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RESEARCH ARTICLE

Age-related loss of VGLUT1 excitatory, but not VGAT inhibitory, immunoreactive terminals on motor neurons in spinal cords of old sarcopenic male mice

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Abstract Age-related changes in ventral lumbar spinal cord (L3–L5) were compared in young [3 month, (M)] and old (27 M) C57BL/6J male mice. The aged mice had previously been shown to exhibit sarcopenia and changes to peripheral nerve morphology. The putative connectivity of β-III tubulin positive α-motor neurons was compared in immunostained transverse sections using excitatory and inhibitory terminal markers vesicular glutamate transporter-1 (VGLUT1) and vesicular GABA transporter (VGAT). Glial fibrillary acidic protein (GFAP) and ionized calcium binding adaptor molecule 1 (Iba1) immunostaining was used to monitor changes in astrocyte and microglial phenotype respectively. For a given motor neuron, the neuronal perimeter was outlined and terminals immunoreactive for VGLUT1 or VGAT in close apposition to the soma were identified. By 27 M, the percentage coverage and total number of VGLUT1 immunoreactive terminals immediately adjacent to the soma of α-motor neurons was significantly decreased compared with young mice. However, percentage coverage of immunoreactive VGAT inhibitory terminals did not change significantly with age. The gray matter of 27 M spinal cords showed increased astrocytic and microglial activity. The loss of VGLUT1 terminals on α-motor neurons, terminals known to be derived from proprioceptive muscle afferents, may further impair sensorimotor control of hind limb skeletal muscle function in old mice.

Keywords VGLUT1 · VGAT · Astrocytes · Microglia · Synaptic transmission · Proprioception · Sarcopenia

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Introduction

In humans, old age has adverse effects on the motor unit, in particular the lower motor neurons (Doherty 2003; Kwan 2013; Lexell 1997). Age-related loss of spinal motor neurons in humans is associated with a reduction in myofiber number and size, resulting in impaired muscle function (sarcopenia) (Aagaard et al. 2010). Progressive age-related changes somewhat similar to human sarcopenia have also been documented in mice, including loss of muscle mass, denervation of neuromuscular junctions and accumulation of proteins in peripheral nerves (Chai et al. 2011; Krishnan et al. 2016; White et al. 2016). Building on these data, the focus of the present study is to determine if there are any concomitant changes in

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the aging spinal cord in these same old mice, in particular analysing interactions between sensory neurons and lower motor neurons. It is important to note here that for rodents (rats and mice), where the life span is at most about 30 months (M), aging occurs over a duration of only about 10–15 M, compared with about 20–40 years in humans.

Studies of human lumbar spinal cord regions show that advancing age (60 years and older) is accompanied by a reduced number and diameter of motor neuron soma counts in the ventral horn (Drey et al. 2014; Kawamura et al. 1977; Mittal and Logmani 1987; Tomlinson and Irving 1977). In rodents, age-related loss of motor neurons has been reported in some (Jacob 1998; Valdez et al. 2010) but not all (Chai et al. 2011) studies. In old rats (around 30 M), motor neurons do show age-related changes in dendritic size, decreased synaptic input and signs of neuro-axonal degeneration which are more prominent in lumbar ventral roots innervating lower limb muscles, making these muscles prone to age-related denervation (Edstrom et al. 2007; Hashizume and Kanda 1995; Pannerec et al. 2016). In rodents, any loss of motor neurons is relatively small (10–15%) during aging (Edstrom et al. 2007) compared with old humans where it occurs over many years ($\sim$ 50% above 60 years) (Tomlinson and Irving 1977).

Apart from changes in spinal motor neuron number, there are other potential contributors to impaired function of old motor neurons including decreased synaptic input (Kullberg et al. 1998) and evidence of altered synaptic transmission (Ranson et al. 2007). Throughout the central nervous system (CNS), tight regulation of excitatory and inhibitory synaptic inputs is essential for the fine-tuned synaptic communication that underlies normal function (Deak and Sonntag 2012; Hu et al. 2006; Monti et al. 2004; VanGuilder et al. 2010), and any age-related changes in synaptic efficacy will clearly impact on function. In addition, there are changes in the phenotype of astrocytes and microglia with age, changes that are also associated with many CNS pathologies, often associated with sensorimotor impairment (Nakajima and Kohsaka 2001; Ridet et al. 1997). In aging mice, our recent study in sciatic nerves that innervate hind limb muscles revealed age-related decreases in both motor and sensory axons by 27 M (Krishnan et al. 2016) and therefore,we here explored the extent of connectivity of sensory and motor neurons in the ventral horn of old spinal cord. Proprioception deterioration with motor coordination and balance declining during the aging process is also reported in humans (Ribeiro and Oliveira 2007; Suetterlin and Sayer 2014).

Sensory neurotransmission from primary afferents onto spinal neurons appears to be largely mediated by glutamate (Brumovsky 2013). Glutamate transporters (VGLUTs) move glutamate from the cell cytoplasm into synaptic vesicles and in ventral spinal cord it has been reported that the VGLUT1 transporter is contained within the terminals of primary *1a* afferents (Levine et al. 2014; Liu et al. 2014; Todd et al. 2003). On the other hand, the vesicular uptake of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) within nerve terminals is mediated by the vescicular GABA transporter (VGAT) (McIntire et al. 1997; Owens and Kriegstein 2002). To investigate whether age-related CNS changes are also evident in the connectivity of motor neurons that project axons to hind limb muscles in mice, immunohistochemistry was used to examine the ventral horn in lumbar regions L3–L5 of the spinal cord (Bala et al. 2014; Rigaud et al. 2008) in young (3 M) and old (27 M) male C57BL/6J mice. Afferent connectivity of presumed (β-III tubulin positive) α-motor neurons was investigated by quantifying putative contacts made on their soma by terminals immunoreactive for either VGLUT1 or VGAT. To evaluate changes in glial cells with aging, astrocytes in young and old spinal cords were visualised using antibodies to detect either glial fibrillary acidic protein (GFAP) or ionized calcium binding adaptor molecule 1 (Iba1), and the anti-CD68 marker ED1 was also used to identify microglia/macrophages (Damoiseaux et al. 1994; Sasaki et al. 2001). In summary, we found a 50% decrease in the number of VGLUT1 but not VGAT immunoreactive terminals in close apposition to motor neurons in old spinal cord, providing novel insight into the altered roles of sensory and motor neurons in the context of sarcopenia, shown by us previously to be evident in hind limb muscles in these same old mice (Soffe et al. 2016).

