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Age-dependent decline in density of human nerve and spinal ganglia neurons expressing the $\alpha 3$ isoform of Na/K-ATPase

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Abstract

Ambulatory instability and falls are a major source of morbidity in the elderly. Age-related loss of tendon reflexes is a major contributing factor to this morbidity, and deterioration of the afferent limb of the stretch reflex is a potential contributing factor to such age-dependent loss of tendon reflexes. To evaluate this, we assessed the number and distribution of muscle spindle afferent fibers in human sacral spinal ganglia (S1) and tibial nerve samples obtained at autopsy, using immunohistochemical staining for the $\alpha 3$ isoform of Na⁺,K⁺-ATPase ($\alpha 3$ NKA), a marker of muscle spindle afferents. Across all age groups, an average of 26±4% of myelinated fibers of tibial nerve and 17±2% of ganglion neuronal profiles were $\alpha 3$ NKA-positive (n=8 per group). Subject age explained 85% of the variability in these counts. The relative frequency of $\alpha 3$ NKA-labeled fibers/neurons starts to decline during the 5th decade of life, approaching half that of young adult values in 65-year-old subjects. At all ages, $\alpha 3$ NKA-positive neurons were among the largest of spinal ganglia neurons. However, as compared to younger subjects, the population of $\alpha 3$ NKA-positive neurons from advanced-age subjects showed diminished numbers of large (both moderately and strongly labeled), and medium-sized (strongly labeled) profiles. Considering the critical significance of ion transport by NKA for neuronal activity, our data suggest that functional impairment and, also, most likely atrophy and/or degeneration of muscle spindle afferents, are mechanisms underlying loss of tendon reflexes with age. The larger and more strongly $\alpha 3$ NKA-expressing spindle afferents appear to be proportionally more vulnerable.

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Disclosure Statement

The authors have no conflicts of interests to disclose

Keywords

Age; Stretch reflex; Muscle spindle afferents; Sodium-potassium ATPase isoforms

1.0 Introduction

By 2030 the number of people older than 65 years of age is predicted to nearly double (Wild et al., 2004). Difficulty with maintaining balance during upright stance or walking is a major cause of falls and morbidity in the elderly (Goble et al., 2009; Shaffer and Harrison, 2007). Loss of deep tendon, and specifically ankle plantar flexor, reflexes has long been established as an important contributor to balance and gait disturbances in people with peripheral neuropathy (Krosnick, 1964; Shehab et al., 2012) and in subjects of advanced age (Bowditch et al., 1996; Vrancken et al., 2006).

Aging affects deep tendon reflexes at different levels of the proprioceptive reflex arc, including degenerative changes in muscle spindle receptors, functional deficiency within the muscle spindle primary afferent pathway, changes in corticospinal excitability, loss of motor neurons and loss of muscle mass and strength ((Baudry et al., 2014; Benjuya et al., 2004; Chung et al., 2005; Liu et al., 2005; Obata et al., 2010; Raffalt et al., 2015; Swash and Fox, 1972), and see (Shaffer and Harrison, 2007) for a review). Of these deficiencies at least two -- a decrease in the number of intrafusal fibers per spindle and delayed and weakened primary afferent input into the spinal cord (longer latency and weaker H-reflex) -- are consistent with the idea of age-dependent atrophy and degeneration of muscle spindle afferent neurons (Liu et al., 2005; Raffalt et al., 2015). This question, however, remains open. Sensory nerve conduction studies do not distinguish between afferent fibers of similar diameter in vertebrate species, such as Ia and Ib (muscle spindle primary and tendon organ) afferents or various group II afferents (muscle spindle secondary and many other mechanoreceptive afferent fibers). H-reflex measurements do not discriminate between structural and functional changes in fibers/neurons and motor neurons.

Histological studies of lumbar and cervical DRGs from 60- to 70-year-old human subjects have reported neuronal count decreases of 35% compared to those of 10- to 30-year-old people (Nagashima and Oota, 1974). Another study of human lumbar ganglia suggested that large afferent neurons (the group of mechanoreceptive neurons that includes muscle spindle afferents) might be more affected by age-dependent loss than small, nociceptive and temperature afferents (Holford et al., 1994). Similarly, selective loss of the largest fibers has been observed in studies of human anterior tibial and sural nerves (O'Sullivan and Swallow, 1968; Swallow, 1966). In lumbar DRG of aged rats (30 months old, which corresponds to the average lifespan of vivarium rats), there is a mild but statistically significant 16% atrophy of neurons that is, again, selective to large neurons (Bergman and Ulfhake, 1998). However, neuronal/axonal profile size is not an absolute criterion for distinguishing muscle spindle from other mechanoreceptive primary afferent neurons in DRG or from other large afferent or motor fibers in peripheral nerves (Lawson, 1992). As a consequence of the lack of a specific marker for muscle spindle afferents, none of these previous studies were able to directly assess the effects of aging on muscle spindle afferent neurons.

