular muscles to coordinate the vestibular ocular reflex and to cervical motor neurons of axial muscles modulating head and neck motion. Jointly with the superior vestibular nucleus, the medial vestibular nucleus is responsible to coordinate the vestibular ocular reflex, which is crucial to stabilize the gaze while moving. For example, turning the head to the right will trigger conjugate eye motions to the left,

Fig. 10: Anatomy of the vestibular nuclei (Ve) complex



ensuring that retinal images do not blur during the movement (Khan & Chang, 2013; Tascioglu, 2005). The inferior vestibular nucleus projects to the other vestibular nuclei and to the cerebellum (Khan & Chang, 2013).The lateral vestibular nucleus sends descending projections via the vestibular spinal tract to excite monosynapticly limb extensor motor neurons and to inhibit disynapticly flexor motor neurons (Basaldella et al., 2015; Liang, Bacskai, Watson, & Paxinos, 2014). It is believed that the main function of the lateral vestibular nuclei (LVe)is to project to the spinal neurons, contributing to postural reflexes, balance and gait (Andrew A. McCall, Miller, & Yates, 2017). Furthermore, it has recently been shown that the LVe project monosynapticly to type Ia inhibitory spinal interneurons (see 3.2.2 The proprioceptive spinal circuits), indicating a role for vestibular input to proprioceptive spinal circuits. How these vestibular projections are established during development is not at all well understood. However, gravity seems to play a major role. Interestingly, mice raised in hypergravity displayed a reduced input to lumbar moto neurons (Brocard, Clarac, & Vinay, 2003). Deletion of gravity sensing receptors in animal studies further confirmed that gravity most likely is an important factor for the development of the vestibular system (Jamon, 2014).

For a long time, the existence of the spinovestibular tract, which sends projections from the lumbar spinal cord to the vestibular nuclei, has been overlooked and therefore the function of these projections remains elusive (Pompeiano, 1972). Interestingly, passive and active movements of limbs stimulates vestibular neuron firing in decerebrated and conscious cats (Arshian et al., 2014; Bankoul, Goto, Yates, & Wilson, 1995; A. A. McCall, Miller, DeMayo, Bourdages, & Yates, 2016). Furthermore, there is evidence that the LVe receives indirect input from neck proprioceptors (Sato, Ohkawa, Uchino, & Wilson, 1997). Combined, these findings might suggest that input from proprioceptors are involved in vestibular nuclei integration in response to limb movements, which could represent an important modulatory feedback. Indeed, the vestibular nuclei integrate different input from a variety of different brain regions, such as the cerebellum, oculomotor and cortical, as wells as sensorimotor areas (Cullen, 2016).

Strikingly, aside from its classical involvement in balance, gait and posture, the vestibular system has also been implicated in the regulation of sleep/wake cycles (Besnard et al., 2018), blood pressure (Mori, Cotter, Arendt, Olsheski, & Yates, 2005) or adipose tissue homeostasis (McGeoch, 2019), emphasizing that the vestibular contribution to basal bodily functions need to be investigated more.

3.3.2 Vertigo and vestibular compensation

Vertigo is a form of dizziness involving a spinning or swaying sensation leading to difficulties in walking. It is an episodic acute response caused by an unbalanced vestibular function that could have resulted from defects in the inner ear labyrinth, Ménière's disease, vestibular neuritis and labyrinthitis, or strokes (Whitman, 2018). Interestingly, loss of vestibular function can be partly compensated (Sjögren, Fransson, Karlberg, Magnusson, & Tjernström, 2018). However, the plasticity underlying the mechanism of

vestibular compensation is not well understood. A study done in monkeys showed that neck proprioceptors could substitute gaze stabilization after a complete loss of vestibular function (Sadeghi, Minor, & Cullen, 2012). In rats, complete labyrinthectomy increased hind limb input to vestibular neurons concomitantly with improvements in gait and posture (Andrew A. McCall, Moy, Puterbaugh, DeMayo, & Yates, 2013). Strikingly, in response to vestibular injury, neurogenesis in the vestibular nuclei was increased (Tighilet & Chabbert, 2019), suggesting that input from proprioceptors and potential neurogenesis could both contribute to vestibular compensation.

3.3.3 Aging of the vestibular system

In humans, vestibular dysfunction with age is often not diagnosed, in part because of low awareness in clinicians and because the underlying causes for loss of balance and dizziness with age are multifactorial including, muscular weakness, loss of proprioception and medication (Rubenstein, 2006). In fact, the incidence for benign paroxysmal positional vertigo (BPPV) in the elderly population is high (Oghalai, Manolidis, Barth, Stewart, & Jenkins, 2000). Nevertheless, it is believed that the gradual deterioration in vestibular function and not BPPV itself contributes to decreased balance in the elderly and high latency in the vestibular ocular reflex response (Aalto, Pyykkö, Juhola, & Jänttil, 1997; Baloh, Jacobson, & Socotch, 1993; Kingma & van de Berg, 2016; Paige, 1992).

Several studies done in humans and animals have shown that the vestibular system experiences morphological and functional age-associated degeneration (Brosel, Laub, Averdam, Bender, & Elstner, 2016). First, otoconia in the saccule and utricle (Saito, Mizukoshi, & Alford, 1993) decrease and a high percentage of mechanosensitive hair cells are lost with age (Rauch, Velazquez-Villasenor, Dimitri, & Merchant, 2001). However, other investigators have found a comparatively low amount of sensory hair cell

degeneration (Ivan Lopez et al., 2005), suggesting that loss of hair cells is likely not the primary cause for decreased vestibular function. Second, the number of vestibular myelinated nerve fibres (Bergström, 1973) and the number of neurons in the vestibular ganglion decrease with age (Park, Tang, Lopez, & Ishiyama, 2001; Velázquez-Villaseñor et al., 2000). Third, several studies done in humans and mice showed that the number and size of neurons in the vestibular nuclear complex are decreasing in an age dependent manner (J. C. Alvarez et al., 1998; Diaz, Suarez, Navarro, Gonzalez del Rey, & Tolivia, 1993; I. Lopez, Honrubia, & Baloh, 1997; Sturrock, 1989). Although the underlying cause for this neurodegeneration is not known it has been suggested that accumulated lipofuscin, granules containing lipid residuals from lysosomes, especially in the lateral vestibular nucleus could be implicated in the neurodegeneration of vestibular complex neurons (J. C. Alvarez et al., 2000).

Taken together, the gradual loss of vestibular function likely involves a deterioration of vestibular sensory organs in the inner ear, as well as neurodegeneration in the vestibular ganglion and in, the vestibular nuclear complex. However, the effects of age on central vestibular network integration has so far not been investigated.

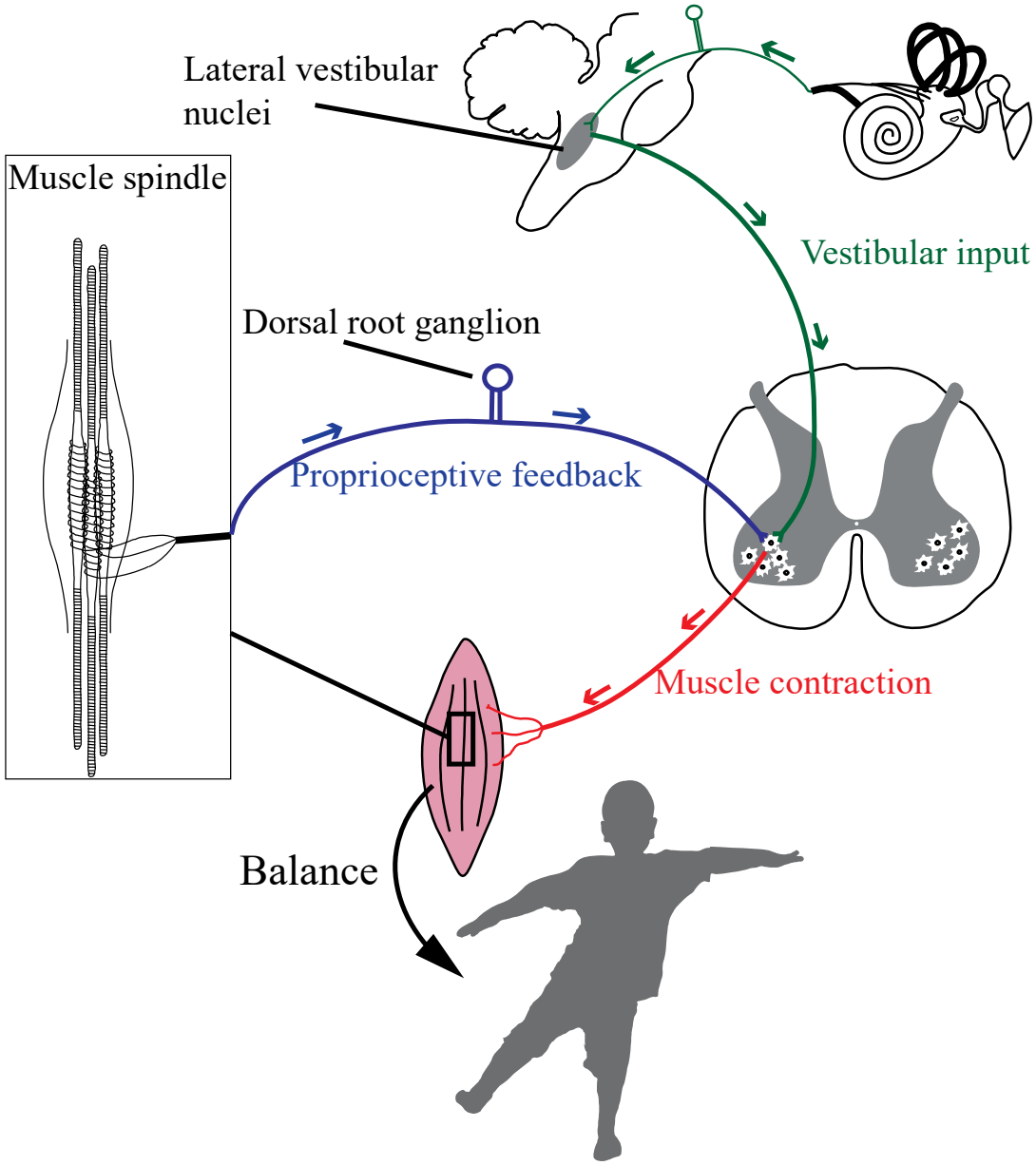
4 Aims of the study

Aging is associated with a loss of muscle function but also declining sense of balance (Eibling, 2018; Lei & Wang, 2018; Lord & Ward, 1994). Statistically, every third person older than 65 falls at least once per year (Fuller, 2000). The high incidence of falls in the elderly is among the main drivers for hospitalization, admission to nursing homes and loss of independence (Fuller, 2000). Alarmingly, in the elderly population the main cause of accidental deaths are falls (Fuller, 2000; Riley, 1992). Considering that the age group above 60 is the fastest growing population (World population aging 2015, United Nations) it is vital to understand in depth the mechanisms leading to falls in this age population.

Remarkably, physical activity improves gait, posture and balance in the elderly (Cadore et al., 2013; Carter, Kannus, & Khan, 2001; Lopopolo, Greco, Sullivan, Craik, & Mangione, 2006; Perrin, Gauchard, Perrot, & Jeandel, 1999; Shigematsu et al., 2002; Simmons & Hansen, 1996). Of interest, exercise improved the vestibular ocular reflex, vestibular postural reflex and proprioception (Gauchard et al., 2003; Petrella, Lattanzio, & Nelson, 1997; Tsang & Hui-Chan, 2003), indicating that exercise could act on central processing of the vestibular and proprioceptive systems. Both the LVe as well as the proprioceptive muscle spindle receptor send monosynaptic projections to motor neurons, where feedback from both sensory systems are integrated by motor neurons (**Fig. 11**) (see 3.2.2 The proprioceptive spinal circuits and 3.3.1Anatomy and function of the vestibular system) to generate an appropriate motor output to regain balance. However, despite the importance of vestibular and proprioceptive feedback to motor neurons, the effect of age and exercise on vestibular and proprioceptive connectivity to motor neurons is only

rudimentarily understood. Therefore, the aim of this study is to relate the effect of age and exercise on balance in mice to components and networks of the proprioceptive and vestibular systems.

Fig. 11: Overview of proprioceptive and vestibular connectivity to motor neurons



To that end, physiologically aged C57BL/6J mice were trained for 6 to 12 weeks on running wheels and treadmill. The use of retro- and anterograde neuronal and synaptic

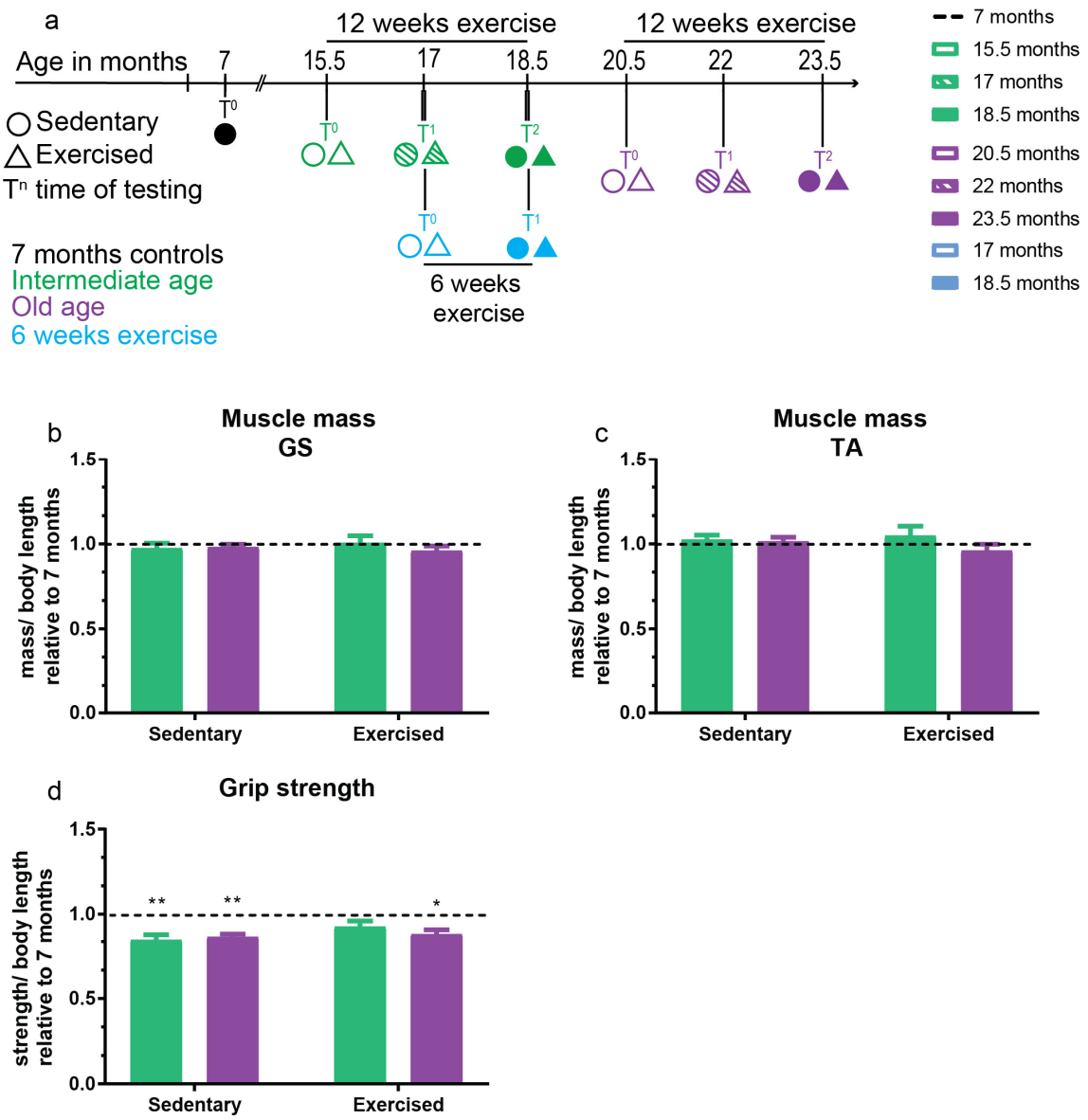
tracing techniques combined with *in vivo* balance and gait testing revealed how age and exercise affect proprioceptive and vestibular input to motor neurons in these contexts.

5 Results

5.1 Muscle function in aged mice

Three different groups of physiologically aged C57BL/6J mice were exercised with running wheels and on treadmill. The intermediate group, aged 18.5 months, and old age group, aged 23.5 months, were trained for 12 weeks starting at 15.5 months and 20.5 months of age, respectively. An additional group aged 17 months was exercised for 6 weeks. (**Fig. 12a**). In order to follow the in vivo behaviour over time, sedentary as well as exercised mice were retested 4 days before, 6 weeks after and 12 weeks after the start of the training (**Fig. 12a**). To exclude muscle mass loss as a confounding factor for the observed phenotypic changes, we measured gastrocnemius (GS) (**Fig. 12b**) and Tibialis anterior (TA) (**Fig. 12c**) muscle mass and found no significant decrease between any of the groups. In addition, we assessed muscle function by measuring all four limbs grip strength. Although muscle mass was not yet significantly reduced we found a small reduction in grip strength in aged sedentary and exercised mice (**Fig. 12d**). Moreover, we did not observe an effect of endurance exercise on muscle mass or grip strength.