Materials and methods

Mice and tissue collection

All animal experiments were conducted in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the Animal Ethics Committee at the University of Western Australia (UWA). The present study was carried out on spinal cord tissues from young (3 M) and aged (27 M) male C57BL/6J mice (n = 4/5 for each age group) that had been killed by cervical dislocation while under terminal anaesthesia (2% v/v Attane isoflurane, Bomac, NSW, Australia, 400 mL NO_2 and 1.5LO_2). It is important to note that immersion fixation of spinal cords was necessary because these same aged mice were used in parallel studies on sarcopenia (Soffe et al. 2016), and both the 3 and 27 M old mice were also analysed for age-related changes to peripheral sciatic nerves (Krishnan et al. 2016). For this current work, the vertebral column containing the spinal cord was dissected from each animal and immediately fixed in diethylpyrocarbonate (DEPC) treated 4% (w/v) paraformaldehyde and stored at 4 °C.

Processing of spinal cord for immunofluorescence studies

Post-fixation, spinal cords were taken out of the vertebral column, re-fixed in fresh 4% (w/v) paraformaldehyde overnight, then cryoprotected with 30% (w/v) sucrose in phosphate buffered saline (PBS) for 24 h. The entire lumbar region of the spinal cord (L1–L5) was dissected out and embedded in 10% (w/v) gelatin in plastic moulds. The gelatin block was treated with 4% (w/v) paraformaldehyde followed by 30% (w/v) sucrose. Frozen blocks were sectioned transversely at 20 µm thickness using a Leica CM3050 cryostat, and sections were collected into PBS containing 0.1% (w/v) sodium azide in 24 well plates (Hodgetts et al. 2013).

Toluidine blue staining of motor neurons to identify the lumbar (L3–L5) region

From each spinal cord, every 24th transverse section was mounted onto a gelatin coated slide and stained with 0.05% (w/v) toluidine blue (45 s), rinsed in water and dehydrated in ethanol series (70%, 90%, and 100% for 3 min each), left to dry overnight and then coverslipped in DPX (Fronine laboratory supplies, NSW, Australia).

These sections were photographed at ×10 magnification using a Nikon Eclipse T1 inverted microscope. The images were compared with the C57BL/6J mouse spinal cord images from the mouse spinal atlas (http://mousespinal.brain-map.org/imageseries/showref.html) to identify the lumbar (L3–L5) region and then sections from this region were used for the immunofluorescence studies.

Immunofluorescence studies on free floating spinal cord sections

Sections were washed in PBS for 10 min (×3) and blocked in antibody diluent containing 10% (v/v) NGS (Normal goat serum) and 0.2% (v/v) Triton X-100 in PBS for 1 h. The sections were then incubated with appropriate primary antibodies (details in Table 1) overnight at 4 °C. After washing in PBS for 10 min (×3) sections were incubated in appropriate secondary antibodies (see Table 2) for 1 h, again washed for 10 min (×3) and mounted with Prolong™ Diamond Antifade Mountant (ThermoFischer Scientific, USA) and coverslipped. Every immunostaining run included control sections to which no primary antibody was added (Hodgetts et al. 2013).

Confocal microscopy and image analysis for VGLUT1 and VGAT immunoreactive terminals

Sections of L3–L5 spinal cord (5 sections per animal) were stained with β-III tubulin to label the cell somata and VGLUT1 and VGAT to label excitatory and inhibitory immunoreactive terminals respectively (Liu et al. 2014; Zhu et al. 2016). We also tested some sections for VGLUT2 immunoreactivity (You et al. 2016), however unlike the other antibodies used here, the staining was unreliable and inconsistent, and therefore not amenable to semi-quantification. Successfully stained sections were viewed using a Nikon laser scanning confocal microscope C2/C2S1. Putative α-motor neurons in the ventral horns, with soma size between 360 and 1200 µm^2 (Friese et al. 2009), were selected for the study since α-motor neurons receive direct *1a* afferent input from proprioceptive

Table 1 Primary antibodies used for immunofluorescence in C57BL/6J male mouse lumbar spinal cord

Antigen	Host	Product number	Dilution	References
VGLUT1	Rabbit	Synaptic systems; 135303, Germany	1:1000	Liu et al. (2014)
VGAT	Rabbit	Synaptic systems; 131002, Germany	1:200	Owens and Kriegstein (2002)
β-III tubulin	Mouse	Covance; MMS-435P, USA	1:1000	Jouhilahti et al. (2008)
GFAP	Rabbit	Dako; Z0334, USA	1:200	Triolo et al. (2006)
Iba1	Rabbit	Wako; 019-19741, USA	1:500	Brettschneider et al. (2012)
ED1	Mouse	Serotec; MCA341R, USA	1:400	van der Poel et al. (2011)

Table 2 Secondary antibodies used for immunofluorescence in C57BL/6J male mouse lumbar spinal cord

Secondary antibody	Product number	Dilution
Anti-Rabbit Cy3	Jackson Immuno Research Labs; 111-166-004	1:400
Anti-Rabbit Alexa Flour 488	Life Technologies; A11008	1:500
Anti-Mouse Cy3	Jackson Immuno Research Labs; 115-166-006	1:400
Anti-Mouse-Alexa Flour 488	Life Technologies; A11001	1:500

muscle spindles (Liu et al. 2014). Motor neurons were selected only if they contained β-III tubulin immunolabel that filled the somata and extended to proximal dendrites, and their cell borders could be recognised. Images of labelled motor neuron cell bodies were taken at ×20 magnification (Liu et al. 2014) at a confocal Z dimension thickness of 2 μm from the ventral horns of 4–5 lumbar spinal cord sections from each mouse.