To address this problem, we utilized immunostaining for the $\alpha 3$ isoform of the sodium-potassium ATPase ($\alpha 3$ NKA). NKA is an ion transporter vital for all animal cells, and specifically so for neurons facing the task of fast restoration of Na^+ and K^+ gradients during and immediately after neuronal discharges. The transporter is composed of α , β , and γ protein subunits, of which the α subunit determines the major catalytic and transport properties of the entire complex. This subunit of mammalian NKA has four known isoforms ($\alpha 1$ through $\alpha 4$), which are expressed in a cell type- and developmental stage-dependent manner (Lingrel, 1992; Sweadner, 1995). Of critical importance for this study is that -- among primary afferent neurons of a variety of vertebrate species, including humans -- the transporter complex that includes the $\alpha 3$ isoform of the α subunit (or $\alpha 3$ NKA) is expressed only by stretch-receptor afferent neurons (Dobretsov et al., 2003; Dobretsov and Stimers, 2005; Edwards et al., 2013; Parekh et al., 2010; Romanovsky et al., 2007). Thus, in spinal ganglia of humans, $\alpha 3$ NKA expression can be used as a reliable identifier of muscle spindle afferents. The same is applicable to human peripheral nerve, provided that small (4-5 μm O.D. or less) myelinated fibers are excluded from analysis (to exclude processes of γ -motoneurons) and with consideration that there is a relative paucity of human β -motor neurons; the other two classes of peripheral neurons expressing $\alpha 3$ NKA (Edwards et al., 2013; Romanovsky et al., 2007)). Human plantar flexion muscles, primarily gastrocnemius and soleus (triceps surae), receive innervation from the S1 ganglion through the tibial nerve. Therefore our study focused on analysis of tissue samples obtained at these two specific locations.

2.0 Experimental procedures

2.1. Tissue sample processing

S1 DRG samples were obtained from human autopsy material within 12 hours of death *via* the_National Disease Research Interchange Program_(NDRI). Samples of the tibial nerve were obtained from routine University of Arkansas for Medical Sciences hospital autopsies (6 - 24 hours post-mortem intervals) of patients without known neural disease. Tibial nerves were obtained at the popliteal fossa just below the bifurcation of the distal sciatic nerve (see (Gustafson et al., 2012)). After isolation, nerve and DRG samples were immediately immersed in fixative (10% formaldehyde in phosphate buffered physiological saline; pH = 7.2), and letter- and number-coded. DRG samples from NDRI were shipped (FedEx overnight) to our laboratory. All samples were characterized by subject age, gender, race, cause of death and immediate medical history.

Upon receipt in the laboratory, samples were cleaned of fat tissue, inspected for integrity, trimmed, post-fixed in 4% formaldehyde for 5-7 days at 4°C and then processed for immunostaining using an established protocol of our laboratory (Romanovsky et al., 2005). In studies of tibial nerve samples, several sequential 5- μm thick cross-sections were cut and each immunostained for $\alpha 3$ NKA and then stained with haematoxylin. In studies of DRGs, 3 pairs of 5-micron-thick, transverse sections were cut from the center of each ganglion at intervals of 100 - 300 μm . For each pair, one of the sections was immunolabeled for $\alpha 3$ NKA while another section was stained with eosin/haematoxylin.

2.2. Antibodies and reagents

α 3NKA was detected with a monospecific rabbit polyclonal IgG raised against rat α 3 Na⁺,K⁺-ATPase ((#06-172; EMD Millipore; Billerica, MA, formerly Upstate Biotechnology, Lake Placid, NY). This antibody recognizes the intracellular isoform-specific domain of the α 3 subunit of NKA, and has been previously extensively characterized, including through immunochemistry experiments using higher primate and human tissue by our and by other groups (Blaustein and Juhaszova, 1997; Dobretsov et al., 2003; Dobretsov et al., 1999; Edwards et al., 2013; Romanovsky et al., 2005; Romanovsky et al., 2007; Shyjan and Levenson, 1989). Biotinylated secondary antibodies were obtained from Jackson Laboratories (West Grove, PA). Other reagents required for the immunoperoxidase detection method were obtained from Sigma (St. Louis, MO) and from Vector Laboratories (Burlingame, CA).