Fig. 12 Muscle function in aged mice

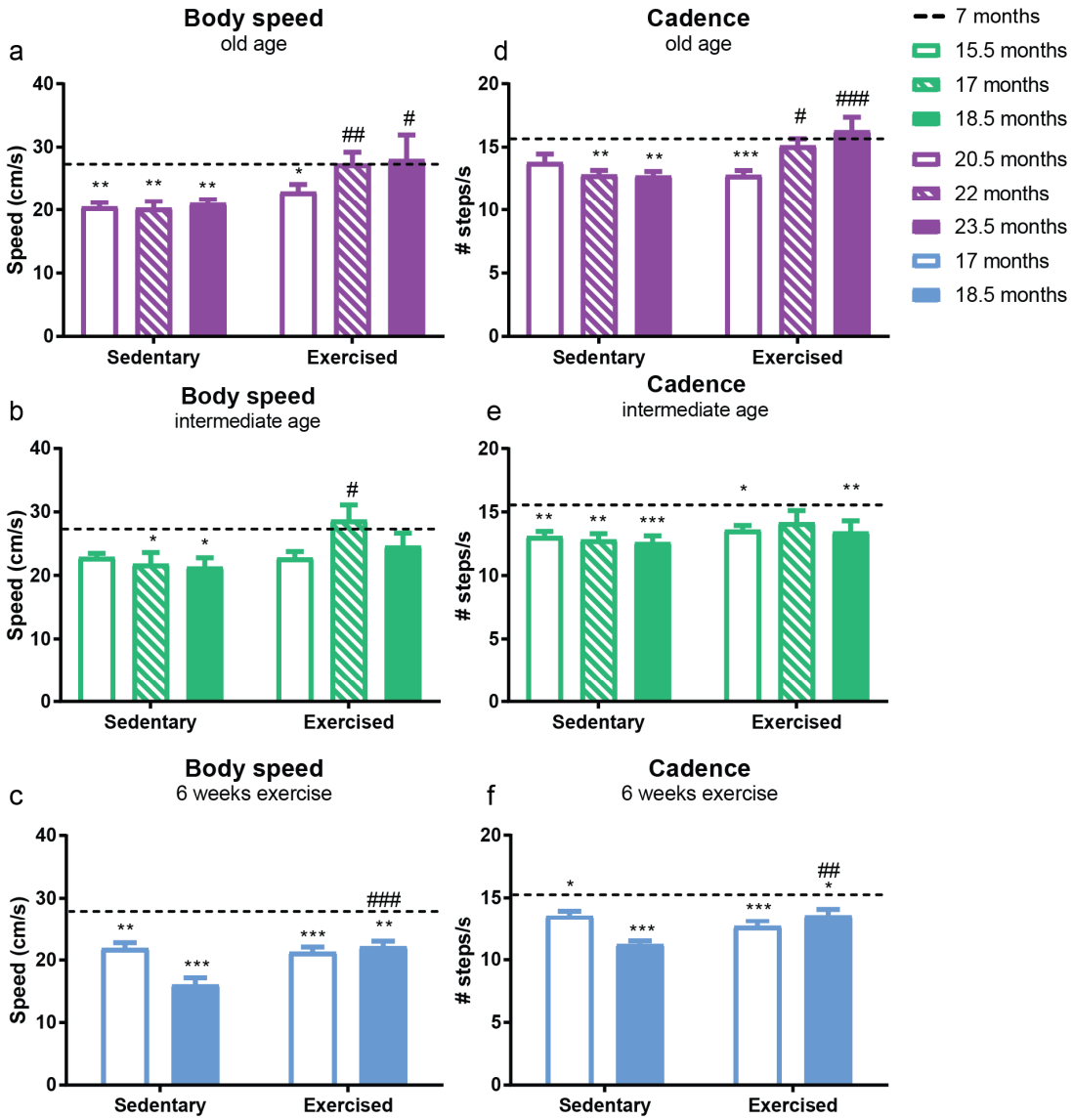


a Representation of different age groups and timeline of study. **b** GS muscle mass and **c** TA muscle mass normalized to body length and relative to 7-month-old controls. **d** All four limbs grip strength normalized to body length and relative to 7-month-old controls. n=6 to 9 animals per group. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

5.2 Changes in gait were ameliorated by exercising

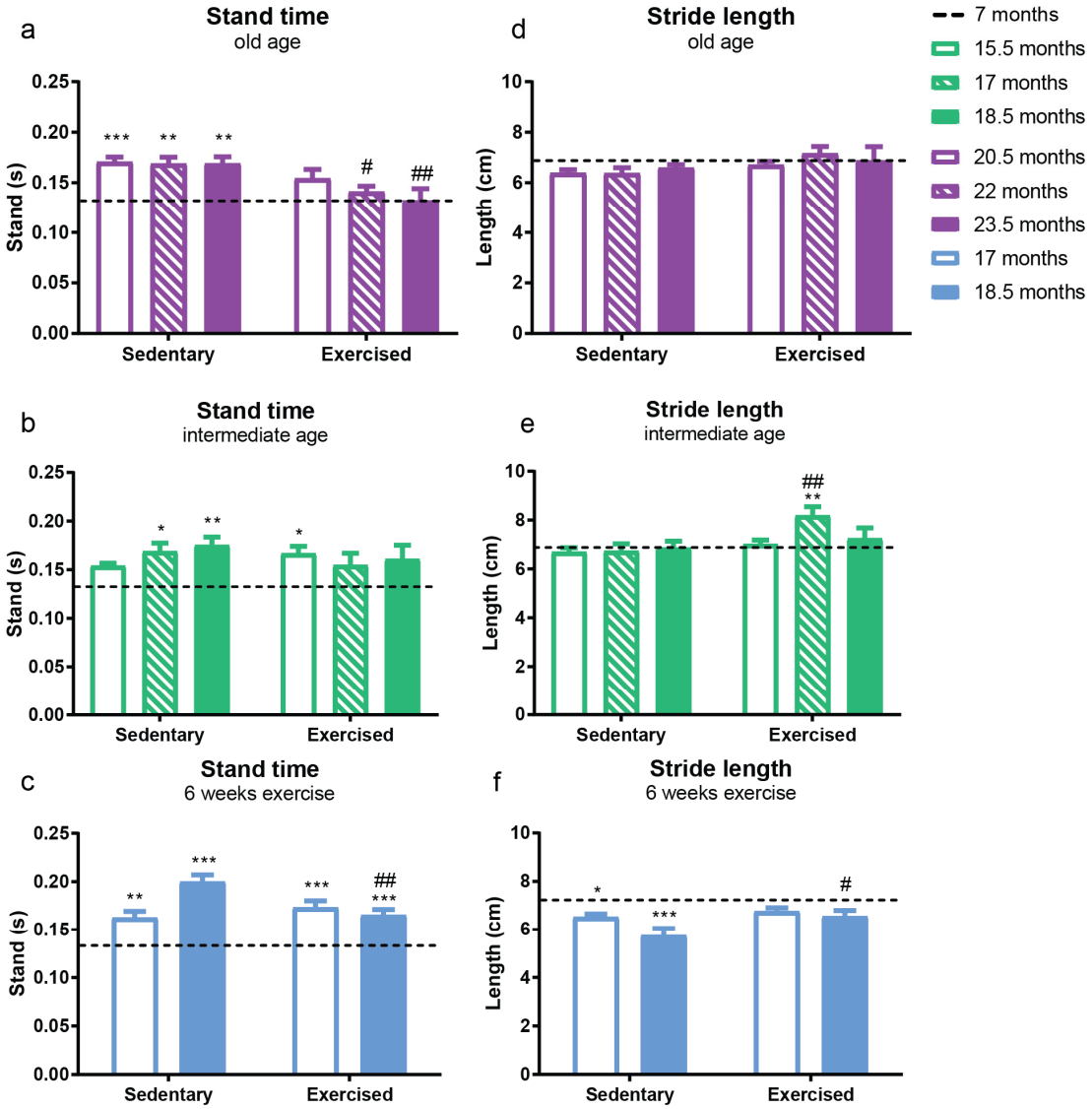
Since elderly humans undergo changes in gait (Gillain & Petermans, 2013; Verghese, Ambrose, Lipton, & Wang, 2010; Verghese et al., 2008), we wondered if aged mice would show a similar phenotypic changes with age. To measure gait in aged sedentary and exercised mice, animals walked voluntarily through a catwalk tunnel connected to a camera tracking paw prints. All aged mice moved significantly slower across the platform (**Fig. 13a to c**), and made significantly fewer steps per seconds than 7-month-old mice (**Fig. 13d to g**). In contrast, aged mice that were exercised moved significantly faster (**Fig. 13a to c**) and made more steps per second (**Fig. 13d to g**) than age-matched sedentary controls. In addition, the stand-time increased with age (**Fig. 14a to c**). Strikingly, exercised aged mice increased in the stand- time (**Fig. 14a to c**) and took bigger steps than sedentary age-matched control mice (**Fig. 14d to g**), showing that age-related gait differences were amended by exercise.

Fig. 13: Changes in locomotive speed



a to c Body speed during locomotion. **d to f,** Cadence, the number of steps per second. n=6 to 9 animals per group. In **a to c** values for both front paws were combined. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

Fig. 14: Changes in stand time and stride length



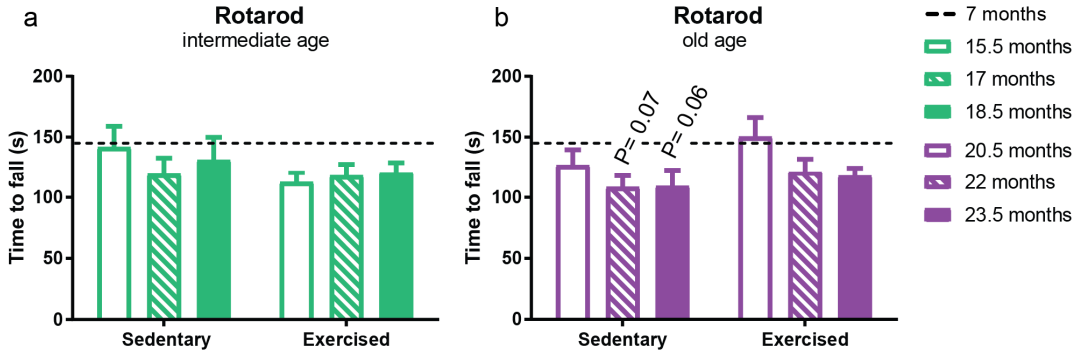
a to c Stand time and **d to f** stride length. n=6 to 9 animals per group. Values for both front paws were combined. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

5.3 Age-associated loss of balance was improved by exercise

Since exercise ameliorated changes in gait, we next asked if also age-related loss of motor coordination and balance could be improved by exercise. To assess motor coordination in aged exercised and sedentary mice we conducted a Rotarod test. Intermediate aged mice did not perform significantly worse than 7-month-old mice (**Fig. 15a)** in the Rotarod test. However, old age sedentary mice tended to fall sooner than 7-month-old mice while exercise did not affect performance in any of the groups (**Fig. 15b)**. To measure balance, old age and intermediate aged mice were tested on a round balance beam. Before the start of the exercising period, intermediate mice, aged 15.5 months, did not perform worse than 7-month-old controls on the balance beam. However, at 17 and at 18.5 months of age, intermediate sedentary mice needed longer to cross the beam (**Fig. 16a)** and slipped more than 7-month-old mice (**Fig. 16b**). Remarkably, intermediate aged mice that exercised crossed the beam faster (**Fig. 16a)** and made significantly fewer slips than sedentary controls (**Fig. 16b**). Moreover, even old age mice that were exercised for 12 weeks traversed the beam significantly faster than sedentary mice of the same age (**Fig. 16c**). To strengthen these findings, we tested 18.5-month-old mice on a square balance beam. Also in this setting, aged mice that exercised crossed the square balance beam significantly faster than sedentary aged matched controls. (**Fig. 16e**).

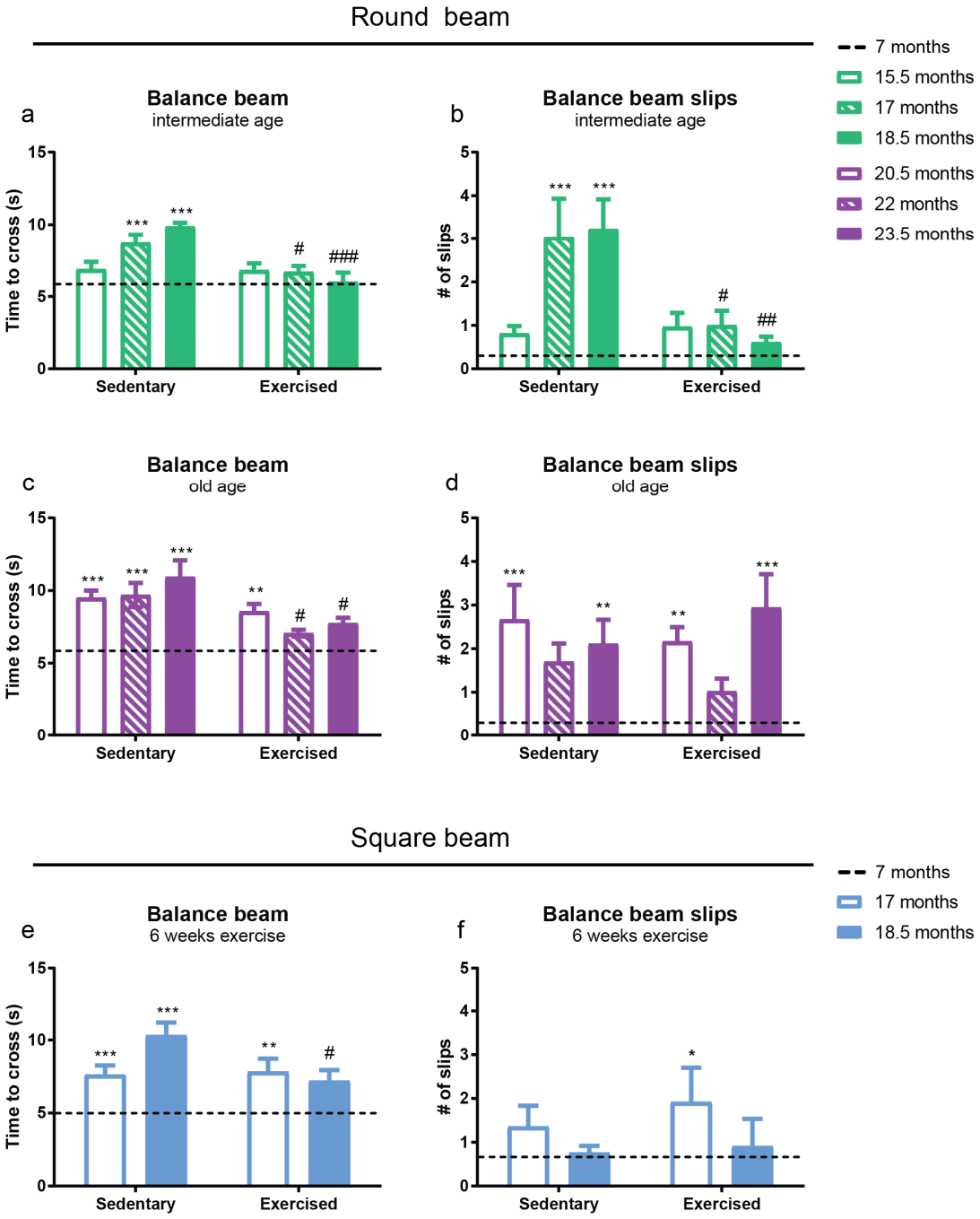
Overall, exercise improved balance in aged mice but did not affect motor coordination.

Fig. 16: Motor coordination in aged mice



a, b Time to fall from Rotarod as a measure for motor coordination. n=6 to 9 animals per group. Data represent the mean ± SEM. Trends are indicated by stating the P-value and are relative to 7-month-old controls, represented as the dotted line. Significance was determined using Anova followed by Sidak's test.

Fig. 16: Balance in aged mice



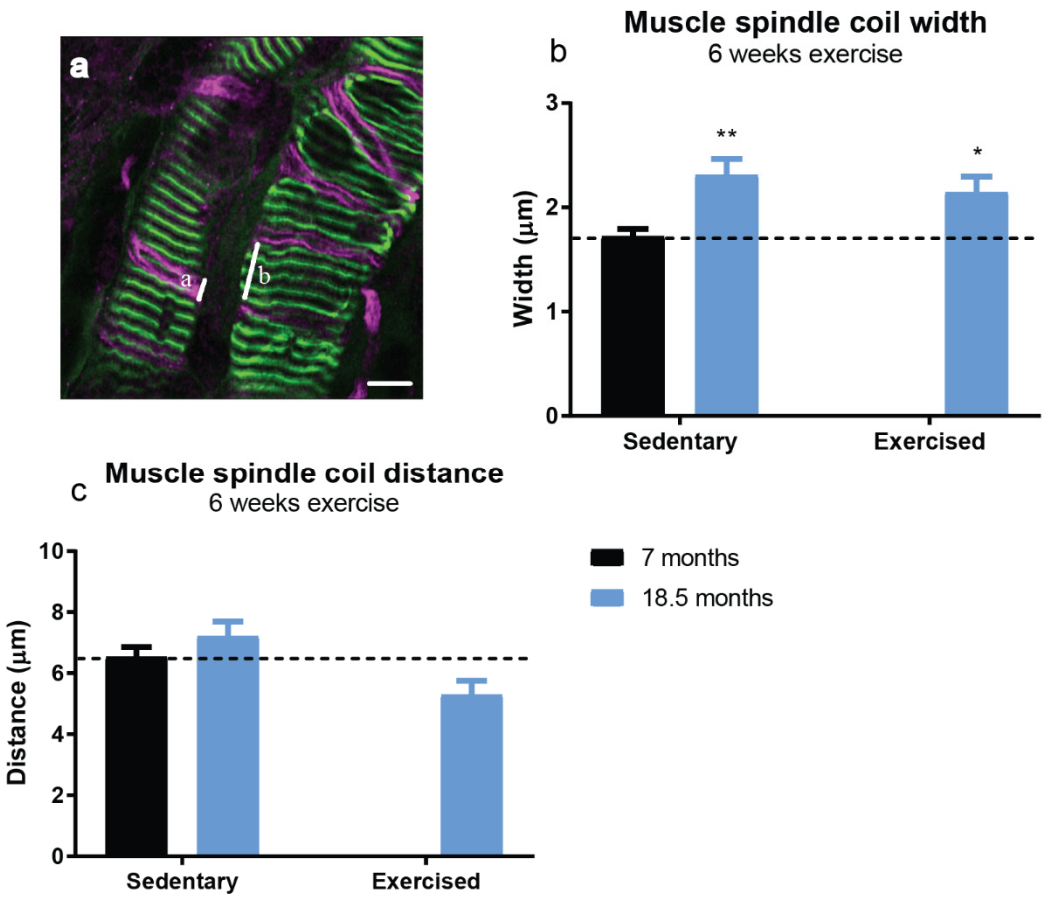
a, c The time to cross the round balance beam. **b, d** The number of slips while crossing the round balance beam. **e** The time to cross the square balance beam and **f** the number of slips. n=6 to 9 animals per group. Data represent the mean ± SEM. . *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

5.4 Age but not exercise affected muscle spindle morphology

Since proprioceptive feedback is crucial for both balance and gait (Proske & Gandevia, 2012; Windhorst, 2007), we next asked if there are changes in muscle spindle receptor morphology that correlate with the observed improvement in balance. To assess muscle spindle coil morphology, we analysed whole-mounted fragments of the extensor digitorum longus (EDL) muscle that were labelled for the intrafusal fibre marker, s46 in green and Neurofilament H (NFH) in purple. **Fig. 17a** shows the longitudinal view of a muscle spindle and how coil width (a) and coil distance (b) were measured. While there was no difference in muscle spindle coil distance (**Fig. 17c**), we found that the width of muscle spindle coils increased significantly with age (**Fig. 17b**). However, we observed no effect of exercise on coil width or distance (**Fig. 17b, c**). In addition, serial cross sections from GS muscle were also analysed. **Fig. 18a to e** shows the cross-sectional view of a muscle spindle labelled for s46 (green), NFH (purple) and laminin (white). We detected no difference in the number of muscle spindles per 20 serial sections in any of the groups (**Fig. 18f**). There was also no significant difference between aged sedentary and exercised mice in the size of $s46^{+}$ fibres. However, muscle spindle fibre diameter was decreased in an age dependent manner (**Fig. 18g**).