Quantification of VGLUT1 and VGAT immunoreactive terminals

To quantify the percentage synaptic coverage of VGLUT and VGAT immunoreactive terminals from spinal cord sections stained with VGLUT1 and VGAT (Fig. 1a, b), a 2 μm section from the Z stack that contained the nucleolus of the cell was chosen for analysis. A region of interest (ROI) was created around the perimeter of each β-III tubulin positive motor neuron (Fig. 2f), and terminals immunoreactive for VGLUT1 or VGAT protein were identified that were in close contact with the cell (as indicated by arrows in Fig. 2f). Based on a previous study (Liu et al. 2014), the length of the cell perimeter covered by VGLUT1 or VGAT immunoreactive terminals was measured and expressed as a percentage coverage of the total perimeter of the neuron (i.e. length of cell perimeter covered by immunoreactive terminals/total cell perimeter of the motor neuron × 100). Additionally for VGLUT1 only, the *total number* of immunoreactive terminals per neuron was calculated. This was done by counting the VGLUT1 positive terminals in every 2 μm slice through each cell. If a positive VGLUT immunoreactive terminal was located in exactly the same location in adjacent 2 μm slices, this bouton was counted only once to avoid double counting errors. A total of 68 and 114 motor neurons was analysed for VGLUT1 immunoreactive terminals in 3 and 27 M mice respectively, and 88 and 111 motor neurons were quantified for VGAT immunoreactive terminals in the young and aged mouse groups.

To check for consistency in immunostaining of immersion fixed spinal cord material, a semiquantitative measurement of fluorescence intensity for VGLUT1 in the dorsal horns was undertaken for young and old lumbar spinal cords. Consistent with previous studies in mouse (Brumovsky 2013) and rat spinal cord (Alvarez et al. 2004), VGLUT1 immunoreactivity in the dorsal horn was strong in laminae II–IV, medial laminae V/VI and dorsal laminae VII (Fig. 1a). In contrast, VGAT was densely and homogenously distributed throughout the gray matter of both the

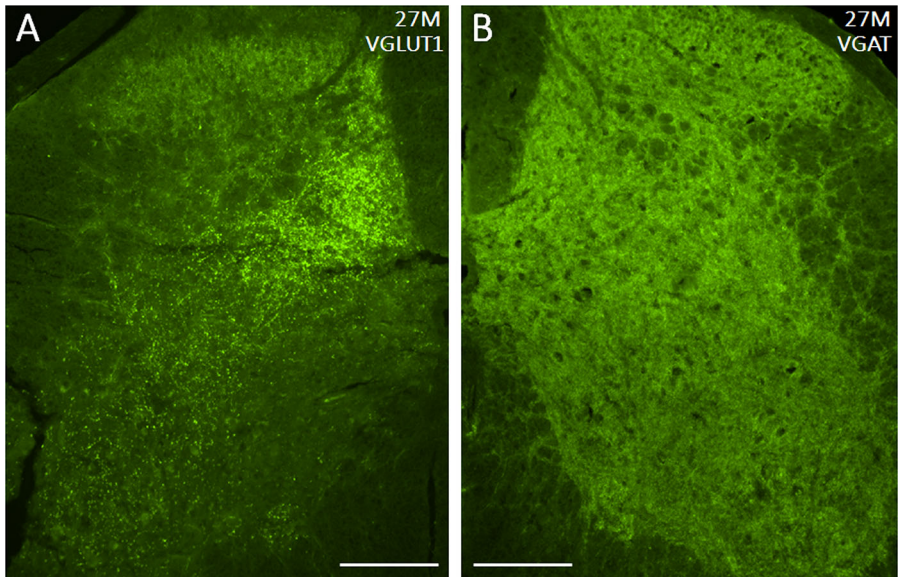

Fig. 1 VGLUT1 and VGAT immunofluorescent staining in lumbar spinal cords of C57BL/BJ mice. Representative images of VGLUT1 (**a**) and VGAT (**b**) staining in 27 M mice. Scale bars: **a**, **b** = 200 µm

dorsal and ventral horns (Fig. 1b) (Sunagawa et al. 2017).

For semi-quantitative measurement of fluorescence intensity for VGLUT1 in the dorsal horns, stained sections were observed and photographed using Nikon Eclipse E-400 fluorescent microscope. Imaging paramaters (brightness, illumination) were kept constant for all images. Using NIS elements imaging software (version 4.51) a ROI was created that included all the dorsal horn containing VGLUT1 staining. A low threshold was set for the green channel to eliminate the background, a procedure that was routinely applied to each of the sections analysed. Within the selected ROI (the entire dorsal horn in this case), the mean fluorescence intensity above threshold was measured and documented. Immunostained sections from 3 young and 3 old mice were quantified (10 sections in total).

GFAP immunohistochemistry and quantification of fluorescence intensity

Transverse sections of lumbar spinal cords of young and old mice were stained with GFAP (4 or 5 sections per animal) to analyse changes in astrocyte phenotype with aging. Stained sections were observed, and photomontaged using Nikon Eclipse N*i* fluorescent microscope. Using NIS elements imaging software (version 4.51), a semiquantitative measurement of fluorescence intensity for GFAP in the ventral horns was undertaken for young and old lumbar spinal cords. The ventral horn of the rodent spinal cord is regarded as starting in the ventral part of Rexed's Lamina 7 (Watson et al. 2009). In the present study, for each spinal cord section a ROI was identified that included all ventral horn gray matter contained within laminae 7–10. A low threshold was set for the red channel to eliminate background. The mean fluorescence intensity above threshold within the specific range for the selected ROI (ventral horn gray matter) was then measured and documented. Twenty-three and 16 lumbar GFAP immunostained sections were quantifed in 3 and 27 M mice respectively.