2.3. Immunohistochemistry

Immunostaining of tissue sections for α 3NKA was conducted as previously described (Romanovsky et al., 2005; Romanovsky et al., 2007). Briefly, after deparaffinization, microwave unmasking, and endogenous peroxidase and nonspecific antibody binding site blocking with hydrogen peroxide (3% in methanol, for 10 minutes at room temperature) and heat-inactivated 10% fetal bovine serum (Sigma; 30 min at 37°C), sections were incubated overnight with primary antibody (dilution 1:800–1:1,600 in phosphate-buffered saline (PBS) containing 1% donkey serum, 4°C), following by incubation with secondary biotinylated antibody (1:600 in PBS), peroxidase-conjugated streptavidin label (1:400 in PBS), and finally 3,3'-diaminobenzidine chromogen solution. The immunoreaction was monitored under a microscope and stopped by washing with PBS. In negative control experiments, the incubations with primary antibody were substituted by an equivalent time of incubation in 1% bovine serum albumin in PBS.

2.4. Image analysis and processing

Stained and immunolabeled sections were evaluated using an upright BX51WI Olympus microscope (Olympus America, Melville, NY). Images were captured using 20x, water-immersion (DRG) or 40x, water-immersion (tibial nerve) objectives, a Coolsnap Cf digital camera (Photometrix, Tucson, AZ) and analyzed with Image-Pro 5 (Media Cybernetics, Rockville, MD)) and “clock-scan” protocol software (Dobretsov and Romanovsky, 2006). The minimal image resolution (20x objective) was 0.33 μ m per pixel or 0.1 μ m² of area per pixel.

For the tibial nerve studies, the entire nerve cross-sectional profile was scanned to count all visible large (4–5 μ m in outer diameter), α 3NKA-ring-labeled myelinated fiber profiles (Fig. 1A, arrows) in all fascicles present (3 to 11 per sample). The small/large fiber diameter cut-off value was selected based on previous studies of paraffin-embedded human formaldehyde-fixed lumbar ventral roots (Romanovsky et al., 2007) and Flemming fixative-fixed anterior tibial nerve samples (Swallow, 1966). Designation of some axons as α 3NKA-positive or negative in cases of weak staining may be subjective. Therefore, each nerve fascicle in each sample was assessed by two independent observers and average values of

these two determinations were then used to calculate a mean percentage of labeled fibers ($\pm$ between-fascicular counts SE) per nerve sample.

In studies of DRG, in each section, only neuronal profiles containing nuclei with nucleoli were counted and measured. Measurements of neuronal profile areas require outlining of neuronal somata, which is difficult without counterstaining for neurons expressing no α 3NKA. On the other hand, counterstaining interferes with measurement of intensity of neuronal plasma membrane labeling for α 3NKA. To address this issue sections were prepared in pairs as described above; one of each pair was stained with H&E to obtain a total neuron count and data for total neuron size distributions, while the matching parallel section was immunostained for α 3NKA count and characterize α 3NKA-labeled neurons only (**Fig.1, B,C**). The ratio of these two counts was used to determine percentage of α 3NKA neuronal profiles at a given level in the ganglion. A mean of three determinations per ganglion ($\pm$ SE, except for subject 2002-05 for which only two pairs of sections were available) was calculated and reported.

Cross sectional areas of all counted neuronal profiles were measured to build and analyze neuronal profile size distributions (85 – 240 profiles per subject). Intensity of cytoplasmic and membrane labeling of α 3NKA-positive neuronal profiles was measured with a clock-scan procedure as described previously (Dobretsov and Romanovsky, 2006). Briefly, the plasma membrane of the neuron of interest was outlined on the regular light image (**Fig. 1D**, inner dashed outline). A center of outline was set in the center of the cell's nucleus, and radial scanning of pixel intensity was started in a direction from the center to the first pixel of the cell outline and continued clockwise (**Fig. 1D**; straight vector and curved arrow, respectively) until all cell radii were scanned. The length of each radial scan exceeded the radius of the cell in the direction of scan by a preset fractional number (0.3 of the radius in this study; outer dashed white line in **Fig. 1D**). Collected radial profiles were aligned by scaling to corresponding radii and averaged to produce the integral clock-scan intensity profile in 256 shades of grey scale units. Examples of individual clock-scan profiles of α 3NKA-positive neurons (#3, #4, and #8; **Fig. 1B-D**) α 3NKA-negative cell (#5 **Fig.1B-C**) are shown in **Fig. 1E**.

One of the powerful features of the clock-scan protocol is that it collects information on integral pixel intensity in several cell compartments (nucleus, cytoplasm and membrane) and over the territory immediately surrounding the cell (**Fig.1E**). In this study, intensity of plasma membrane labeling for α 3NKA (I_{mem}) was measured as the clock-scan profile peak intensity (100% of profile X-axes) corrected by subtraction of average intensity of pixels of cytoplasmic region (I_{cyt} ; pixels between 40% and 80% of the profile X-axis; **Fig 1E**, labeled arrows). Subtraction of this latter effectively corrects I_{mem} value for variations associated with experiment-to-experiment variations in microscope light intensity, variations in staining procedure or local variations in the section thickness (see (Dobretsov and Romanovsky, 2006; Lacroix-Fralish et al., 2009)).