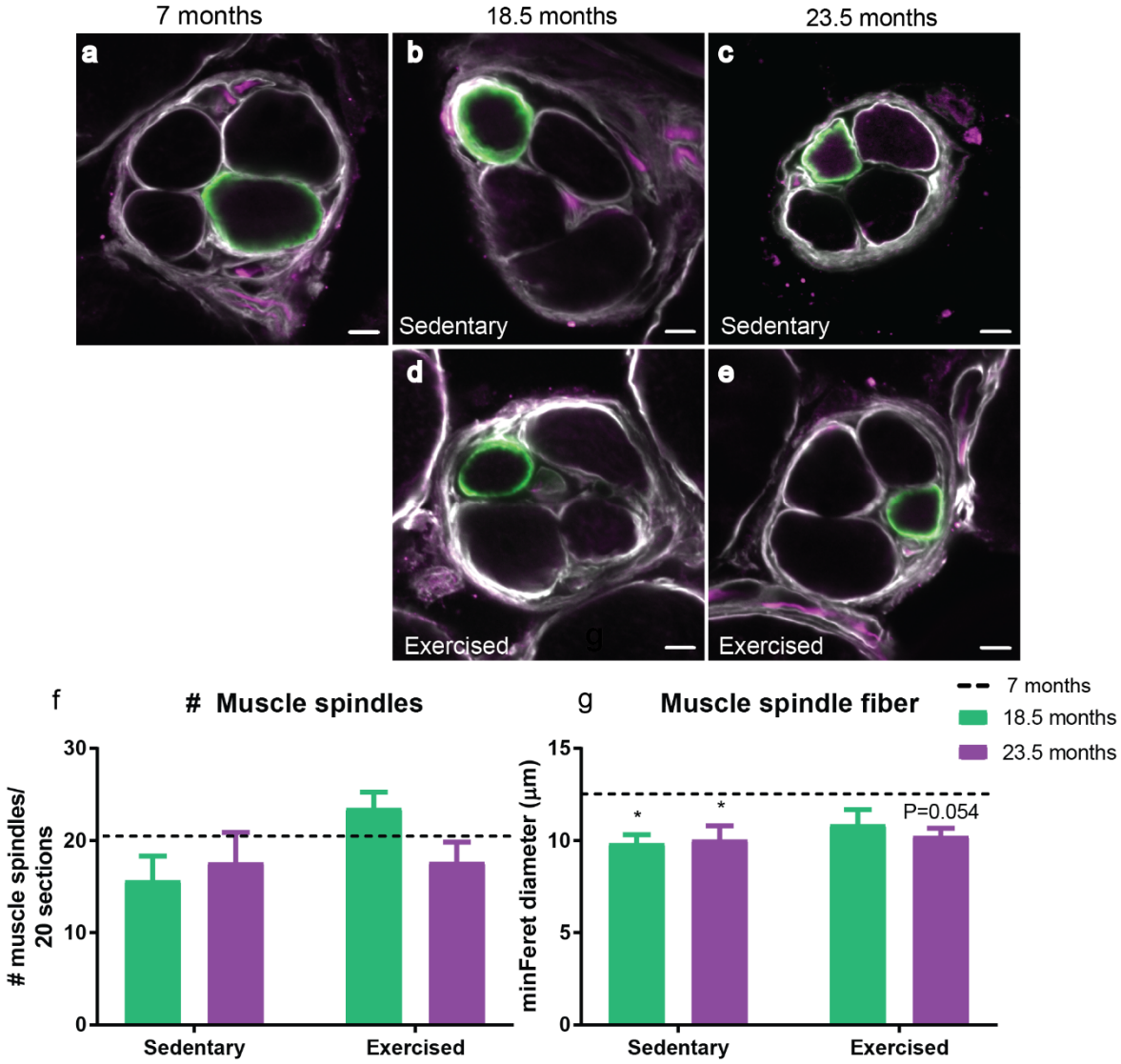
In sum, muscle spindle morphology was altered with age but exercise had no effect on muscle spindle fibres or coils.

Fig.17: Muscle spindle coil morphology



a Longitudinal view of muscle spindle from EDL whole-mount labeled with s46 in green and NFH in purple. a and b on the image show how coil width and distance was measured. **b** Muscle spindle coil width and **c** distance between coils. n = 16 muscle spindles from a minimum five animals. Scale bar = 5 µm. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

Fig. 18: Muscle spindle fibre size



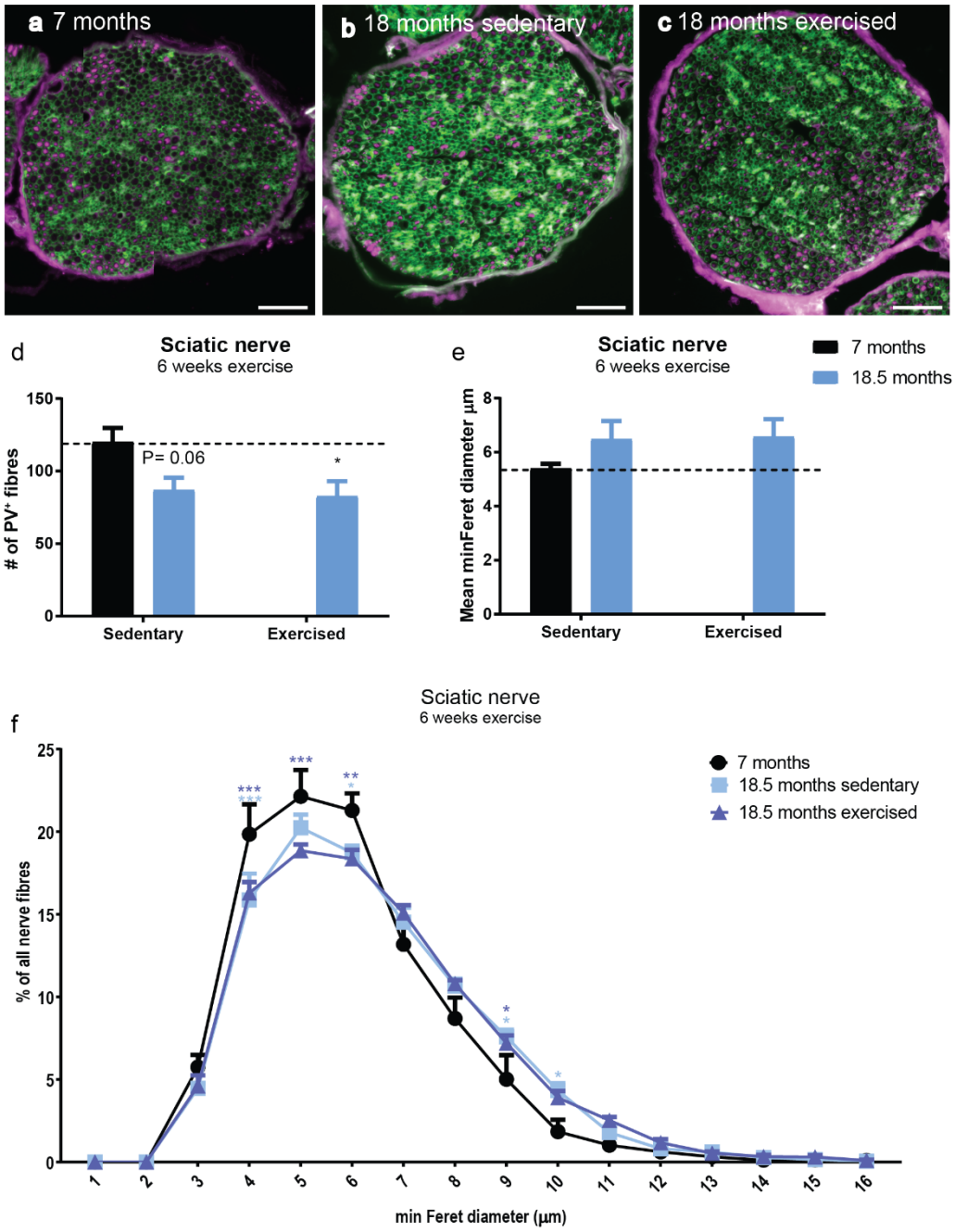
a to b Cross-sectional view of muscle spindle from GS sections labelled with s46 in green, NFH in purple and laminin in white. **f** The number of muscle spindles per 20 serial sections. **g** The minimal Feret's diameter of $s46^{+}$ muscle spindle fibres. n = 2 to 3 muscle spindles per animal from a minimum five animals. Scale bar = 5 µm. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Trends are indicated by stating P-value and are relative to 7-month controls. Significance was determined using Anova followed by Sidak's test.

5.5 The number of Parvalbumin$^+$ nerve fibres was decreased with age but not with exercise

Since we observed changes in the sensory neuron innervating muscle spindles, we next wondered if the sciatic, nerve supplying innervation to muscle spindles as well as to hind limb muscles would be affected by age or exercise. To that end, we analysed cross-sections from sciatic nerve of aged sedentary and exercised mice. **Fig. 19a to c** show representative images of sciatic nerve cross sections labelled for parvalbumin (PV) in purple and laminin in white. Neither the number of PV$^+$ nerve fibres (**Fig. 19d**), nor the fibre size distribution (**Fig. 19f**) were significantly different between sedentary and exercised mice. Moreover, the average size of nerve fibres was not significantly changed between any of the groups (**Fig. 19e**). However, with age the fibre size distribution was shifted towards larger nerve fibres (**Fig. 19f**) and we detected fewer PV$^+$ nerve fibres in aged sedentary mice than in 7-month-old mice (**Fig. 19d**).

In sum, nerve fibres from aged sedentary mice had a shifted size distribution and fewer PV$^+$ nerve fibres.

Fig. 19: Size and composition of nerve fibres in the sciatic nerve



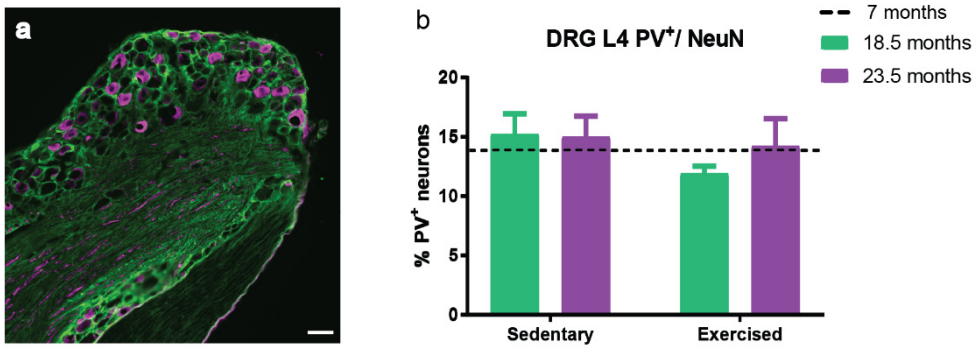
a to c Representative images of sciatic nerve cross-sections labelled with laminin in green, and PV in purple. **d** The number of PV^+ nerve fibres. **e** The minimal Feret's fibre diameter. .**f** The nerve fibre size distribution in percentage of all nerve fibres. n = 2 to 3 sections per animal from a minimum five animals. Scale bar = 50 µm. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

5.6 The number of proprioceptive sensory neurons was unchanged with age or exercise

Aside from proprioceptive sensory axons, other types of axons in the sciatic nerve also express PV. Consequently, we next asked if the changes in the number PV^+ nerve fibres could be reflected by a decreased amount of PV^+ proprioceptive sensory neurons in the DRG. To answer that question, we counted the number of PV^+ proprioceptive sensory neurons on cross sections from L4 DRGs (**Fig. 20a**). Unlike in the sciatic nerve, the number of PV^+ cells was unchanged by age or exercise (**Fig. 20b**).

Collectively, we showed that muscle spindle receptor morphology and sciatic nerve composition were affected by age. However, in response to exercise we found no effect in the number of proprioceptive sensory neurons or muscle spindle morphology that could explain the improved balance in exercised aged mice.

Fig. 20: Proprioceptive sensory neurons in the DRGs



a Representative image of L4 DRG labelled with laminin in green, and PV in purple. **b** The number of PV^+ proprioceptive sensory neurons were counted and the ratio relative to the total number of sensory neurons was calculated. n = 2 to 5 sections per animal from a minimum of four animals. Scale bar = 50 µm. Data represent the mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

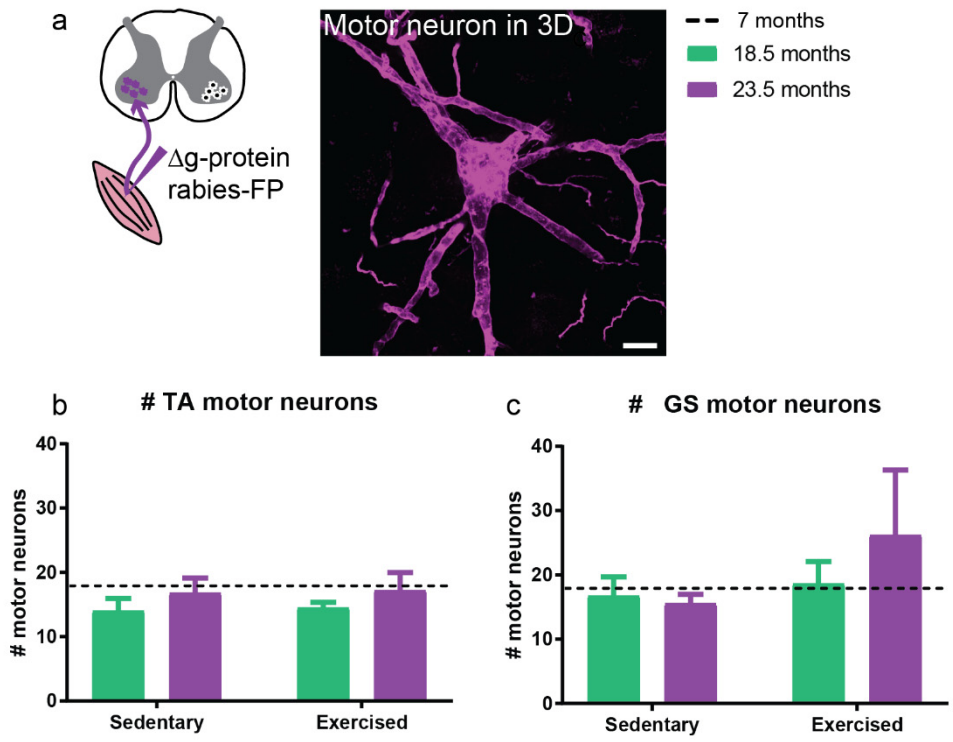
5.7 Proprioceptive input to motor neurons was decreased with age but not with exercise

Since proprioceptive sensory neurons have bifurcating axons, one projecting to the muscle spindles and the other projecting centrally to connect directly to motor neurons (see 3.2 The proprioceptive system), we next asked if there could be age or exercise related changes in the muscle spindle feedback to motor neurons. In order to pursue this question, we used g-protein deleted rabies virus to retrogradely trace the GS and TA motor neuron pools (**Fig. 21a**). To exclude loss of motor neurons as a contributing factor for balance loss, we counted the number of motor neurons from the GS and TA motor neuron pool. Neither the number of TA motor neurons (**Fig. 21b**) nor the number of GS motor neurons (**Fig. 21c**) were reduced with age. In order to quantify proprioceptive synapses on traced motor neurons, we co-stained spinal cord cross sections with the proprioceptive synapse marker vGlut1. **Fig. 22a and b** shows an example of a 3D projection of a motor neuron area in purple co-stained with vGlut1 staining in green and the corresponding reconstruction of synapses on a motor neuron. The number of vGlut1 synapses that were touching the motor neuron surface was normalized to the surface area of the reconstructed motor neuron, which resulted in the synaptic density. We found that irrespective of exercise, TA and GS motor neurons from aged mice had a lower synaptic density than 7-month-old mice (**Fig. 22c, d**). Consistently, we observed significantly fewer synapses on the soma of TA and GS motor neurons from aged sedentary mice. Moreover, between sedentary and exercised mice of the intermediate age group, we did not detect differences in the amount of vGlut1 synapses on motor neuron soma from both motor neuron pools. However, we counted fewer vGlut1 synapses on TA and GS motor neurons from exercised old age mice than from sedentary age-matched controls (**Fig. 22e,**

f). To investigate if exercise affected the vGlut1 synaptic distribution we measured the distance between synapses and the dendrite root for TA and GS motor neuron trees. In GS motor neurons from both age groups, we found fewer synapses between the first 100 µm from the dendrite root than in 7-month-old mice (**Fig. 23a, b)**. While there was no difference in synaptic distribution between sedentary and exercised mice in GS motor neurons (**Fig. 23a, b)**, we observed a significantly higher percentage of synapses between 60 to 160 µm in TA motor neurons from exercised old age mice than from sedentary age-matched controls. However, in the intermediate age group we did not detect differences between sedentary and exercised mice (**Fig. 24a, b)**.

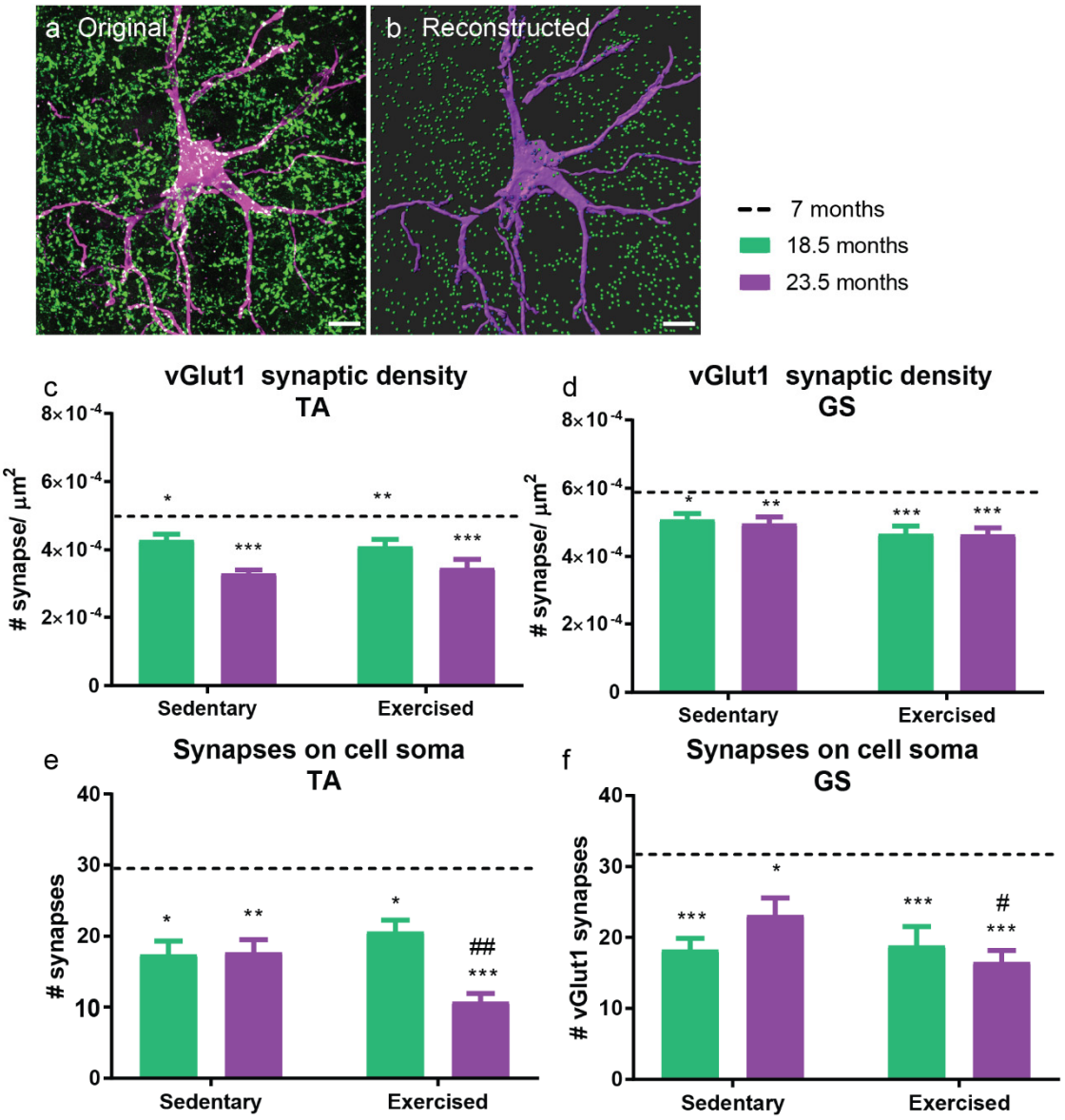
Taken together, proprioceptive synaptic density and distribution were changed with age but not with exercise. In TA motor neurons, exercise did not alter synaptic density but slightly shifted vGlut1 synaptic distribution farther away from the dendrite root.