Iba1 and ED1 immunohistochemistry

To further analyse age-related glial changes, tranverse sections of lumbar spinal cords of young and old mice were stained with Iba1. To help differentiate between normal (ramified) and activated microglia, young and old lumbar spinal cords were double immunostained with Iba1 and ED1. Stained sections were observed and high magnification images were photographed using a Nikon Eclipse E-400 fluorescent microscope.

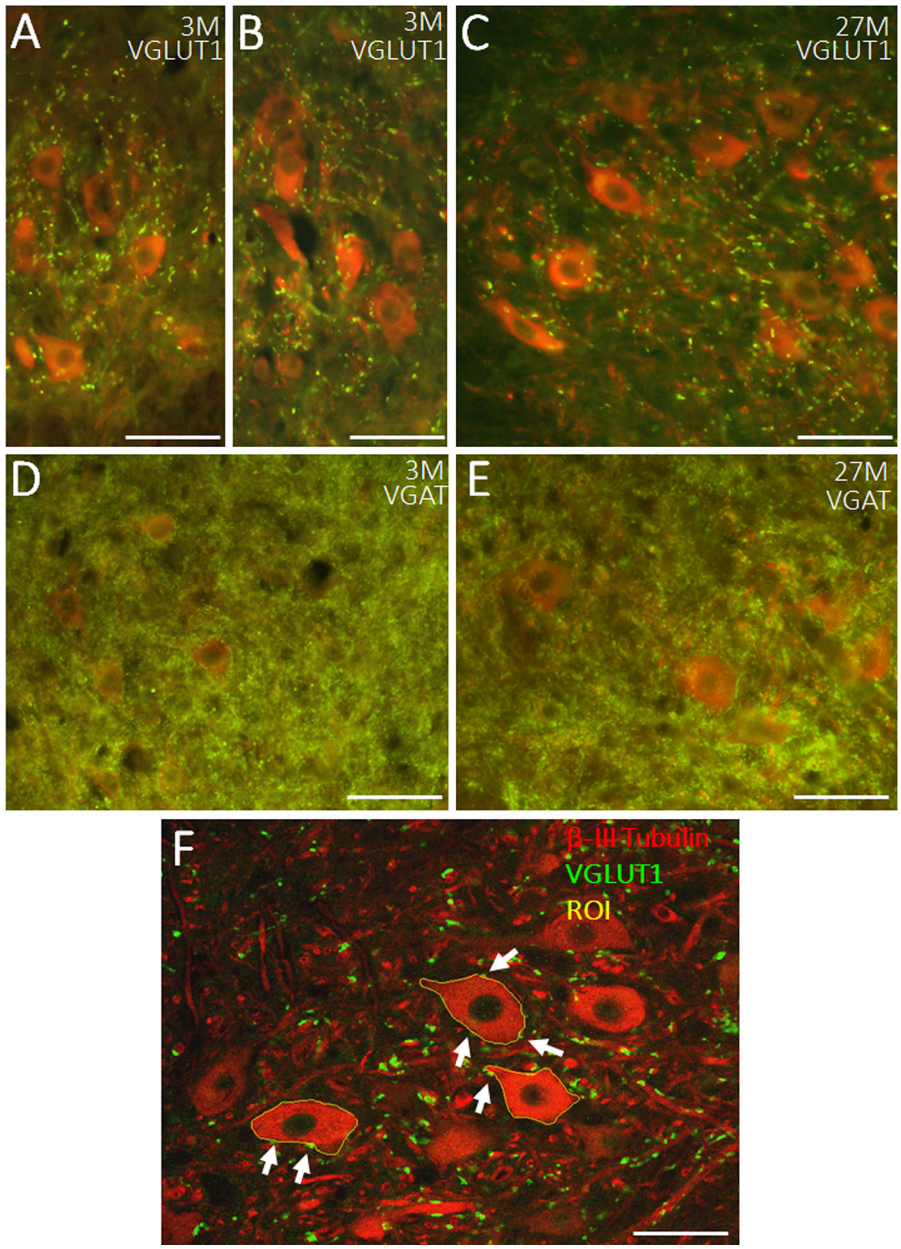

Fig. 2 Fluorescent immunostaining for VGLUT1 and VGAT (green), along with β-III tubulin (red) to identify motor neurons, in ventrolateral spinal cords of C57BL/6J male mice. Examples of VGLUT1 (**a**–**c**) and VGAT (**d**, **e**) immunolabelling in 3 and 27 M mice; **f** Confocal image showing a region of interest (ROI) (indicated in yellow line), defining the perimeter of the β-III tubulin positive (red) motor neuron, used for studying VGLUT1 (green) immunoreactive terminals in a 3 M old lumbar spinal cord. Terminals that were in close apposition to labelled motor neuron (indicated by white arrows) were counted and expressed as percentage terminal coverage. Scale bars: **a**–**f** = 50 μm

Statistical analyses

GraphPad prism (version 7) was used as the statistical package. Data obtained from quantification of VGLUT1 and VGAT immunoreactive terminals on motor neurons in 3 and 27 M male C57BL/6J mice were compared using two tailed unpaired *T* test. VGLUT1 and GFAP fluorescence intensity between

young and old mice was compared using two tailed unpaired *T* test. Significance threshold was set at $P \leq 0.05$.

Results

Age-related decrease in VGLUT1 immunoreactive terminal coverage in old 27 M spinal cords

Immunostaining for VGLUT1 (Fig. 1a) revealed a significant decrease in the percentage terminal coverage of motor neuron soma in old 27 M ($P = 0.024$) (Fig. 2c) (mean of 5.68 ± 0.26) compared with 3 M (Fig. 2a, b) (mean of 10.9 ± 1.5) spinal cords (Fig. 3a). The total number of VGLUT1 immunoreactive terminals per motor neuron was also significantly decreased in 27 M old spinal cords ($P = 0.004$) (mean of 14.08 ± 1.35) compared with tissue for 3 M old mice (mean of 21.4 ± 1.14) (Fig. 3b). Importantly however, no age-related changes were observed in VGLUT1 fluorescence intensity in the dorsal horns of 3 and 27 M mice (Fig. 3c).