Fig. 21: The TA and GS motor neuron pool



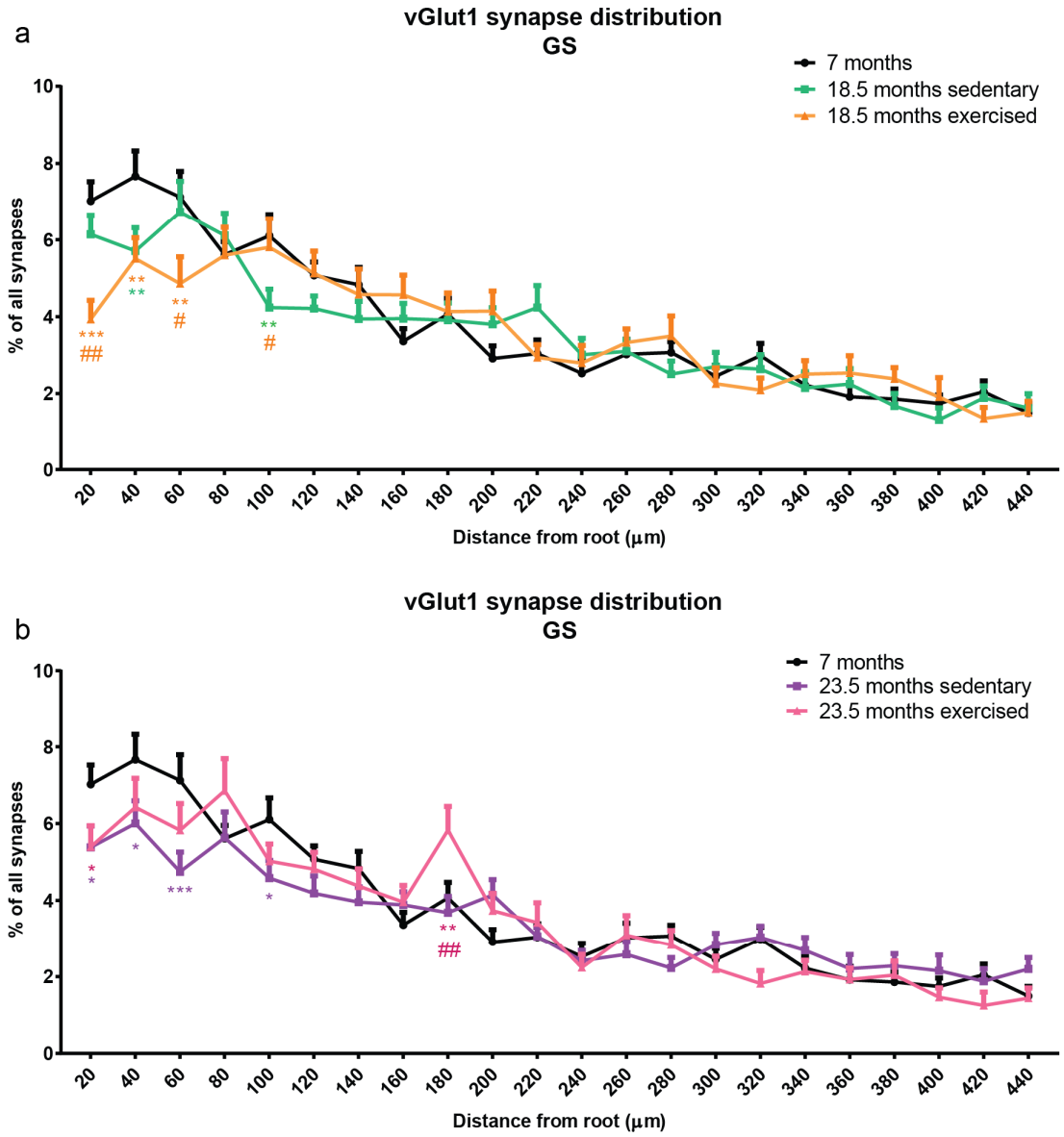
a Schema of retrograde tracing approach and representative image of a motor neuron (purple) in 3D. **b** The number of TA and **c** GS motor neurons. n = 5 to 8 animals. Scale bar = 100 µm. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0,001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

Fig. 22: The proprioceptive input to motor neurons with age



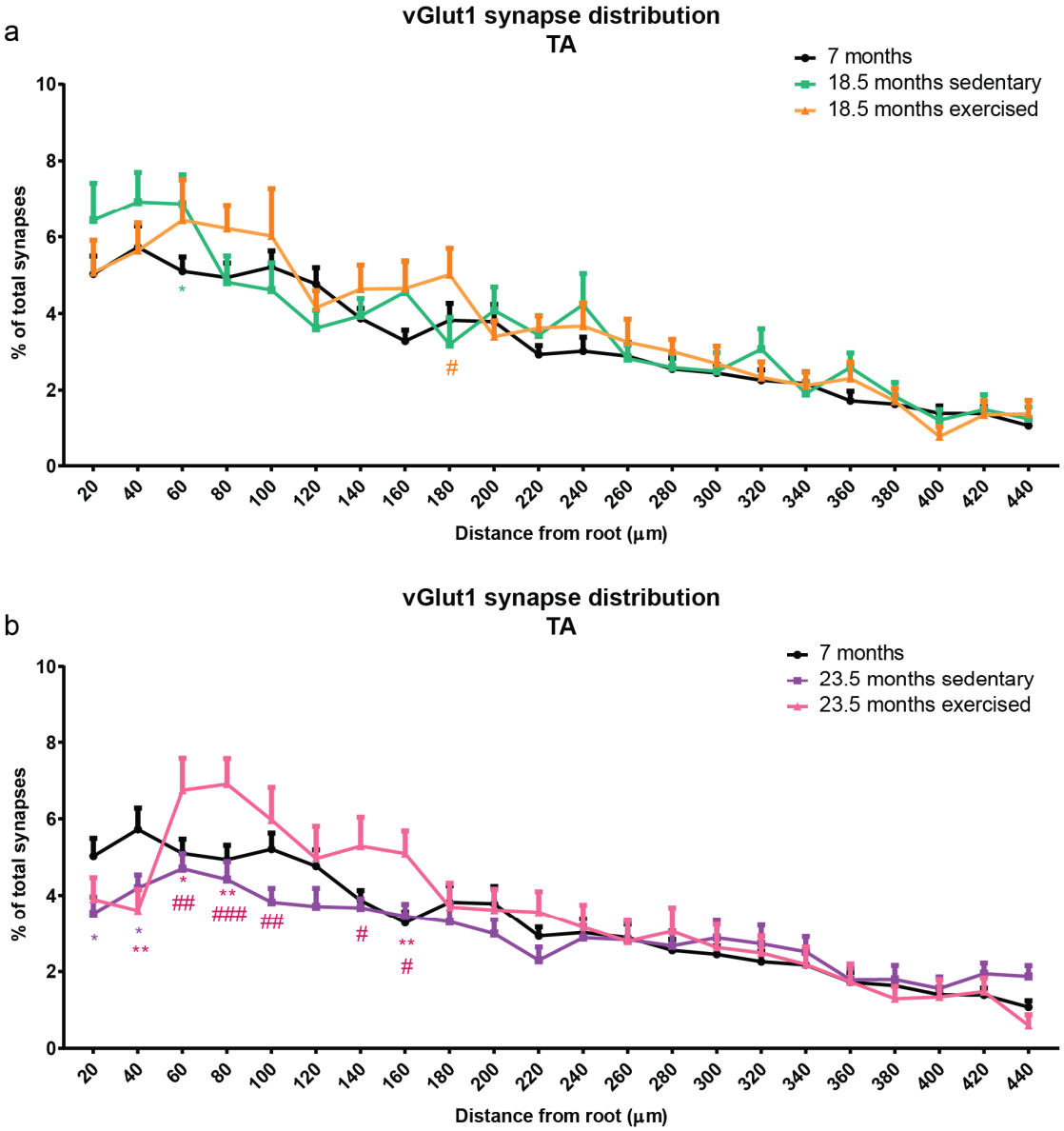
a Example of motor neuron (purple) in 3D projection labelled with vGlut1 (green) and **b** the corresponding reconstruction. **c, d** The calculated number of synapses was normalized to the surface area of the reconstructed motor neuron, resulting in the synaptic density. **e, f** The number of synapses on the motor neuron soma. A minimum of 20 motor neurons from a minimum of five different animals per group were analysed. Scale bar = 100 µm. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0. indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

Fig. 23 vGlut1 synaptic distribution of GS motor neurons



a, b Distribution of vGlut1 synapses along GS motor neuron branch relative to the motor neuron root in percentage of all synapses. A minimum of 20 motor neurons from a minimum of five different animals per group were analysed. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

Fig. 24: vGlut1 synaptic distribution of TA motor neurons



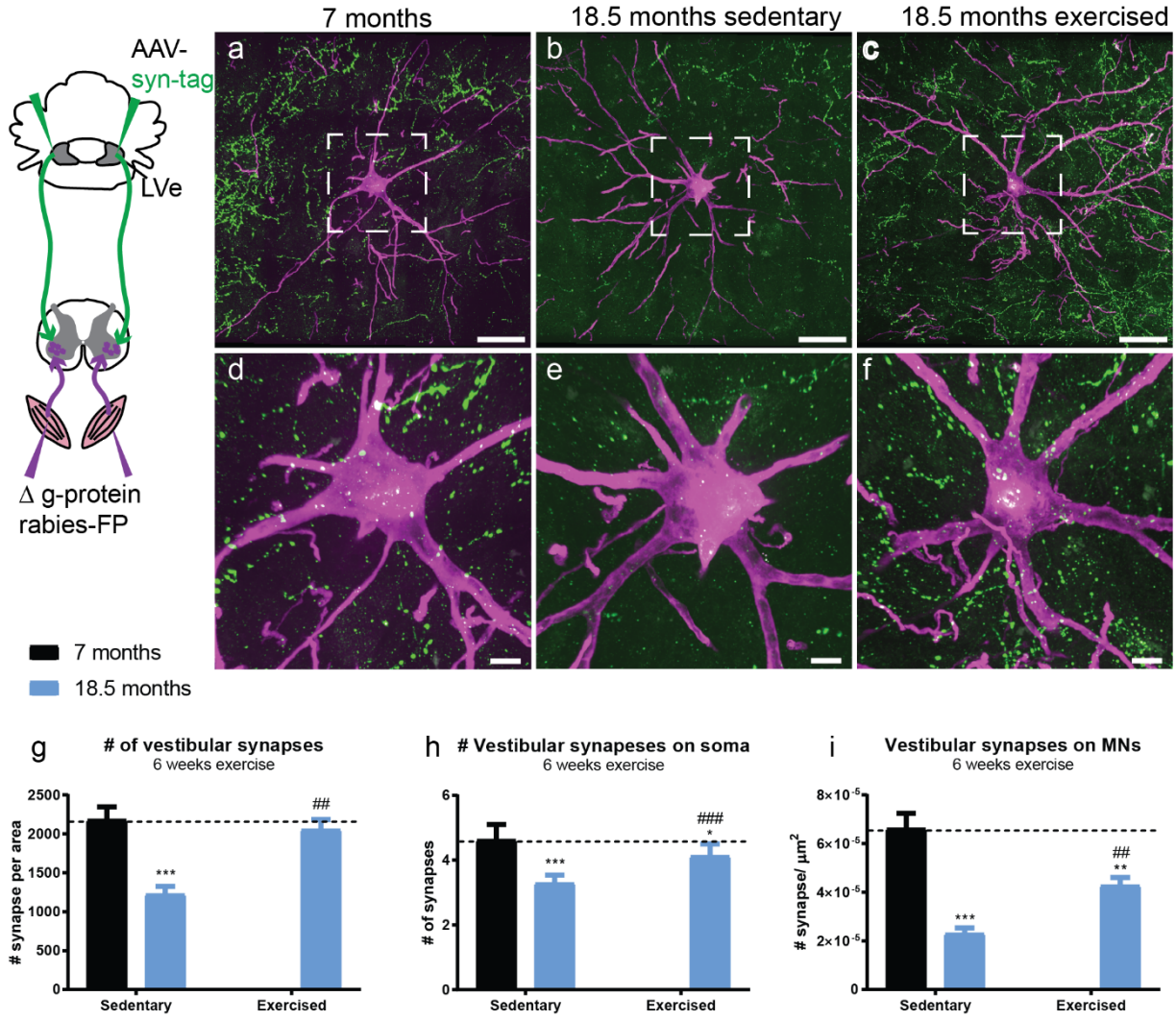
a, b Distribution of vGlut1 synapses along TA motor neuron branch relative to the motor neuron root in percentage of all synapses. A minimum of 20 motor neurons from a minimum of five different animals per group were analysed. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

5.8 Exercise increased the vestibular input to motor neurons.

Since not only proprioceptive but also vestibular input is necessary to ensure proper balance, we next asked if the improvement in balance in response to exercise could be mediated by an enhanced vestibular input to motor neurons. To pursue this question, we traced vestibular monosynaptic contacts to motor neurons by injecting an AAV-virus containing a plasmid with a synaptophysin-GFP-tagged fusion (AAV-Syn-tag) protein into the LVe and traced the soleus motor neuron pool using g-protein deleted rabies virus. **Fig. 25a to f** show representative images of soleus motor neurons in purple and vestibular synapses in green. We detected fewer synapses over the whole motor neuron area (**Fig. 25g**) and fewer vestibular synapses on the cell soma (**Fig. 25h**) in aged sedentary mice compared to 7-month-old controls. Moreover, in aged mice the vestibular synaptic density was significantly decreased (**Fig. 25i**). In contrast, motor neurons from aged exercised mice had more synapses over the whole motor neuron area (**Fig. 25g**), a higher synaptic density (**Fig. 25i**) and more synapses on the cell soma (**Fig. 25h**) than aged sedentary mice. In addition, we analysed the synaptic distribution along motor neuron branches. We found fewer synapses between 20 and 120-µm distance from the motor neuron root in aged sedentary mice compared to 7-month-old controls but the overall distribution was not shifted by age or exercise (**Fig. 26a**). To exclude that changes in synapse content on the level of motor neurons could be due to a different virus infection rate we analysed coronal sections from AAV-syn-tag injected brains that were labelled for NeuN in red, and Dapi in blue (**Fig. 27a**). We counted the number of GFP infected LVe neurons and observed no significant difference in the number of infected neurons between aged sedentary and exercised mice (**Fig. 27c**).

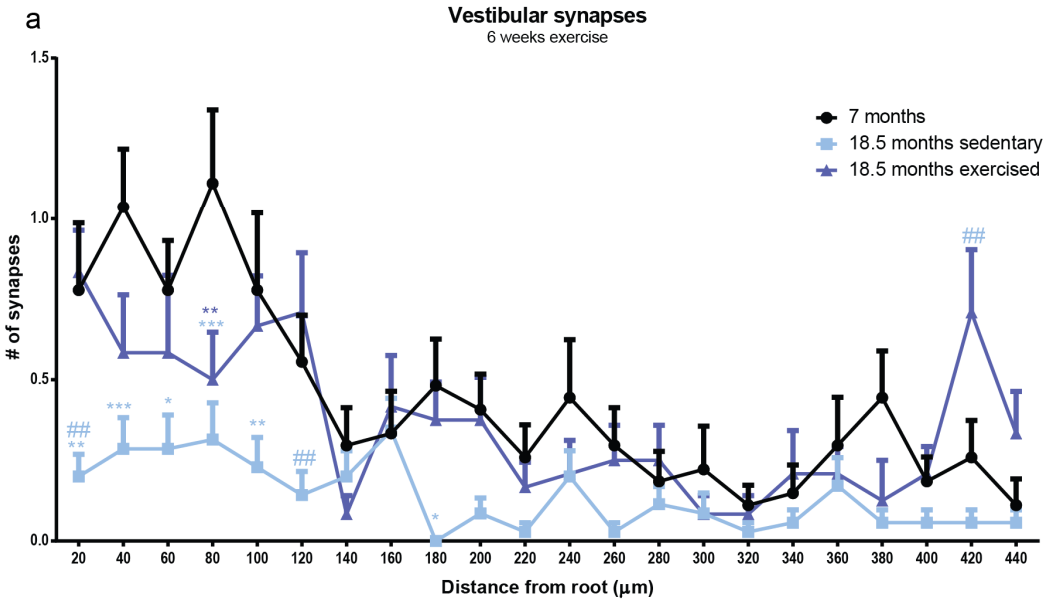
Taken together, these results show that exercise ameliorated the age-related loss in vestibular synaptic connections to motor neurons.

Fig. 25: Vestibular input to soleus motor neurons



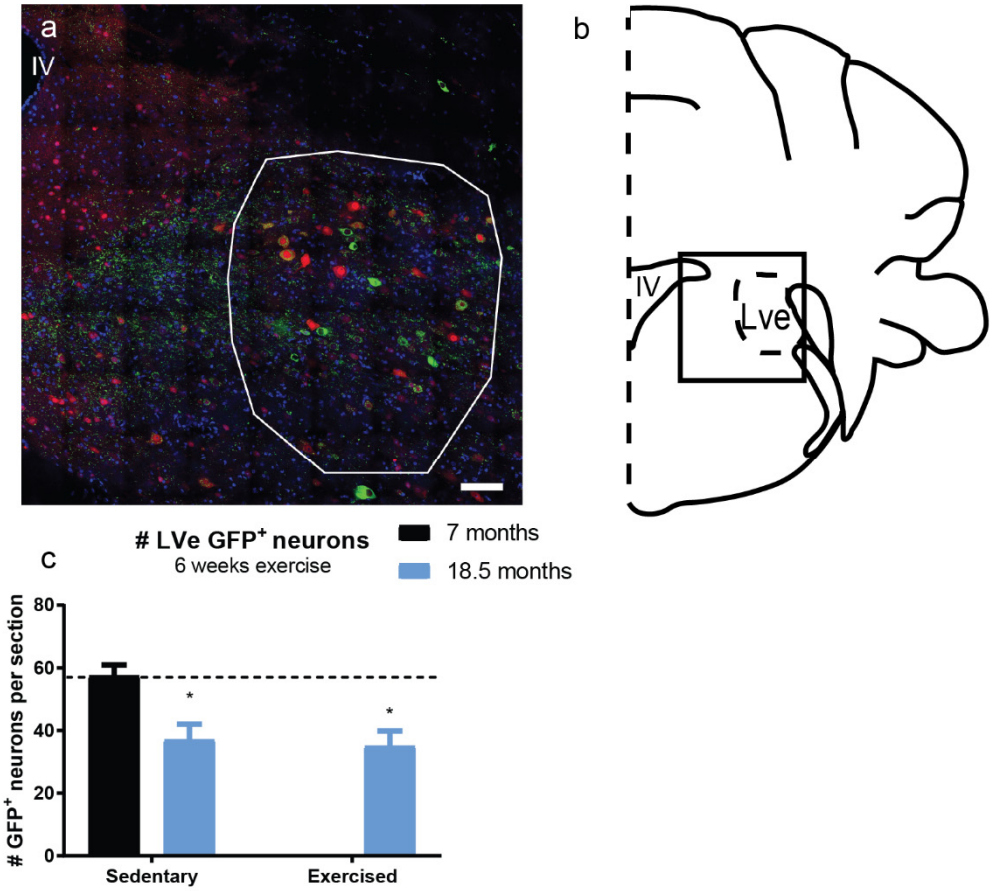
Vestibular synapses were traced by injecting an AAV-virus containing a synaptohysin-GFP fusion protein into the LVe. **a to f** Representative images of soleus motor neurons (purple) with vestibular synapses (green). **g** The number of synapses over the whole motor neuron area. **h** The number of synapses on motor neuron soma and **i** the synaptic density. **j** The distribution of vestibular synapses along soleus motor neuron branch relative to the motor neuron root. A minimum of 41 motor neurons from a minimum of five different animals per group were analysed. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

Fig. 26: Distribution of vestibular synapses along motor neuron branches



Vestibular synapses were traced by injecting an AAV-virus containing a synaptohysin-GFP fusion protein into the LVe. **a** Distribution of vestibular synapses along soleus motor neuron branch relative to the motor neuron root. A minimum of 41 motor neurons from a minimum of five different animals per group were analysed. Data represent the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. #p < 0.05; ##p < 0.01; ###p < 0.001 indicate statistically significant differences between sedentary and exercised animals of the same age group. Significance was determined using Anova followed by Sidak's test.

Fig. 27: The number of LVe neurons infected with AAV-synaptophysin-GFP-tag



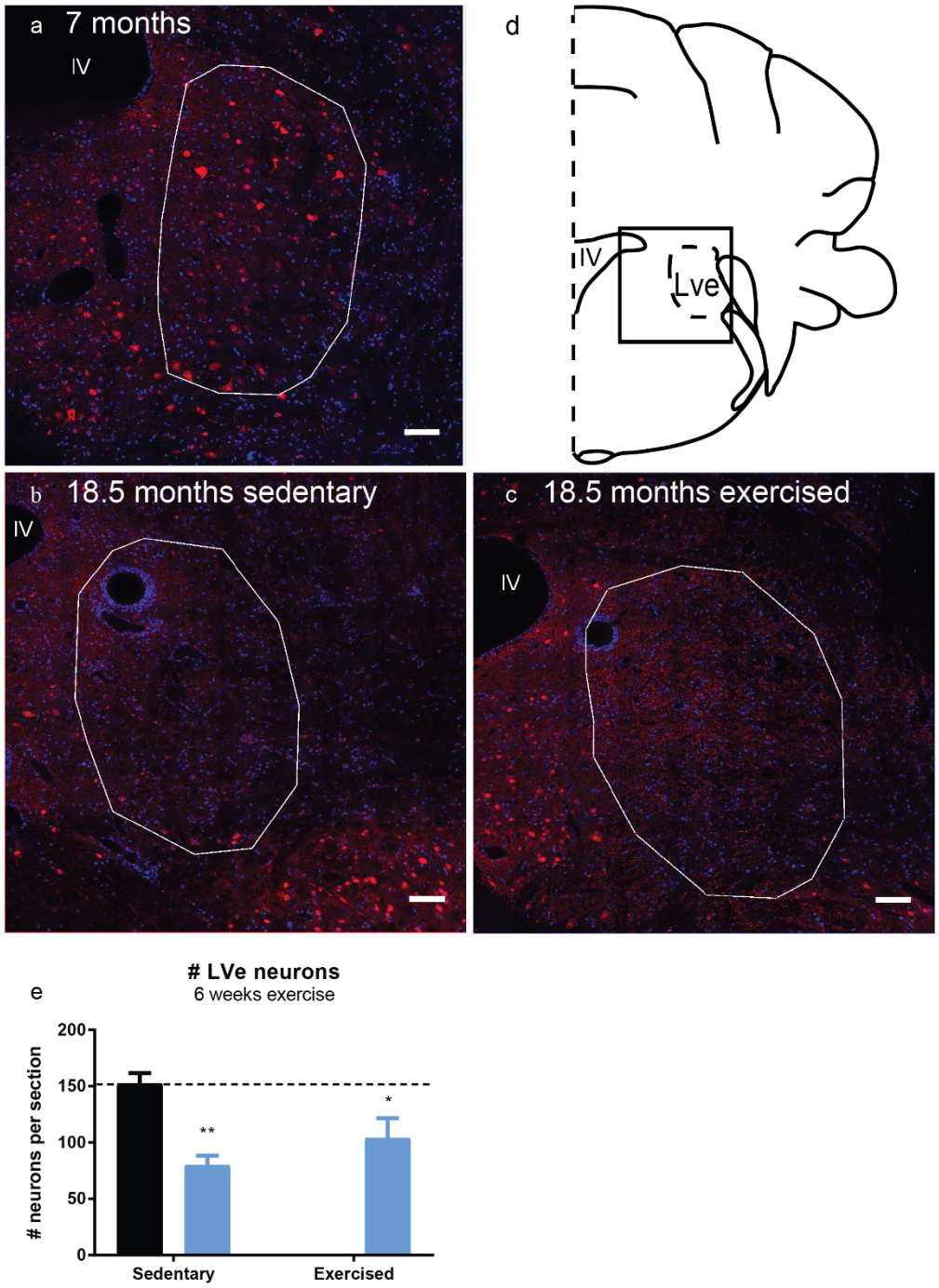
An AAV- virus containing a plasmid with a synaptophysin- GFP tag was injected into the LVe. **a** Representative image of LVe region from coronal brain section labelled with NeuN (red) and dapi. **b** Schema of coronal brain section with IV ventricle and the LVe region (dotted line). The square represents the field of view of the image. **c** The number of infected LVe neurons per section. n = 3 to 4 sections per animal from a minimum of five animals. scale bar = 100 µm. Data show mean ± SEM. *p < 0.05 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals.

5.9 The number of LVe neurons decreased with age.

Since the loss in vestibular synaptic connections to motor neurons was ameliorated by exercise, we next asked if an increased number of LVe neurons could be responsible for the improvements in response to exercise. **Fig. 28a to c** show representative images of coronal brain sections that were labelled for NeuN (red) and Dapi (blue). On sections from aged mice, we counted fewer LVe neurons compared to 7-month-old mice. However, we observed no significant difference in the number of LVe neurons between aged sedentary and exercised mice (**Fig. 28c**).

Collectively, we showed that although the number of LVe neurons was decreased with age there was no significant change between sedentary and exercised mice, showing that the improvement in vestibular synaptic content on motor neurons in response to exercise was not due to an increased LVe neuron number.

Fig. 28: The number of LVe neurons with age and exercise



a to c Representative images of the LVe from coronal brain sections labelled for NeuN (red) and dapi. **d** Schemaic overview of brain section with IV ventricle and the LVe region (dotted line). The square represents the field of view of the images. **e** The number of LVe neurons per section. n = 3 to 4 sections per animal from a minimum of five animals. Scale bar = 100 µm. Data show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month controls, represented as dotted line, and aged animals. Significance was determined using Anova followed by Sidak's test.

6 Discussion and conclusions

6.1 Effects of age and exercise on the neuromuscular system

Old age affects all cells and organs, leading to accumulation of DNA damage, increased cellular and mitochondrial ROS release, disturbed proteostasis and loss of regenerative capacity due to stem cell senescence (Aunan et al., 2016), (see 3.1.1Molecular mechanisms of aging). In the neuromuscular system, old age is characterized by a net loss of muscle mass and function, changed fibre type distribution, and decreased neuromuscular transmission exacerbating loss of muscle function leading to skeletal muscle fragility and increased morbidity and mortality in affected elderly people (Nair, 2005), (see 3.1.3 Sarcopenia). However, the sequence of events in the progression of sarcopenia is still a matter of debate and the question if the loss of muscle mass is the cause or the consequence of altered neuromuscular integrity is still debated.

Similar to the NMJ, the sciatic nerve also experiences age-associated changes. Here, we show that the amount of axons in the sciatic nerve that are positive for the calcium binding protein PV, is decreased with age. Interestingly, reduced levels of PV in peripheral nerves and motor neurons have been suggested to increase the vulnerability to neurodegeneration in diabetic neuropathy and motor neuron disease conditions, respectively (Endo & Onaya, 1986; Ince et al., 1993). Since PV has been suggested to be involved in neuronal calcium buffering (Chard, Bleakman, Christakos, Fullmer, & Miller, 1993; Seto-Ohshima, 1994), the decreased amount of PV in the aged sciatic nerve could represent early age-associated signs of calcium-induced toxicity, contributing to neurodegeneration, as has been suggested for other neurodegenerative diseases like Alzheimer's or motor neuron diseases

(Duchen, 2012; Sattler & Tymianski, 2000). Expression levels of proteins, involved in axonal transport, were already altered in the sciatic nerve at 18-months of age (Krishnan et al., 2016). Similarly, the NMJ also shows early age-associated changes in morphology (Michael R. Deschenes, Roby, Eason, & Harris, 2010; Valdez et al., 2010), further suggesting that the peripheral nerves experience age-associated changes before onset of muscle mass loss.

Surprisingly, we observed an increase in sciatic nerve fibre size in the absence of motor neuron loss in aged mice, contrasting studies done in humans, where old age has been reported to be accompanied by a loss of large myelinated fibre size and decreased lumbar motor neurons (Tomlinson & Irving, 1977; Ugrenović et al., 2016). In line with our findings, an increase in peripheral nerve fibre size with age was also observed in rodents (Hashizume & Kanda, 1995; Krause Neto et al., 2017). Thus, the discrepancy could be due to differences in rate of aging between human and animals. However, in other rodent studies, a regression in peripheral nerve fibre size with age was also reported (Jeronimo, Jeronimo, Filho, Sanada, & Fazan, 2008; Sakita, Murakami, & Fujino, 2016). Therefore, it is more likely that the opposing observations concerning neve fibre size could be due to the in-between differences in peripheral nerves and to differences in the age of animals. Indeed, we have observed alterations in nerve fibre size already in 18.5-month and 23.5.-month old mice. Although muscle mass in these mice was not yet significantly reduced, we observed a reduction in grip strength. The decreased force in aged mice could be due to lower neuromuscular transmission, as it was observed that the amplitude of sciatic nerve excitability is already lower in 20-month old mice (Walsh et al., 2015). Therefore, increased nerve fibre size in the sciatic nerve could represent a compensatory response to cope with decreased conduction velocity due to altered quality of nerve integrity.

Endurance exercise has been reported to increase grip strength, improve recovery and increase the sciatic nerve fibre size after nerve-crush injury (Bobinski et al., 2011; van Meeteren, Brakkee, Hamers, Helders, & Gispen, 1997). Consistently, voluntary wheel running improves NMJ integrity in aged rats (Valdez et al., 2010), showing the potential of endurance exercise to ameliorate neuromuscular age-related alterations (see 3.1.4 Exercise as a therapeutic intervention). However, we failed to detect any effect on the sciatic nerve composition and size or grip strength in aged mice. It is possible, that the duration or the timing of the exercise intervention was insufficient to illicit changes in the sciatic nerve. However, nerve-crush injury leads to a stark remodelling of muscle fibres and function, whereas our mice did not yet have a muscle mass loss and did not yet show severe aging symptoms. Nevertheless, it is possible that endurance exercise during midlife could delay the onset of severe aging pathologies. Therefore, it would be interesting to assess the efficacy of endurance exercise during midlife in ameliorating the decay of the sciatic nerve integrity in very old sarcopenic animals.

In sum, endurance exercise was ineffective in preventing these early neuromuscular degenerative events. Nevertheless, based on the evidence presented her, we hypothesize that age-associated alteration in the sciatic nerve size, in the amount of PV expressing nerve fibres and in NMJ integrity contribute to decreased neuromuscular transmission and loss of muscle fibre innervation, consequently leading to muscle atrophy.

To conclude, these findings indicate that sarcopenia likely is of neurogenic origin. Future studies should investigate the potential role of PV in calcium-induced toxicity leading to neurodegenerative events in the sciatic nerve.

6.2 Effects of age on the proprioceptive system

The sensory feedback of the muscle spindles to motor neurons are crucial for motor coordination, balance and gait. Elderly humans experience a loss of balance and proprioception (Lei & Wang, 2018; Lord & Ward, 1994) which is reflected in the risk of falls in this age population. An improved understanding of the processes that control balance in aging is therefore instrumental to mitigate the dramatic loss in quality of life, and the increase in morbidity and mortality of elderly individuals.

We now show that the amount of proprioceptive feedback to motor neurons decreases with age in the absence of a loss of motor neurons, confirming that age-related remodelling of the motor neuronal synaptic tree occurs before motor neuronal loss, as has been suggested before (Maxwell et al., 2018). In addition, we show that the size of intrafusal fibres decrease in an age-dependent manner in the absence of overall decreased muscular mass, suggesting that extrafusal fibres do not exhibit atrophy at the observed age and that intrafusal fibre atrophy could represent early age-associated degeneration of muscle spindles independently of extrafusal fibres. Furthermore, proprioceptive sensory neuron coil width, innervating intrafusal fibres, are widened further indicating functional decay of muscle spindle firing. These age-associated alterations in the muscle spindle morphology are similar to the observation of human and rodent studies (Kararizou et al., 2005; Kim et al., 2007; Liu et al., 2005; Swash & Fox, 1972; Vaughan et al., 2016), confirming deterioration of the aged muscle spindle. In elderly humans, the balance capacity and gait parameters are changed with age, which is consistent with our observation in aged mice, where balance, locomotive speed and cadence were altered with age. Thus, it is likely that altered muscle spindle morphology and decreased proprioceptive input to motor neurons are mainly responsible for loss of proprioception

in elderly humans. However, what is driving these age-associated changes on the proprioceptive system is unknown.

Interestingly, conditional satellite cell depletion in skeletal muscle of adult mice leads to changes in gait, reduced running wheel activity, and loss of balance concomitantly with increased capsular thickness of muscle spindles and intrafusal fibre atrophy (Jackson et al., 2015) reminiscent of aged muscle spindles. Therefore, it is possible that decreased satellite cell activation, as is known to occur during the aging process (Rando, 2005), could reduce the regenerative capacity of intrafusal fibres, which could contribute to aberrant proprioceptive sensory neuron innervation of intrafusal fibres leading to a loss of proprioceptive input to motor neurons. However, proprioceptive coil width widening could also point towards denervation induced atrophy of intrafusal fibres. Interestingly, in rats one year after peripheral nerve injury and subsequent reinnervation proprioceptive vGlut1$^+$ synapses on motor neurons were not restored even though motor innervation was re-established. Importantly, the loss of other synapse, such as vGlut2$^+$ synapses, were restored after re-innervation (F. J. Alvarez et al., 2011), indicating that the selective loss of proprioceptive vGlut1$^+$ synapses is responsible for the diminished stretch reflex in nerve-injured rats (Bullinger et al., 2011). Thus, it is conceivable that aging could lead to denervation of muscle spindle afferents and decreased muscle spindle firing. Absence of muscle spindle firing could have promoted the selective loss of vGlut1$^+$ proprioceptive synapses on motor neurons further leading to decreased stretch reflex activity.

Consistent with this chain of thought, sciatic denervation leads to intrafusal fibre atrophy accompanied by a reduction in NT3 expression (Copray & Brouwer, 1997; Schroder, Kemme, & Scholz, 1979). Interestingly, expression of NT3 in intrafusal fibres has been shown to be necessary for normal muscle spindle maturation, but the role of NT3

signalling in muscle spindle maintenance during adulthood is not known. The finding that NT3 mRNA levels in intrafusal fibres decrease after sciatic denervation and increase again after subsequent reinnervation in adult animals suggest that NT3 could also play a role in muscle spindle maintenance and axon guidance during adulthood. In addition, during muscle spindle development, NT3 has been shown to be important to modulate the strength of proprioceptive feedback to motor neurons. Strikingly, intramuscular supplementation or muscle specific overexpression of NT3 increases the number of innervated intrafusal fibres and improves recovery after injury (Taylor et al., 2005; Wright et al., 2002). Because NT3 levels within the whole muscle are lower with age (Y. Ming et al., 1999b), reason stands that also intrafusal derived NT3 might be reduced with age. Therefore, lower levels of NT3 in intrafusal fibres could contribute to aberrant proprioceptive sensory neuron innervation of intrafusal fibres, which could lead to denervation, fibre atrophy and reduced proprioceptive input to motor neurons. Furthermore, to what extent γ-moto neuron innervation changes during the aging process and if it is needed for intrafusal fibre maintenance remains to be investigated. However, neuromuscular chemical denervation in rats showed that the majority of muscle spindles lacked γ-moto neuron innervation while most of the proprioceptive sensory connections were re-established four weeks after (Wolf & English, 2000), suggesting that γ-moto neuron innervation might be more vulnerable to insults than proprioceptive sensory axons. Thus, it is conceivable, that also age-related degeneration of γ-moto neuron innervation could also contribute to intrafusal fibre atrophy.

Finally, inhibitory interneurons, such as Renshaw cells and Ia inhibitory interneurons also modulate the strength of the stretch reflex. However, it is not known how interneuron connectivity to motor neurons changes during old age. Data from ALS mouse models

suggest that decreased inhibition from Renshaw interneurons are implicated in the disease-associated motor neuronal loss (Ramírez-Jarquín, Lazo-Gómez, Tovar-y-Romo, & Tapia, 2014; Wootz et al., 2013), suggesting that this neuronal subpopulation could be vulnerable to damage. Therefore, it is possible that altered Ia and Renshaw interneuron connectivity could also contribute to decreased proprioceptive function with age.

Taken together, the muscle spindle experiences age-associated degeneration that could be explained by altered satellite cell activation and NT3 levels in intrafusal fibres. Reduced NT3 levels could have contributed to denervation of muscle spindles leading to decreased muscle spindle firing and a loss of proprioceptive input to motor neurons.

Future studies should consider the role of muscle spindle derived NT3 in modulation of the stretch reflex, muscle spindle integrity and proprioceptive input to motor neurons during the aging process.

6.3 Exercise improves the vestibular input to motor neurons

Proprioceptive muscle spindles and the lateral vestibular nuclei both project directly to motor neurons, where input from both systems is integrated to adapt motor output (Basaldella et al., 2015; Liang et al., 2014; Proske & Gandevia, 2012). As discussed in the previous subchapter, the muscle spindle experiences age-associated detrimental changes and proprioceptive input to motor neurons is decreased, likely contributing to the decreased balance capacity in elderly people. Interestingly, physical activity improves balance, posture and gait in elderly humans (Cadore et al., 2013; Carter et al., 2001; Lopopolo et al., 2006; Perrin et al., 1999; Shigematsu et al., 2002; Simmons & Hansen, 1996). However, despite the importance of these sensory feedback systems involved in

balance, the effect of age and exercise on vestibular and proprioceptive circuits remains elusive.