No significant difference in VGAT immunoreactive terminal coverage between young and old mice

Immunostaining for VGAT (Fig. 1b) revealed no significant changes in the percentage coverage of VGAT immunoreactive terminals on individual β-III tubulin positive motor neurons between young 3 M (Fig. 2d) and old 27 M (Fig. 2e) spinal cords (3 M:

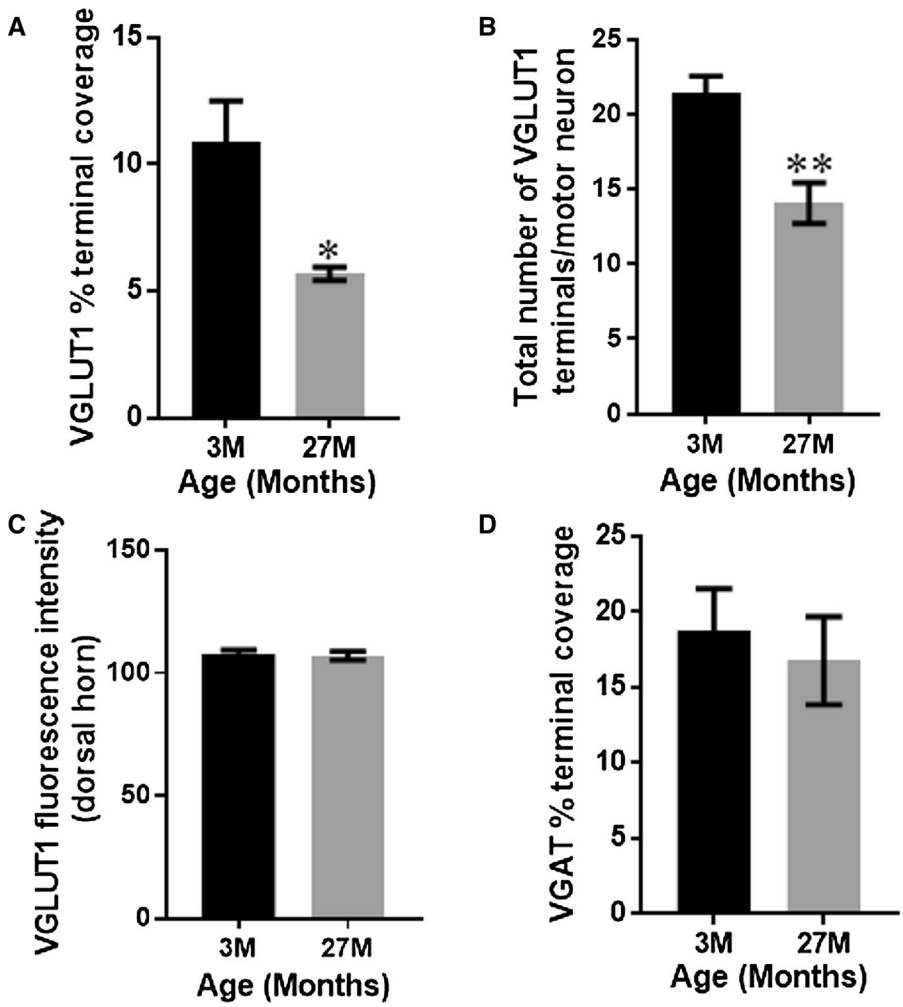

Fig. 3 Quantification of VGLUT1 and VGAT in young (3 M) and old (27 M) lumbar spinal cords of male C57BL/6J mice. **a** Percentage coverage of terminals immunoreactive for VGLUT on (β-III-tubulin positive) motor neuron somata. **b** Total number of terminals immunoreactive for VGLUT1 per motor neuron (β-III-tubulin positive). **c** Quantification of VGLUT1 fluorescence intensity in dorsal horns of young and old lumbar spinal cords; Y-axis shows arbitrary unit values of mean fluorescence intensity above threshold. **d** Percentage coverage of terminals immunoreactive for VGAT on (β-III-tubulin positive) motor neuron somata (mean ± SEM). n = 5; *$P \leq 0.05$, **$P \leq 0.01$

18.75 ± 2.78; 27 M: 16.75 ± 2.9) (P = 0.6385) (Fig. 3d).

Significant increase in GFAP staining intensity in the gray matter in 27 M spinal cords

Compared with young (3 M) spinal cords (Fig. 4a, c), a qualitative increase in GFAP immunostaining was observed in the gray matter of 27 M spinal cords (Fig. 4b, d) indicating increased astrocyte reactivity. Quantitatively, there was a significant age-related increase in GFAP fluorescence intensity in old compared to young spinal cords (P = 0.0242) (Fig. 4e). GFAP positive astrocytes were more evenly distributed throughout the spinal tissue, with more pronounced staining in gray matter and intense reactivity around the central canal and in peripheral white matter.