In humans, it has been reported that the amount of sensory neuron hair cells in the otolithic organs and semicircular canals in the inner ear decrease with age (Rauch et al., 2001). Concomitantly, the vestibular ganglia experience neurodegeneration (Park et al., 2001; Velázquez-Villaseñor et al., 2000). However, the extent of sensory hair cells loss is being questioned (Brosel et al., 2016; Gopen, Lopez, Ishiyama, Baloh, & Ishiyama, 2003; Ivan Lopez et al., 2005). Evidence suggests that degeneration of sensory hair cell might only contribute very little to the loss of vestibular function and that altered central processing within the vestibular system might be more involved in the deterioration of vestibular function.

We now show that in aged mice, descending vestibular input to motor neurons is substantially decreased, even more so than proprioceptive input to motor neurons, suggesting that decreased vestibular input could be one of the main drivers for loss of balance in elderly humans. Strikingly, exercise mitigated the decrease in vestibular input for some parameters to levels that were indistinguishable from young control mice, indicating that the improved balance performance in exercising mice is likely due to elevated vestibular input to motor neurons. Interestingly, this exercise-mediated boost in vestibular synapses in the spinal cord occurred in the absence of a protective effect of training on the neurodegeneration in the LVe that was also observed in mice (Sturrock, 1989) and humans (Diaz et al., 1993). In line with degeneration of LVe neurons with age, another study also showed decreased regenerative capacity of LVe axons after spinal cord lesion (Roozbehi et al., 2015), suggesting a high vulnerability of LVe axons to age-associated degeneration. Although the underlying cause for this neurodegeneration is not

known it has been suggested that accumulated lipofuscin, granules containing lipid residuals from lysosomes, especially in the lateral vestibular nucleus could be implicated in the neurodegeneration of vestibular complex neurons (J. C. Alvarez et al., 2000). Strikingly, in response to vestibular injury, neurogenesis in the vestibular nuclei was increased (Tighilet & Chabbert, 2019). Since exercise is known to increase neurogenesis in other brain regions in the hippocampus (van Praag et al., 2005) and the subventricular zone (G.-L. Ming & Song, 2011), we wondered if training could also promote neurogenic events in the vestibular nuclei. To that end, we injected aged exercising mice for three consecutive days with bromodeoxyuridine (BrdU) to label proliferating cells. However, after 6 weeks of exercise we failed to detect any proliferating cells in the vestibular nuclear complex (data not shown).

Interestingly, loss of peripheral vestibular function can be partly compensated (Sjögren et al., 2018). However, the neuronal plasticity underlying the mechanism of vestibular compensation is unknown. Our observation of increased vestibular input could suggest that modulation of vestibular input to motor neurons in response to exercise could represent a response involved in vestibular compensation. Interestingly, the spinovestibular tract projects from the lumbar spinal cord to the vestibular nuclei (Pompeiano, 1972), but the functional implication of these projections are unknown. Strikingly, limb movement generate vestibular neuron firing in decerebrated and conscious cats (Arshian et al., 2014; Bankoul et al., 1995; A. A. McCall et al., 2016) and it has also been reported that the LVe receives indirect input from neck proprioceptors (Sato et al., 1997). These findings raise the possibility that limb proprioceptors could feedback on the LVe to modulate LVe firing. Therefore, future studies should investigate the effect of age and exercise on spinovestibular projections to vestibular neurons.

The observation that vestibular input is increased, overcoming the overall loss in the LVe neuron number, supports a model in which endurance exercise could promote the regeneration of descending vestibular projections by promoting axonal sprouting and synapses formation, compensating for the decline in overall neuronal number. Functionally, the restoration of vestibular contacts to motor neurons seems sufficient to significantly enhance balance and gait in old, trained animals. Exercise-induced axonal sprouting as a potential explanation for the increased synaptic connections despite reduced neuronal numbers would be in line with observations that treadmill training promotes axonal sprouting leading to improved motor recovery after spinal cord hemi-section (Houle & Cote, 2013). The mechanistic basis of our results on vestibular connectivity or the axonal sprouting in spinal cord lesions is unknown, but could involve neurotrophic factors. First, mice lacking BDNF exhibited balance and motorcoordination issues reflected in neurodegenerative events in the vestibular connective networks (Patrik Ernfors, Lee, & Jaenisch, 1994). Second, exercise increases neurotrophic factors in muscle and spinal cord (English et al., 2014; Gomez-Pinilla et al., 2001; Molteni et al., 2004; Sakuma & Yamaguchi, 2011). Third, BDNF, NT3 and GDNF have been shown to promote axonal recovery and to increase synapse formation in spinal cord-injured animals (Deng et al., 2013; Ye & Houle, 1997). Therefore, future studies will aim at investigating the regulation and role of neurotrophic factors in motor neuron innervation by the vestibular system with age and exercise.

Unlike the vestibular system, exercise training did not improve proprioceptive input to motor neurons or muscle spindle morphology in old mice. Consistently, proprioceptive input to motor neurons with age was lower in aged sedentary as well as in aged exercised mice. However, surprisingly, the amount of proprioceptive synapses on motor neurons

was even lower in aged exercised mice than in aged-matched sedentary controls, while the synaptic density was similar between the two groups. These findings suggest that exercise could slightly shift the vGlut1 synaptic distribution. Altered vGlut1 synaptic distribution, has been observed in SMA mouse models (Mentis et al., 2011). However, exercised mice still perform substantially better on the balance beam, indicating that increased vestibular input could be sufficient to overcome the supposed detrimental effects of changed proprioceptive synapse distribution.

In contrast to vestibular input, proprioceptive input was not improved in response to exercise, at least at old age. It is conceivable that the training paradigm, such as resistance exercise, might be important to differentially modulate the vestibular and the proprioceptive system. However, it has been reported that the adaptation to resistance training in the H-reflex latency, used to evaluate the strength of proprioceptive feedback to motor neurons, (see 3.2.2 The proprioceptive spinal circuits) was lost in aged human subjects compared to young, although the overall muscular strength was improved (Scaglioni et al., 2002). These findings suggest that resistance exercise is probably also not the ideal strategy to modulate the proprioceptive system. Finally, it is also possible that the training protocol used in this study was insufficient to affect the proprioceptive synaptic density, e.g. in terms of volume intensity or duration. It will thus be interesting to see whether future studies reveal an effect of life-long endurance exercise on proprioceptive and vestibular circuitry.

To conclude, age-related changes in gait and balance are reflected by deterioration of morphological proprioceptive and vestibular synaptic networks. Improvements in balance due to exercise are not mediated by enhanced proprioceptive feedback but by increased

vestibular input to motor neurons that could involve the promotion of synaptic sprouting on the level of spinal circuits.

7 Future prospective

Elderly people above age 65 have a very high prevalence to fall, greatly reducing the quality of life and independence in affected people (Lei & Wang, 2018; Rubenstein, 2006). Unfortunately, pharmacological treatment is so far not possible because knowledge about the neuronal mechanisms are lacking. Exercise has been shown to have positive effects on balance and body posture (Lelard & Ahmaidi, 2015; Rooks, Kiel, Parsons, & Hayes, 1997; Ruffieux et al., 2017; Shigematsu et al., 2002). However, especially elderly people tend to exercise less. Therefore, it is important to promote physical activity in the elderly (Hughes et al., 2018). Since the age group above 60 is the fastest growing population (World population aging 2015, United Nations), it is vitally important to improve our understanding of why aged people fall and to improve treatment strategies. Despite the existing knowledge about the principle components and functions of the proprioceptive and vestibular system in balance, motor coordination and body posture (Angelaki & Cullen, 2008; Proske & Gandevia, 2012), the role of age and exercise on the proprioceptive and vestibular connectivity to motor neurons is not well understood.

Here we show that endurance exercise increased the vestibular input to motor neurons concomitant with a striking improvement in balance capacity and gait in aged exercising mice. Although proprioceptive input was not affected by exercise, the moderate training of aged mice was sufficient to overcome the age-associated decrease in vestibular and proprioceptive input and neurodegeneration in the vestibular nuclear complex. Therefore, it is likely that balance in aged exercising mice is improved by increased vestibular input. Of note, endurance training at old age, at moderate dose (3 times per week), and for different times, was sufficient to improve balance and gait. It will be interesting to

compare this outcome on neuronal connectivity and phenotypic adaptation to life-long training studies and to evalutate the persistence of this effect when the training ceases.

It is still unclear how exercise increases the number of vestibular synapses on motor neurons. As it has already been reported that exercise training increases axonal sprouting after spinal cord injury (Houle & Cote, 2013), increased vestibular synapses could be explained by exercise-induced sprouting of descending vestibular axons to motor neurons. To follow up on this hypothesis, the LVe of aged trained mice could be injected with an anterograde neuronal tracer, thereby labelling LVe axons and motor neurons could be retrogradely traced using rabies virus. By use of light sheet imaging, the number of descending axonal sprouts connecting to motor neurons could be determined.

Furthermore, it is unknown what triggers these plastic neuronal changes in the vestibular connective networks in response to exercise. Exercise increases neurotrophic factors in muscle and spinal cord (English et al., 2014; Gomez-Pinilla et al., 2001; Molteni et al., 2004; Sakuma & Yamaguchi, 2011). In turn, neurotrophic factors can enhance axonal recovery and synapse formation in spinal cord-injured animals (Deng et al., 2013; Ye & Houle, 1997). Therefore, it is plausible that neurotrophic factors could be involved in modulating the increased vestibular input in response to exercise. To follow up on these hypotheses, supplementation of neurotrophic factors to the spinal cord of aged mice could be used to ascertain if neurotrophic factors would have a similar beneficial effect on the vestibular input to motor neurons and balance than exercise. Strikingly, loss of BDNF leads to decreased balance capacity and motorcoordination difficulties that are related to neurodegenerative events in the vestibular connective networks (Patrik Ernfors et al., 1994). Therefore, BDNF could be a likely candidate to test in this experimental setting.

In addition, mass spectrometric analysis of the spinal cord of aged exercised mice could help to identify new factors that are regulated by exercise in the spinal cord.

In addition to proprioceptive and vestibular contacts, motor neurons also interconnect with a complex network of inhibitory interneurons, such as Ia interneurons and Renshaw cells modulating the strength of the proprioceptive stretch reflex (Rosales & Dressler, 2010; Windhorst, 2007). To what extent altered interneuron connectivity is contributing to age-associated degeneration of proprioceptive and vestibular reflexes is unknown. Interestingly, spinal interneuron activity is needed for the generation of rhythmic locomotive patterns (Ramírez-Jarquín et al., 2014). Therefore, the question can be raised if altered gait in response to aging could be due to altered interneuronal connectivity and if the improvement in gait with exercise could be reflected on the level of spinal interneuron networks. Therefore, it would be interesting to investigate the effect of age and exercise on spinal inhibitory interneurons.

Combined with our findings, the results of the studies proposed above could help to enhance our understanding of why elderly people fall. Because of technical restraints, changes in proprioceptive and vestibular synaptic networks with age and exercise cannot be studied in human patients. This is why experiments with mice such as described above will be key in understanding the underlying neuropathological mechanism. Importantly, studying the process of balance improvement in response to exercise could prove useful in advancing pharmacological treatment strategies against age-associated loss of balance and could thus prevents falls in elderly humans.

8 Methods

Animals

All animal experiments were done with male C57BL/6J mice that were obtained from Janvier. Animals were fed ad libitum with regular chow diet and kept under a 12-h/12-h light–dark cycle at 23°C. All experiments were performed in accordance with the Swiss federal guidelines for animal experimentation and were approved by the Kantonales Veterinäramt of the Kanton Basel-Stadt.

Exercising protocol

Mice aged 15.5 and 20.5 month were trained on a treadmill for 12 weeks, 3 times per week 1 hour. Additionally, 17- month- old mice were exercised on a treadmill for 6 weeks, 3 times per week for 45 min. All exercising animals had free access to running wheels during the intervention period.

The treadmill speed was determined prior to the intervention by testing the animal´s maximum speed. Accordingly, the training speed was gradually increased from 50% to 80% maximum speed at an angle of 5º or until mice could not keep up pace during training sessions.

The protocol to assess the maximum speed was set at 8m/min for 3 min and was increased every 2 min by 2m/min at an angle of 5º until mice were exhausted.

In vivo testing

Unless otherwise stated all in vivo test were done 4 days before, 6 weeks after, and 12 weeks after the start of the exercise intervention. When animals were exercised for 6 weeks, in vivo test were done 4 days before and 6 weeks after the intervention.

Rotarod

Mice were put on a horizontal rotating rod (Ugo Basile) suspended from the ground. Since mice are naturally afraid to fall they will try hard to remain on the rotating rod. Mice were acclimatized for three consecutive days. On the first day, mice were put on the rotating rod three times for 3 min at 5rpm, the second day at 8rpm and third day at 12rpm. On the day of testing, the rotation speed was gradually increased from 5rpm to 68rpm during 7 min. The time until a mouse falls was recorded and the average of three trials was calculated as a measure for motor coordination.

Balance beam test

Mice were taught to cross an inclined beam. They were familiarized with the task 1 day before the testing by giving them three practice trials. Doing this, mice learned to expect a red hiding box at the end of the beam, which ensured the animal´s compliance. The time to cross the beam and the number of slips were measured to assess balance performance. The intermediate and old age group were tested on a round balance beam system while the 6-weeks-exercise group was tested on a square beam. Thus for the 6-weeks-exercise group an additional control group aged 7 months was used for baseline testing.

Gait analysis

The gait of mice was assessed using the CatWalk XT system by Noldus. Briefly, mice walked voluntarily across a glass platform. Several paw prints sequences were captured by a camera located under the platform and gait parameters were calculated using the Noldus software.

Grip strength

Mice all fours were put on a grid connected to a force meter. To measure all limbs muscle strength mice were gently pulled by the tail against the grid to measure how much force was needed to break their grip on the grid.

Proprioceptive tracing

Motor neuron pools from GS muscles were retrogradely traced using a g-protein deleted rabies virus as done previously (Basaldella et al., 2015). Briefly, GS and soleus muscles of anesthetized mice were injected with 5 µl of virus. After 4 days of incubation mice were anesthetized and transcardially perfused.

Vestibular tracing

To trace descending vestibular mono-synaptic contacts to motor neurons, an AAV-virus containing a plasmid with a synaptophysin-GFP-Tag fusion protein was bilaterally injected into the lateral vestibular nucleus as done previously (Basaldella et al., 2015). Briefly, the AAV-virus was injected into the LVe using the coordinates 0.24mm antero-posterior, 0.134mm medio-lateral and 0.355mm dorso-ventral from lambda. The virus was incubated for 14 days after which the animals were perfused. 4 days before perfusion

the soleus muscle was injected with g-protein deleted rabies virus to trace soleus motor neuron pools.

Imunohisto-chemistry

Spinal cord DRG and sciatic nerve

Mice were anesthetized and transcardially perfused with 20 ml saline solution followed by 70 ml 4% PFA in PB buffer. After perfusion, spinal cords, DRGs and sciatic nerves were dissected and post-fixed overnight in 4% PFA. After fixation, they were sucrose protected for 24 hours in 30% sucrose in PBS and then frozen and cut in a cryostat. Spinal cord sections 60 µm thick were blocked 1 hour in 4% BSA 5% goat serum in 0.5% Triton-X-100 at room temperature and then incubated overnight in primary antibody at 4 ºC followed by three washes in PBS. Spinal cord sections were incubated overnight in secondary antibody at 4 ºC and then washed three times with PBS before sections were mounted.

DRGs and sciatic nerves were cross-sectioned at 10-µm thickness were blocked 1 hour in 4% BSA 5% goat serum in 0.5% Triton-X-100 at room temperature and then incubated overnight in primary antibody at 4 ºC followed by three washes in PBS. Then they were incubated 1 hour in secondary antibody and washed three times with PBS before mounting.

Brains

Mice were anesthetized and transcardially perfused with 20 ml saline solution followed by 70 ml 4% PFA in PB buffer. After perfusion, brains were dissected and post-fixed overnight in 4% PFA and stored in PBS until sectioning. Brains were cut at 40 µm on a vibratome and blocked 1 hour in 4% BSA 5% goat serum in 0.5% Triton-X-100 at room temperature and then incubated for two nights in primary antibody at 4 ºC. Sections were

washed three times in PBS and incubated in secondary antibody at RT for 1 hour. After three washes of PBS brain sections were mounted with mounting medium on slides.

Muscle sections

Muscles were fresh-frozen in isopentane cooled by liquid nitrogen. To assess muscle spindles, 60 µm cross- sections from GS muscle were blocked 1 hour in 4% BSA 5% goat serum in 0.5% Triton-X-100 at room temperature and then incubated overnight in primary antibody at 4 ºC followed by three washes in PBS. Muscle sections were incubated 1 hour in secondary antibody and washed three times with PBS before mounting.

Muscle whole mounts.

EDL muscle was dissected and separated by their tendons into four fragments. EDL fragments were fixed in 4% PFA in PB buffer for 20 min and then washed three times followed by blocking in 4% BSA 5% goat serum in 0.5% Triton-X-100. Then, EDL fragments were incubated in primary antibody for 4 days at 4 ºC and then washed three times in PBS. After, EDL fragments were incubated for 2 days in secondary antibody at 4 ºC and then washed three times before whole-mounting on slides.

BrdU labelling to stain proliferating cells

Brain sections were incubated for 1h with Triton X100 0.5 % with 4% BSA and 5% Goat serum with Dapi 1:500. Sections were washed 3 times 5min. The DNA was denatured by incubating sections in 2N HCL for 20min at 37 degrees. To neutralize acid, sections were treated with Borax for 10min at RT. Sections were thoroughly washed minimu of three times 15min before sections were blocked with Triton X100 0.5 % with 4% BSA and 5% Goat serum for 1h. Sections were incubated overnight with anti-BrdU antibody 1:500 at 4 degrees. The next day, sections were washed three times 5min with PBS and before

incubation with secondary antibody for 1h. Finally, Sections were mounted on slides with mounting medium.

Target	dilution	Reference	Company
Anti-Laminin alpha 2	1:500	ab11576	abcam
Anit-s46	1:50		Developmental Studies Hybridoma Bank
Anti-NFH	1:200	AB1991	Chemicon
Anti-Parvalbumin	1:500	PV27	Swant
Anti-NeuN	1:500	ab134014	abcam
Anti-NeuN	1:100	MAB377	Millipore
Anti-vGlut1	1:20'000	AB5905	Chemicon
Anti-RFP	1:5'000	600-401-379	Rockland
Anti-GFP	1:1'000	A10262	Invitrogen
Anti-BrdU	1:500	ab6326	Abcam
A488 anti-rat	1:100	A11006	Invitrogen
A568 anti-mouse	1:100	A21124	Invitrogen
A647 anti-rabbit	1:100	711-605-152	Jackson Immuno Research
A568 anti-rabbit	1:100	A10042	Invitrogen
A647 anti-guniea pig	1:100	AP193SA6	Chemicon
A488 anti chicken	1:100	A11039	Invitrogen

Microscopy and image analyses

Motor neurons

20 tiles and stacks of the motor neuron tree were imaged on a spinning disk Nikon eclipse connected to a Visitron system with a 63x objective. For the proprioceptive tracing, we imaged stacks of 1 µm sampling. For the vestibular tracing, we imaged stacks with 300 nm sampling. Stacks and tiles were stitched together in Fiji. The motor neuron surface was reconstructed in Imaris and synapses were detected by the Imaris spot detection function. A matlab extension tool was used to calculate the number of spots touching the reconstructed motor neuron. The number of touching spots was normalized to the motor neuron surface. In the proprioceptive tracing, an area of 650 µm by 650 µm centred on the MN was evaluated.

Additionally, the synapse distribution on the motor neuron tree was analysed using Neurolucida. A blinded observer manually traced the outline of the motor neuron soma and the dendrites. All synapses touching the motor neuron were marked and the software calculated the distance of this mark to the motor neuron soma. The number of synapses contacting directly the cell body were counted manually. For analyses, a minimum of three motor neurons per animal was assessed.

Muscle spindles

To visualize muscle spindles with sensory nerve, muscle cross section and EDL whole mounts were labelled for s46, laminin, dapi and NFH. Muscle spindles were identified by s46 positive muscle spindle fibres surrounded by a capsule, nuclear aggregation within muscle spindle fibre and a NFH staining near muscle spindle fibres.

Muscle spindle from GS cross sections were imaged on a LSM 700 confocal system from Zeiss with a 63x objective. Muscle spindle number was counted per 20 serial sections and the minimum Feret diameter of s46 positive muscle spindle fibres was measured in Fiji. Sections from at least four animals per group were assessed and two to 6 muscle spindle-sections were analysed per animal and the average calculated.

Stacks of muscle spindles from EDL whole mounts were imaged on a LSM 880 confocal system from Zeiss with a 63x objective and 0.75 µm sampling. Muscle spindle coil distance and width were measured in Fiji. The average coil distance and width for each muscle spindle was calculated by measuring at least two coils. Overall, a minimum of two muscle spindles from at least five animals per group was assessed.

Sensory neuron

L4 DRG sections were imaged with an AxioScan.Z1 slide scanner from Zeiss using a 20x objective. Proprioceptive sensory neurons were identified by PV^+ cells. All sensory

neurons were labelled by NeuN. The number of PV and NeuN positive sensory neurons was counted manually by taking the average of minimum two sections from the same animal.

Sciatic nerve

Cross sections from sciatic nerve were imaged with an AxioScan.Z1 slide scanner from Zeiss using a 20x objective. Sections were labelled for PV and laminin. The number of PV^+ axons was counted manually. The average minimum Feret diameter of axons and their size distribution was measured in Fiji using a custom-made macro. The average of minimum two sections per animal was calculated.

Brain sections

Coronal brain sections were imaged on a spinning disk Nikon eclipse connected to a Visitron system with a 63x objective. To visualize neurons from the lateral vestibular nucleus sections were stained for NeuN and anti-GFP. To capture the whole vestibular nuclei area, 12 by 12 tiles were imaged and the lateral vestibular nuclei identified. Using several tiles and stacks with 0.8 µm sampling, for each brain section the number of GFP^+ and the total number of neurons in the lateral vestibular nucleus was counted manually by scrolling through the different image planes. The average neuron number from at least three different sections per animal was used to calculate an average neuron number per section from a minimum of five different animals.

Statistics

Unless otherwise stated, significance between groups was assessed using Anova followed by a Sidak multiple comparison test. In all Fig.s, the mean value and SEM was used to plot the data with error bars.

9 Side project: Effect of PGC-1α on balance and the proprioceptive system

9.1 Introduction

The peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) is transcriptional coactivator, involved among many other functions in the regulation of metabolism, such as gluconeogenesis in the liver, or browning of adipose tissue , as well as mitochondrial function and mitochondrial biogenesis (BAAR et al., 2002; Boström et al., 2012; Handschin, 2010; Yoon et al., 2001). In skeletal muscle, PGC-1α is induced by training favouring skeletal muscle fibre type remodelling (BAAR et al., 2002; Russell et al., 2003). Consistently, overexpression of PGC-1α in skeletal muscle leads to the conversion of fast twitch typ II fibres to slow twitch typ I fibres and a shift towards oxidative metabolism (Lin et al., 2002). On the other hand, loss of PGC-1α in skeletal muscle drives the shift from oxidative type I and IIa toward type IIx and IIb and decreases exercise capacity (Christoph Handschin et al., 2007). Moreover, aside from its effect on the muscle itself, muscle-derived PGC-1α can also promote the remodelling of the NMJ morphology affecting not only the post synapse but also to presynaptic structures (Arnold et al., 2014). In addition, overexpression of PGC-1α increases the frequency of muscle fibre innervated by presumably slow type motor neurons (Chakkalakal, Nishimune, Ruas, Spiegelman, & Sanes, 2010), further suggesting a PGC-1α dependent retrograde signalling between muscle and motor neurons.

In humans, age-related loss of PGC-1α has been implicated to decreased mitochondrial function with age (Ghosh et al., 2011), which could contribute to the development of sarcopenia (Johnson, Robinson, & Nair, 2013). Given the findings that PGC-1α promotes mitochondrial biogenesis, it was inferred that PGC-1α could slow down muscle aging (Anderson & Prolla, 2009; Leick, Lyngby, Wojtasewski, & Pilegaard, 2010). Strikingly, Gill et al, 2017 have previously found that PGC-1α also affects motor coordination and

balance in aged animals (Gill, Santos, Schnyder, & Handschin, 2017), suggesting that with age, PGC-1α could mediate retrograde crosstalk between the muscle and spinal circuits, important to guide motor coordination and balance.

The main sensory pathways that are involved in the regulation of balance are the proprioceptive and vestibular system that send direct monosynaptic input to motor neurons, affecting motor output (see 3.2 The proprioceptive system and 3.3 The vestibular system). Interestingly, the amount of proprioceptive and vestibular input to motor neurons between different motor neuron pools is antagonistically regulated. While fast motor neurons from flexors, such as TA motor neurons, receive more proprioceptive input, slow motor neurons within extensors, such as soleus motor neurons, are mainly contacted by vestibular descending projections but not proprioceptive ones (Basaldella et al., 2015). Since the fast-twitch TA muscle is predominantly glycolytic and the slow-twitch soleus oxidative, the question can be raised if shifting muscle fibre phenotype towards more oxidative metabolism would retrogradely affect proprioceptive feedback to motor neurons, as was already suggested to occur in the motor neuron neve terminals (Chakkalakal et al., 2010)

Thus, in this project we investigate if PGC-1α affects motor coordination and balance in aged animals by retrogradely affecting proprioceptive input to motor neurons. To that end, we used muscle specific knock out mice of PGC-1α, termed mKO, and mice with overexpression of PGC-1 α, termed TG and traced motor neurons from different motor neuron pools by use of g-protein deleted rabies varies as done previously (Basaldella et al., 2015).

9.2 Results

9.2.1 Pilot study with female PGC-1α mKO and TG mice

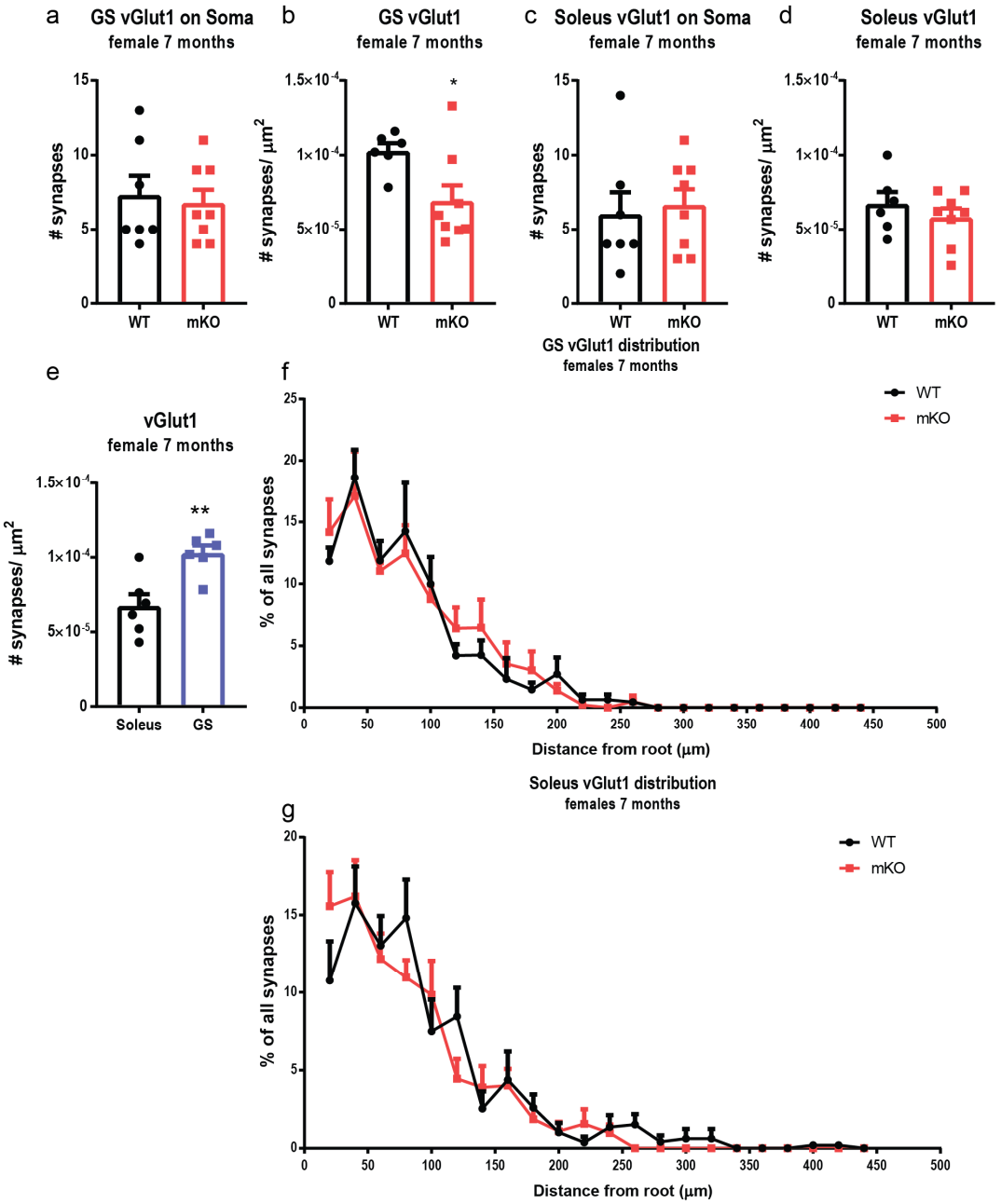
We quantified proprioceptive synapses by tracing motor neurons with rabies virus and co-stained with the proprioceptive synapse marker vGlut1. To establish the technique and to generate some preliminary data the tracing was only done on three female mice per group. Since it was already shown that soleus motor neurons receive less proprioceptive input than GS motor neurons (Basaldella et al., 2015), we compared vGlut1 synaptic density of soleus and GS motor neurons, serving as an internal control. Indeed, also in our hands, GS motor neurons had a higher synaptic density than soleus motor neurons (**Fig. 29a**). In **Fig. 29b to g** we assessed the number of vGlut1 synapses on the motor neuron soma, the synaptic density and the synaptic distribution of GS and soleus motor neuron pools of 7-month old female PGC-1α mKO mice. We did not observe significant differences in the synapse content or distribution on soleus motor neurons between WT and mKOs (**Fig. 29d to g**). Furthermore, the number of synapse on GS motor neurons (**Fig. 29b**) and the synaptic distribution (**Fig. 29f**) were also not altered by the loss of PGC-1α. However, GS motor neurons from PGC-1α mKO mice had a lower synaptic density than WT mice (**Fig. 29b**).

In contrast, in GS motor neurons from11-months-old female PGC-1α TG mice, the synaptic density, number of synapses on cell soma and synaptic distribution were not altered (**Fig. 30a, b, e**). However, we observed fewer vGlut1 synapses on the cell soma of TA motor neurons and synapses were distributed farther away from dendrite root while the overall proprioceptive, synaptic amount was not altered (**Fig. 30c to f**). To investigate if changes in proprioceptive synapse content and synaptic distribution affected the balance and motor coordination, we performed a balance beam and Rotarod test. We did

not detect any significant differences in Rotarod (**Fig. 31a**) and balance beam (**Fig. 31b, c**) performance in any of the groups, which could be due to the low amount of animals used to test in vivo behaviour. To increase *n* -number we repeated the balance beam test again with higher amount of animals in male PGC-1α mKO aged 12 months. However, also with increased *n*-number we failed to detect any significant effect of PGC-1α deletion on balance performance in mice (**Fig. 31g to h**).

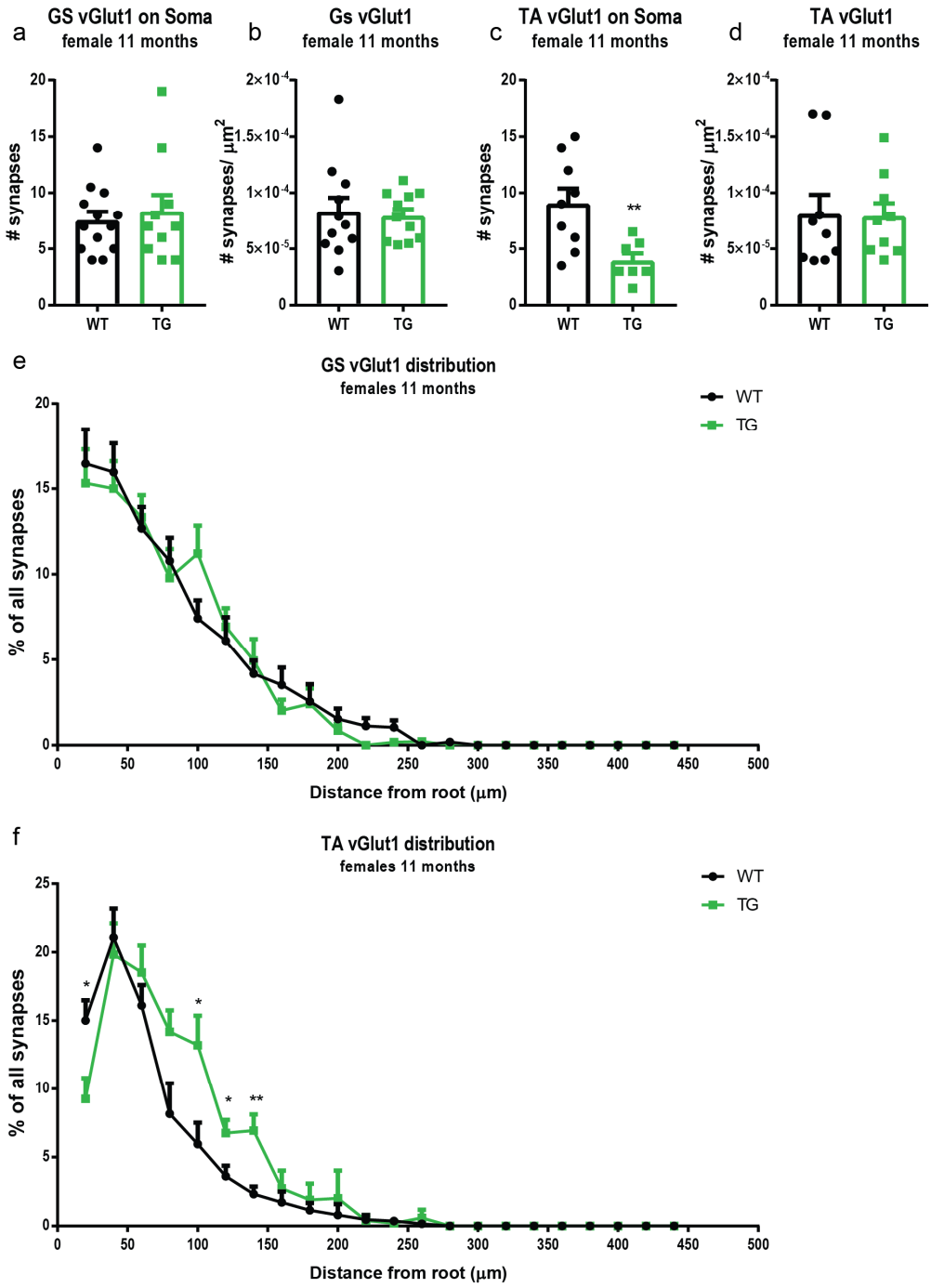
In sum, preliminary data suggested that in young female mice PGC-1α deletion reduced proprioceptive synaptic density while PGC-1α overexpression affected the synapse distribution. However, these changes had no effect on balance performance in 12-month-old male PGC-1α mKO mice.

Fig. 29: vGlut1 synapses in 7-month-old female PGC-1α mKOs



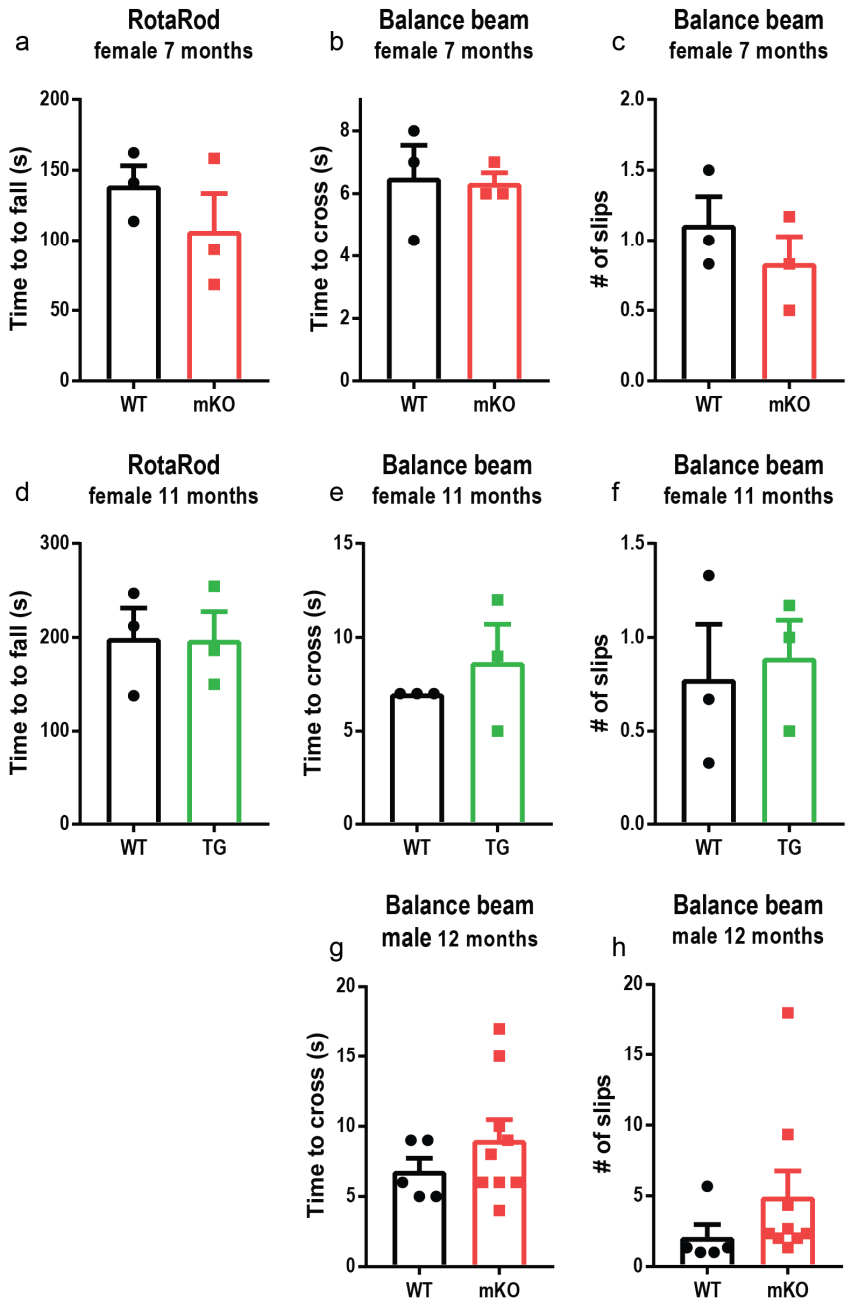
GS and soleus motor neurons from 7-month-old female PGC-1α mKOs were traced using g-protein deleted rabies virus and co-stained with vGlut1. **a** The number of vGlut1 synapses on GS motor neuron soma. **b** The **s**ynaptic density of GS motor neurons. **c** The number of vGlut1 synapses on soleus motor neuron soma. **d** The **s**ynaptic density of soleus motor neurons. **e** The comparison of synaptic density between soleus and GS motor neurons in WT animals. **f** The synaptic distribution along GS and **g** soleus motor neuron branches. A minimum of 9 motor neurons from a minimum of two animals per group were analysed. Data show mean ± SEM. In **a to d**, *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between WT and mKO and in **e** between soleus and GS motor neurons. Significance was determined using a two-tailed t-test.