Increase in Iba1 positive microglial staining in old 27 M spinal cords

Compared with young mice (Fig. 5a), an increase in immunoreactivity for Iba1 positive microglial cells was evident in both the ventral and dorsal horns of old (27 M) spinal cords (Fig. 5b). These highly immunoreactive, Iba1 positive cells in old spinal cords had relatively enlarged cell bodies with multiple, often thickened and ramified, branches (arrows, Fig. 5b). Interestingly these Iba1 positive microglia were not immunoreactive for ED1 (Fig. 5c) and qualitatively there was no obvious age-related change

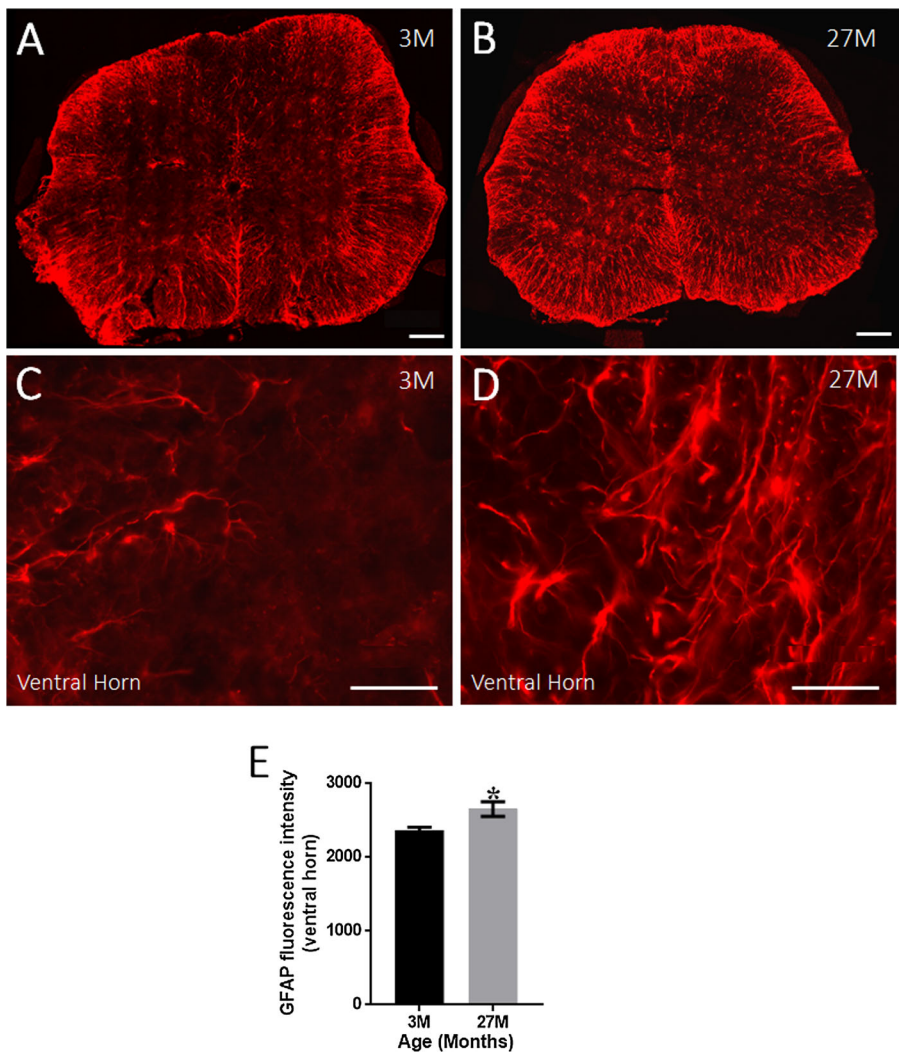

Fig. 4 GFAP immunostaining on transverse sections of lumbar spinal cords from young (3 M) and old (27 M) male C57BL/6J mice. Representative images shown for **a** young 3 M and **b** old 27 M spinal cords. High magnification images of ventral horns are shown **c** for young and **d** old spinal cords. Quantification of GFAP fluorescence intensity in ventral horns of young and old spinal cords is compared in **e**. Mean ± SEM, *P ≤ 0.05; Scale bars: **a**, **b** = 200 μm; **c**, **d** = 50 μm. Y-axis shows arbitrary unit values of mean fluorescence intensity above threshold

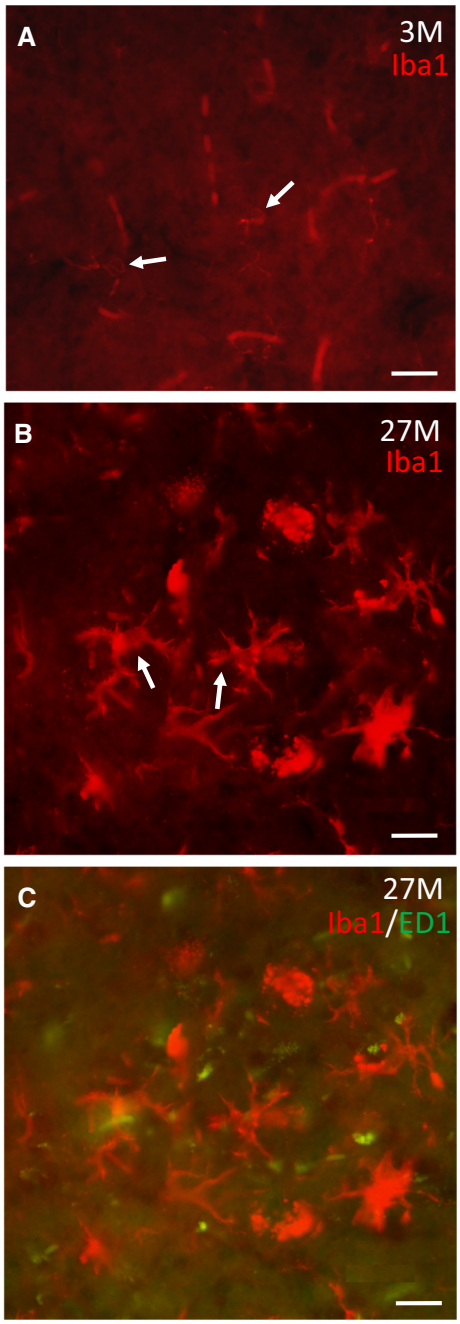

Fig. 5 Iba1 and ED1 immunostaining on transverse section of lumbar spinal cord in 3 and 27 M old male C57BL/6J mice. Iba1 immunostaining **a** in the ventral horn of a young 3 M and **b** old 27 M lumbar spinal cord: note the ramified, intensely immunoreactive microglia with relatively enlarged cell bodies and branching (white arrows) in 27 M old spinal cord compared with cells at 3 M (arrows in **a**). **c** Double immunostaining of Iba1 (red) and ED1 (green) in aged 27 M lumbar spinal cord—same field as 'b'. Scale bar for all images = 20 µm

in overall ED1 immunoreactivity between young and old spinal cords.

Discussion

The present study used spinal cords from the *same* male C57BL/6J mice that had previously been shown to exhibit age-related loss of muscle mass (sarcopenia) (Soffe et al. 2016), as well as changes in sciatic nerves that innervate hind limb musculature (Krishnan et al. 2016). We performed immunohistochemistry to compare the distribution of excitatory and inhibitory terminals, and glial cell reactivity, in L3–L5 segments of young (3 M) versus old (27 M) mouse spinal cords. Together, these combined data obtained from spinal cord, peripheral nerve and muscle for individual aged mice constitute a unique set of analyses of the effects of aging on sensorimotor control of skeletal musculature.

Age-related decrease in VGLUT1 immunoreactive terminals

Quantitative analyses of VGLUT1 immunolabelled terminals on β-III tubulin positive α-motor neurons in sections of 27 M old ventral horns revealed a striking decrease, relative to young (3 M) mice, in the percentage of terminal coverage (∼ 6% vs ∼ 11%) as well as the total number of VGLUT1 immunoreactive terminals in close apposition with each motor neuron (∼ 14 vs ∼ 22). Importantly, the age-related loss of VGLUT1 immunoreactive terminals on motor neurons was specific, because there was no significant change in intrinsic immunoreactive VGAT inhibitory terminals associated with α-motor neurons in aged spinal cords.

Glutamate is the principal excitatory neurotransmitter in the CNS and variations in VGLUT1 levels critically affect the efficacy of glutamatergic synaptic transmission (Balschun et al. 2010; Fremeau et al. 2004; Wojcik et al. 2004). Importantly, sensory neurotransmission from primary *1a* afferents onto spinal neurons appears to be largely mediated by glutamate, and involves VGLUT1 containing terminals (Brumovsky 2013; Levine et al. 2014). Murine motor neurons are extensively driven by premotor neurons in the medial deep dorsal horn, which also receive proprioceptive input (Levine et al. 2014)

nonetheless an age-related decline in VGLUT1 innervation on α-motor neurons in old spinal cords will lead to at least some reduced activation of these cells that may in turn lead to altered excitation and contraction of innervated myofibers. Note that VGLUT1 terminals are also associated with corticospinal tract axons (Alvarez et al. 2004; Brumovsky 2013; Persson et al. 2006), however in rodents the main termination of these axons is in laminae III–V in the cervical and lumbar spinal cord (Casale et al. 1988; D'Acunzo et al. 2014; Levine et al. 2014; Watson et al. 2009; Welniarz et al. 2017). In adult mice there is no cortical innervation of lower motor neurons in ventral horn, although in cervical spinal cord there is evidence of transient direct connections with motor neurons that are eliminated during development (Maeda et al. 2016).

Sensory denervation via experimental dorsal rhizotomy significantly reduces VGLUT1 in the ventral horn in rats (Alvarez et al. 2004; Li et al. 2003; Oliveira et al. 2003; Todd et al. 2003) and mice (Brumovsky et al. 2007; Brumovsky 2013), and these terminals are also lost in regions occupied by cell bodies of motor neurons after more distal peripheral nerve injury (Liu et al. 2014; Rotterman et al. 2014; Zhu et al. 2016). Together, these data suggest that *Ia* afferent fibre collaterals and their synapses retract from lamina IX after peripheral nerve injuries, and the density of VGLUT1 synapses on somata and dendrites of motor neurons is diminished (Alvarez et al. 2011). The impact of aging in glutamatergic inputs was recently studied in C57BL/6J mice and monkeys, both species showing a significant age-related decrease in VGLUT1 terminals on α-motor neurons in old ventral horns (Maxwell et al. 2018). Loss of presumptive VGLUT1 synapses on the soma and proxomal dendrites of lumbar spinal cords was also reported in a mouse model of spinal muscular atrophy (SMA) (Ling et al. 2010; Mentis et al. 2011).

The age-related loss of VGLUT1 immunoreactive terminals associated with α-motor neurons in 27 M old mice seen in the present study is indicative of a loss of normal function in these excitatory terminals (Fremeau et al. 2004; Wojcik et al. 2004), and is most likely linked to a loss of *1a* afferents. This loss is presumably linked in some way to distal degenerative changes in hindlimb skeletal musculature. In this regard, in the 4 aged mice examined here, their hindlimb wet weight muscle mass (gastrocnemius, quadriceps, triceps, extensor digitorum longus, tibialis anterior, and soleus) had previously been recorded (Soffe et al. 2016). We found no correlation between total muscle weight and VGLUT1 synaptic coverage or total number of terminals on motor neurons in these four mice, suggesting that the observed central changes in connectivity may not simply be the result of a loss of peripheral muscle mass.

In mice, the premotor neurons, or motor synergy encoder neurons (MSE) in medial dorsal horn, that provide major monosynaptic input to α-motor neurons, receive both VGLUT1 positive corticospinal and proprioceptive inputs (Levine et al. 2014). While we did not detect a measurable loss of VGLUT1 immunoreactivity in the dorsal horns of old mice, it remains to be determined whether the age-related loss of VGLUT1 immunoreactive *1a* afferent terminals on motor neurons in the ventral horn is also a feature of the innervation of the aged MSE population.