Fig. 30: vGlut1 synapses in 11-month-old female PGC-1α TGs



GS and TA motor neurons from 11-month-old female PGC-1α TGs were traced using g-protein deleted rabies virus and co-stained with vGlut1. **a** The number of vGlut1 synapses on GS motor neuron soma. **b** The **s**ynaptic density of GS motor neurons. **c** The number of vGlut1 synapses on TA motor neuron soma. **d** The **s**ynaptic density of TA motor neurons. **e** The synaptic distribution along GS and **f** TA motor neuron branches. A minimum of 9 motor neurons from a minimum of two animals per group were analysed. Data show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between WT and TGs. Significance was determined using two-tailed t-test.

Fig. 31: Assessment of motor coordination and balance in PGC-1α.mKO and TG mice



Motor coordination and balance in PGC-1α.mKO and TG mice. **a** The time to fall from Rotarod for female mKOs and **d** female TG mice. **b** The time to cross balance beam for female mKOs and **e** TG mice. **c** The number of slips while traversing the balance beam for female mKOs and **f** TG mice. **g** The time to cross balance beam and **h** the number of slips on the beam for male mKO mice. In **a to f** n = 3 and in **g to h** n = 5 to 9 animals. Data show mean ± SEM. Data for each mouse line were treated separately by comparing to its own line internal control. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between WT and KO or WT and TG. Significance was determined using two-tailed t-test.

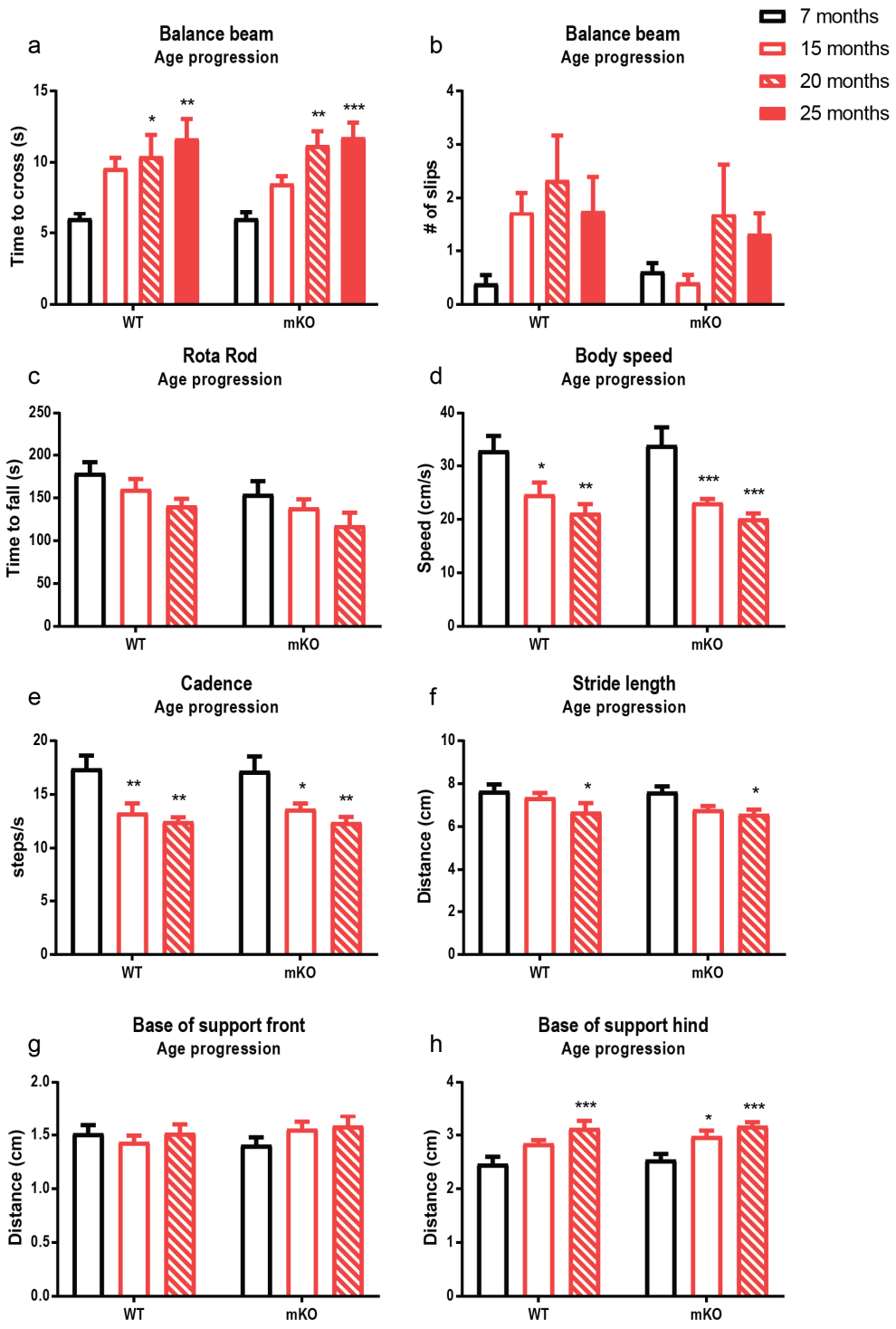
9.2.2 Age progression of male PGC-1α mKO and TG mice

We next wondered if altered proprioceptive synapse content or distribution at young age would affect balance performance at old age. To answer that question, we performed balance beam and Rotarod as well as gait analysis tests with 15-month-old male PGC-1α mKO and re-evaluated them in regular intervals. Independently of the genotype, aged mice crossed the balance beam significantly slower than 7-month-old mice (**Fig. 32a**) and tended to slip more (**Fig. 32b**). Concomitantly, Rotarod performance (**Fig. 32c**) also tended to decline with age. Moreover, aged mice moved significantly slower (**Fig. 32d**) and took less steps per second (**Fig. 32e**). In addition, at 20 months of age, mice took significantly smaller steps (**Fig. 32f**) and their hind paws were located farther away from each other than in your mice (**Fig. 32h**). However, despite significant age differences, we did not detect any changes due the loss of PGC-1α. Consistently, PGC-1α overexpression in aged mice also did not alter balance beam performance (**Fig. 33a, b**) or gait parameters (**Fig. 33c to g**).

Nevertheless, we wanted to reproduce the observed decrease in vGlut1 synaptic content on motor neurons from female mice, in male PGC-1α mKO animals. To that end, we analysed the vGlut1 synaptic density of 7-month-old male PGC-1α mKO mice and found no difference between WT and mKO animals (**Fig. 34a, b**).

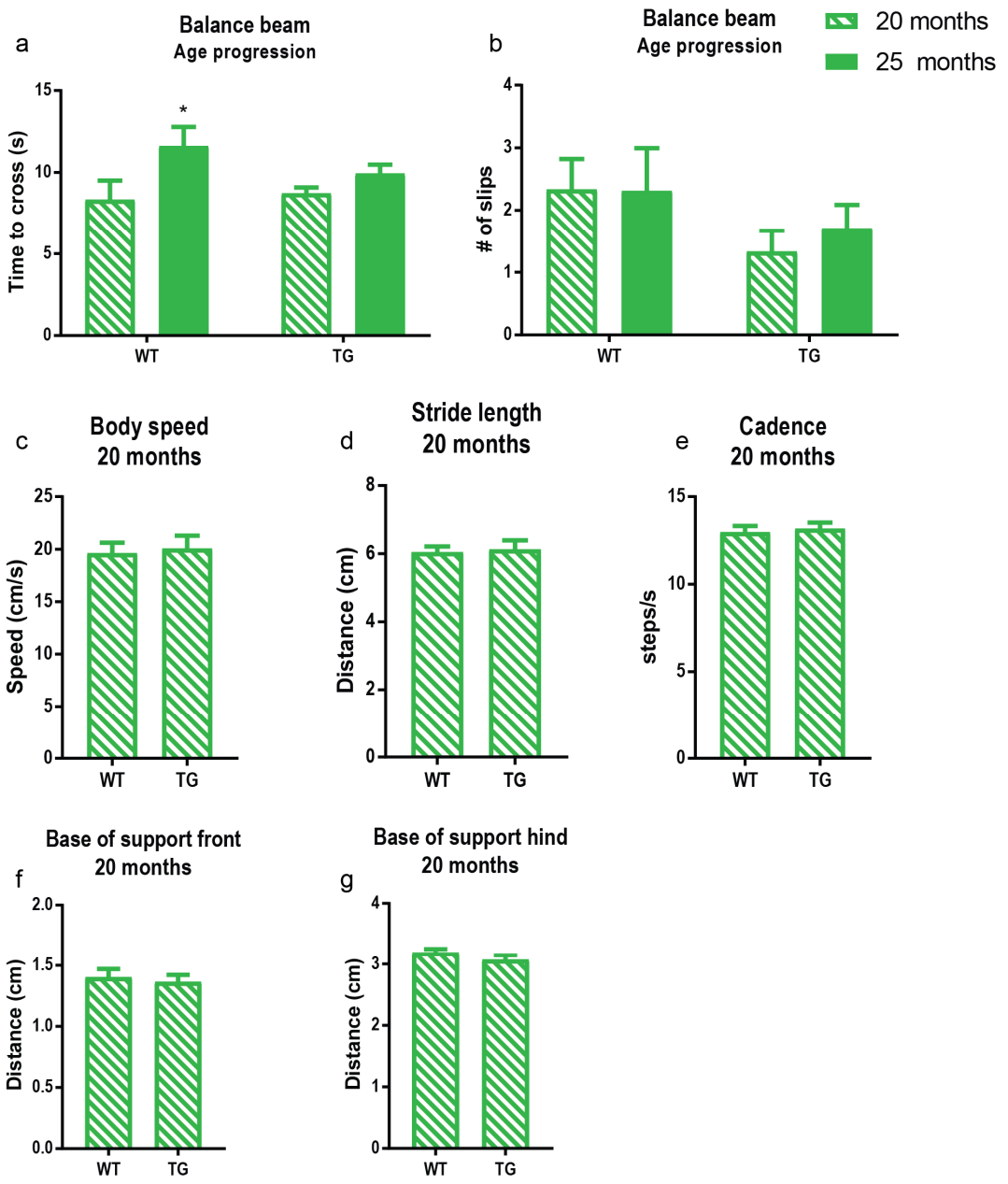
In sum, we were unable to reproduce the previously observed balance phenotype in aged PGC-1α mKO and TG mice. Furthermore, our preliminary results on changed proprioceptive synapse content due to the deletion of PGC-1α in female mice could not be confirmed in male mice.

Fig. 32: Age progression of balance, motor coordination in aged mKO mice



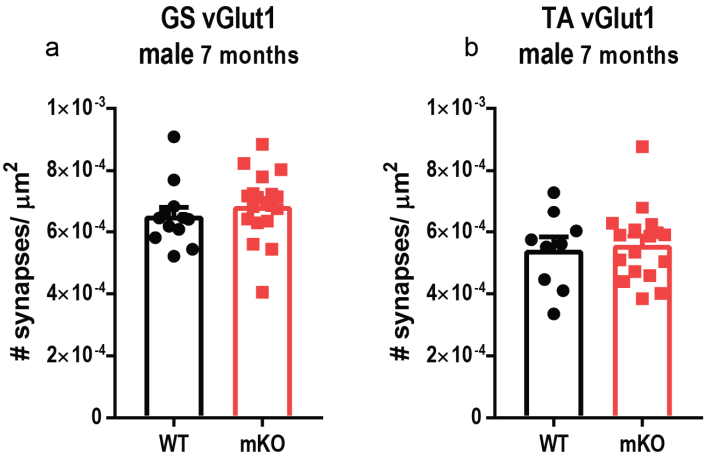
In vivo age progression of a mKO mice. **a** The time to cross and **b** the number of slips on the balance beam. **c** The time to fall from Rotarod. **d** The body speed during locomotion and **e** the number of steps per seconds. **f** The stride length. **g** The horizontal distance between the front and **h** the hind paws. n = 5 to 9 animals per group. Data show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 7-month-old and aged animals. Significance was determined using Anova followed by Sidak's test.

Fig. 33: Age progression of balance, motor coordination and balance in TG mice



In vivo age progression of a TG mice. **a** The time to cross and **b** The number of slips on the balance beam. **c** The body speed during locomotion and **d** stride length. **e** The number of steps per second. **f** The horizontal distance between the front and **g** the hind paws. n = 8 to 9 animals per group. Data show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between 20-month-old and 25-month-old mice from. Significance was determined using Anova followed by Sidak's test.

Fig. 34: vGlut1 synaptic density of GS and TA motor neurons



GS and TA motor neurons from 7-month-old male PGC-1α mKO mice were traced using g-protein deleted rabies virus and co-stained with vGlut1. **a** The **s**ynaptic density of GS and **b** TA motor neurons of 7-month-old male mKO mice. A minimum of 12 motor neurons from a minimum of 4 animals per group were analysed. Data show mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001 indicate statistically significant differences between WT and TGs Significance was determined using two-tailed t-test.

9.3 Discussion

In our preliminary study done with a small cohort of female mice, we found a reduction in proprioceptive synaptic density in the GS motor neuron pool from PGC-1α mKO.s. On the other hand, in TA motor neurons from PGC-1α TGs we observed a shift in the vGlut1 synaptic distribution along the motor neuron branch farther away from the dendrite root. However, these changes on proprioceptive input to motor neurons did not translate to altered motorcoordination or balance in mice. Unfortunately, repetition of the observed reduction in vGlut1 proprioceptive density in male 7-month-old PGC-1α mKO did not reveal the same results as with female mice. This discrepancy could be due to inherent gender differences. However, since in our preliminary data in some conditions a limited amount of motor neurons from only two animals per group could be analysed, it is far more likely that the observed differences are due to sample bias.

Previously, Gill et al, 2017 observed a striking decrease in balance performance already in 12-month old PGC-1α mKO compared to WT aged-matched control mice. On the other hand, 24-month-old PGC-1α TGs performed better on the balance beam than aged-matched WT mice (Gill et al., 2017). However, in a new cohort of 12-months-old PGC-1α mKO we did not observe decreased balance in these mice. An additional cohort of PGC-1α mKO and TG mice was followed over time until the age of 25 months. Also in these groups, we did not detect any alteration in balance or motorcoordination between mKOs and TG compared to their corresponding age-matched WT controls, although age-associated changes in gait and balance compared to young mice were apparent. Since the mKO mice from the Gill et al, 2017 study tended to be heavier than their WT mice, it is possible that the increased body weight in this cohort could have negatively affected the balance beam performance.

To conclude, skeletal-muscle derived PGC-1α does not modulate the proprioceptive input to motor neurons. Furthermore, PGC-1α overexpression or deletion in aged animals does not influence balance or motor coordination.

9.4 Methods

Animals

Mice with muscle deletion (mKO) and overexpression (Tg) of PGC-1α were described previously (C. Handschin et al., 2007; Lin et al., 2002). Animal experiments were done with male and female that were bred in-house. In all experiments, PGC-1α mKO and TG were compared to their line internal WT control animals. Mice were fed ad libitum with regular chow diet and kept under a 12-h/12-h light–dark cycle at 23°C. All experiments were performed in accordance with the Swiss federal guidelines for animal experimentation and were approved by the Kantonales Veterinäramt of the Kanton Basel-Stadt.

Rotarod

Mice were put on a horizontal rotating rod (Ugo Basile) that was suspended from the ground. Since mice are naturally afraid to fall they will try hard to remain on the rotating rod. Mice were acclimatized for three consecutive days. On the first day, mice were put on the rotating rod three times for 3 min at 5rpm, the second day at 8rpm and third day at 12rpm. On the day of testing, the rotation speed was gradually increased from 5rpm to 68rpm during 7 min. The time until a mouse falls was recorded and the average of three trials was calculated as a measure for motor coordination.

Balance beam test

Mice were taught to cross an inclined beam. They were familiarized with the task 1 day before the testing by giving them three practice trials. Doing this, mice learned to expect a red hiding box at the end of the beam, which ensured the animal´s compliance. The time to cross the beam and the number of slips were measured to assess balance performance.

The intermediate and old age group were tested on a round balance beam system while the 6-weeks-exercise group was tested on a square beam. Thus for the 6-weeks-exercise group an additional control group aged 7 months was used for baseline testing.

Gait analysis

The gait of mice was assessed using the CatWalk XT system by Noldus. Briefly, mice walked voluntarily across a glass platform. Several paw prints sequences were captured by a camera located under the platform and gait parameters were calculated using the Noldus software.

Proprioceptive tracing

Motor neuron pools from GS, TA and soleus muscles were retrogradely traced using a g-protein deleted rabies virus as done previously (Basaldella et al., 2015). Briefly, muscles of anesthetized mice were injected with 5 µl of virus. After 4 days of incubation mice were anesthetized and transcardially perfused with 20 ml saline solution followed by 70 ml 4% PFA in Phosphate buffer.

Immunohistochemistry

After perfusion, spinal cords were dissected and post-fixed overnight in 4% PFA followed by sucrose protection for 24 hours in 30% sucrose in PBS. Subsequently, spinal cords were frozen and cut in a cryostat. Spinal cord sections 60 µm thick were blocked 1 hour in 4% BSA 5% goat serum in 0.5% Triton-X-100 at room temperature and then incubated overnight in primary antibody at 4 ºC followed by three washes in PBS. Spinal cord sections were incubated overnight in secondary antibody at 4 ºC and then washed three times with PBS before sections were mounted.

Microscopy and image analyses

20 tiles and stacks of the motor neuron tree were imaged on a spinning disk Nikon eclipse connected to a Visitron system with a 63x objective. Stacks of 200 nm sampling and tiles were stitched together in Fiji. The motor neuron surface was reconstructed in Imaris and synapses were detected by the Imaris spot detection function. A matlab extension tool was used to calculate the number of spots touching the reconstructed motor neuron. The number of touching spots was normalized to the motor neuron surface

Additionally, the synapse distribution on the motor neuron tree was analysed using Neurolucida. The outline of the motor neuron soma and the dendrites were manually traced. All synapses touching the motor neuron were marked and the software calculated the distance of this mark to the motor neuron soma. The number of synapses contacting directly the cell body were counted manually. For analyses, a minimum of three motor neurons per animal was assessed.

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