Peripheral proprioceptors, particularly muscle spindles, play an important role in the detection of both passive and active limb movements (Proske et al. 2000), maintenance of postural stability (Lord and Ward 1994) and control of posture and balance during the swing phase of locomotion (Sorensen et al. 2002) (reviewed in Kirkpatrick et al. 2010; Thornell et al. 2015). Spindles are difficult to investigate and quantify, but age-related changes are reported for humans and other mammals and this is probably related to a decline in sensory and motor nerve function affecting postural and locomotion control (Akay et al. 2014; Thornell et al. 2015). The loss of proprioception with increasing age in humans is widely recognised and is central to a number of geriatric syndromes, in particular falls (Suetterlin and Sayer 2014). In this regard, it is worth noting that in mice aged between 11 and 21 M, *1a* proprioceptive sensory endings undergo morphological changes in extensor digitorum longus and soleus muscles, and neurons are fewer in number in L3 dorsal root ganglia in these mice by 17 M (Vaughan et al. 2017).

Physiological, behavioural and immunohistochemical studies in rodents investigating the impact of aging on the GABAergic system have frequently demonstrated an altered excitatory–inhibitory balance in old animals (Canas et al. 2009; Rozycka and Liguz-Lecznar 2017; Schmidt et al. 2010; Stanley et al. 2012). Age-related loss of VGLUT and VGAT markers has been reported in hippocampus (Canas

et al. 2009) and in cortex (Liguz-Lecznar et al. 2015), although in one other study in rat prefrontal cortex expression of VGAT remained unchanged while levels of other GABAergic proteins were either decreased (GABA receptors) or increased (GAD67) with age (Banuelos et al. 2014). Importantly however, little is known about how VGAT expression changes with age in the rodent spinal cord. In the present study there appeared to be no overall qualitative change in VGAT immunoreactivity in young versus old mice lumbar spinal cord, and quantitatively we found no difference in the percentage coverage of VGAT immunoreactive terminals on individual β-III tubulin positive motor neurons in 3 and 27 M spinal cords.

The present study has not distinguished between subtypes of α-motor neurons including fast-twitch fatigable (FF), fast-twitch, fatigue-resistant (FR), and slow-twitch fatigue-resistant (Kanning et al. 2010) using specific markers such as matrix metalloproteinase-9 (MMP-9) (for FF motor neurons) and osteopontin (OPN) (for FR and slow motor neurons) (Morisaki et al. 2016). In mouse spinal cords 70% of $OPN^{+}/ChAT^{+}$-positive motor neurons and group Ia proprioceptive terminals are immunoreactive for VGLUT1 (Misawa et al. 2012). Hence it is likely that the motor neurons in the present study positive for VGLUT1 were slow/FR motor neurons that are especially important for activities that require sustained muscular contraction, such as the maintenance of an upright posture and balance (Kanning et al. 2010; Purves 2001).

Reactive glia in aged spinal cord

Age-related changes in astrocyte reactivity have been reported in spinal cords (Barbeito et al. 2004; Duan et al. 2009; Kane et al. 1997), characterized by hypertrophy of cell bodies as well as thickening of their processes (Barrett et al. 1981). Consistent with these earlier studies, we observed a significant age-related increase in staining density for GFAP, particularly within the spinal cord gray matter of old mice. Mature astrocytes play important roles in stabilization and maintenance of synaptic contacts, and in the clearance and recycling of glutamate and GABA (Bernardinelli et al. 2014; Karimi-Abdolrezaee and Billakanti 2012; Rodriguez-Arellano et al. 2016; Sykova et al. 2002). Altered astrocyte phenotype towards a more reactive cell type is associated with a deterioration in synaptic function as well as increased levels of pro-infammatory cytokines (Lynch et al. 2010). Interestingly, in a murine model of SMA astrocyte dysfunction has been directly linked to motor neuron pathology (Martin et al. 2017). Overall then, it is likely that age-related astrocyte activation in aged spinal cords will affect neuronal health as well as synaptic (Petralia et al. 2014) and perhaps even extra-synaptic (Sykova et al. 2002) transmission, potentially also contributing to sensorimotor impairment.

Microglia are resident innate immune cells within the CNS and are also involved in the modulation of synaptic activity (Clark et al. 2015; Siskova and Tremblay 2013; Taves et al. 2013). We found an increase in Iba1 positive, process bearing microglial cells (ramified) in both the dorsal and ventral horns of the old (compared with young) spinal cords which were not immunopositive for the CD68 specific marker ED1, indicating that the microglia were not activated (Norden and Godbout 2013; Sutherland et al. 2016). Others have also described increased Iba1 positive microglial cell density in lumbar dorsal horns in aged (17 M) mice, the cells exhibiting thicker, rigid branches and enlarged cell bodies (Lee et al. 2015). In very old (30 M old) rats, a region specific increase in ED1 and GFAP expressing cells was documented, most prominently in the dorsal columns, with little evidence of activated ED1 positive cells in gray matter (Kullberg et al. 2001). Altered microglial reactivity in aged mouse spinal cord is associated with increased cytokine levels and greater blood–brain barrier permeability (Ritzel et al. 2015). Taken together with a likely impact on synaptic function, this observed altered microglial phenotype and increase in Iba1-positive spinal microglia is likely to exacerbate sensorimotor dysfunction in old sarcopenic mice.

In summary, we show for the first time that there is a selective and significant reduction in the number of VGLUT1 terminals, which are likely derived from primary *1a* afferents, associated with α-motor neurons in aged (27 M) mouse spinal cord, potentially compromising feedback from muscle spindles and negatively affecting sensorimotor coordination and neuromuscular performance. Altered glial cell phenotype was also characteristic of aged murine spinal cord but we saw no significant age-related change in VGAT immunoreactive terminal coverage on motor neurons in the ventral horn. The present data were obtained from 27 M old mice previously shown to exhibit

sarcopenia (Soffe et al. 2016) and with significant changes in sciatic nerve biology (Krishnan et al. 2016). It remains to be determined to what extent the observed loss of VGLUT1 immunoreactive terminals on α-motor neurons in aged spinal cord, and perhaps also the reactive glial changes, are a consequence of, or precede, these documented age-related changes in the peripheral neuromuscular system.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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