During counting and morphometric/clock-scan measurements, observers were blinded with respect to subjects' characteristics.

2.5. Curve fitting and statistical data analysis

The area and labeling intensity frequency distributions of counted neuronal profiles were analyzed using a Gaussian function, best-fit procedure and the Levenberg–Marquardt best fit minimization algorithm (Marquardt, 1963). In all cases presented in this work fitting the frequency distributions required using a sum of two Gaussian functions to achieve an overall goodness of fit (R^2) value of 0.8 or more, with the Y-axis offset value set to “0” with all other fit parameters (unless stated otherwise) free to vary. Linear regression analysis was used to evaluate the strength of association between individual subject's age and percentage of α 3NKA-positive fiber and neuronal profiles. Curve fitting and regression analysis were conducted using Origin 9.0 (OriginLab, Northampton, MA) software. To compare frequency distributions, percentages of observations were rounded up and converted into numbers of observations, and contingency tables based on these bin data were constructed and analyzed using χ^2 criterion. When required, data from adjacent distribution bins were combined to allow each cell in the contingency table to contain 6 or more observations. The contingency table analysis and other statistical tests were done with use of a GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA) software program package. A probability of $p < 0.05$ was used as the criterion for significance.

3.0 Results

3.1. Tissue samples

Overall we obtained 11 DRG samples (from NDRI) and an equal number of tibial nerve autopsy samples. Two of the DRG samples and three of the tibial nerve samples were excluded from the study because of records of diabetes (a potential covariate of the effect of “normal aging” ; see (Bowditch et al., 1996)). One additional DRG sample was excluded because of an unexplained failure of several attempts of immunochemistry for α 3NKA in sections from this sample. The characteristics of the final cohort of subjects used in this work are listed in **Table 1**.

3.2. Percentage of α 3NKA-neuronal and axonal profiles

Mean densities of all counted myelinated fibers and neuronal profiles were, respectively 6254 ± 1068 per mm^2 of fascicular area and 14 ± 2 per mm^2 of DRG neuropil area. There was a tendency for these counts to decrease with age, but neither regression reached statistical significance (data not shown). Percentages of α 3NKA-positive profiles of tibial nerve large myelinated fibers and S1 DRG neurons were $26 \pm 4\%$ and 17 ± 2 , respectively. Both tibial nerve fiber and DRG α 3NKA-positive neuron relative counts showed a strong negative correlation with the subject age. Linear regression analysis demonstrates a decline of approximately 5% in both variables *per* decade of human life (**Fig. 2A and B**).

Overall, the ages of the tissue donors did not differ between the two groups (tibial nerve vs. DRG); however male subjects were overrepresented in the former and Caucasian subjects were overrepresented in the latter group. Therefore, to assess potential role of race or gender in the determined relative counts of α 3NKA fibers/neuron profiles, individual neuron and fiber profile counts were normalized to the respective group mean value, pooled together and then grouped by gender or race (**Fig 2C, D**). These new groups all passed Kolmogorov-

Smirnov assessment, but some of them (females and non-Caucasians; less than 6 subjects) were too small for reliable conclusions using D'Agostino and Pearson or Shapiro-Wilk normality tests. Nonetheless, no statistically significant gender or race effect was identified for percentage of α 3NKA-positive fibers or for neuron profiles, using either the non-parametric Mann-Whitney U-test or the parametric -- and more prone to Type I error (false-positive result; (Krzywinski and Altman, 2014)) -- two-population t-test (**Fig. 2C and D**).

3.3. Neuronal profile size and intensity of plasma membrane labeling for α 3NKA distributions

To obtain further insight into these age-dependent changes we have analyzed and compared size and integral membrane labeling intensity of α 3NKA neuronal profiles from ganglia of the three youngest and three oldest subjects from our sample (**Fig. 3**; mean ages 44 ± 5 and 64 ± 3 years; α 3NKA neuronal profile percentages 21.5 ± 0.5 and 13.1 ± 1.4 , respectively).

Age did not affect the shape of total neuronal profile area distributions (X^2 (df = 7) = 2.565; $p = 0.922$). In both age groups, a sum of two Gaussian curves was required to describe these distributions, suggesting that overall population of human S1 DRG neurons is composed of sub-populations of large and small neurons, with large neurons constituting about half of all cells (47% and 52% in ganglia of young and older adult subjects, respectively; **Fig 3A, Table 2**).

With regard to α 3NKA-positive neurons, on average and as compared to younger subjects, the area of these neuronal profiles in aged subjects decreased by 35% and the intensity of their plasma membrane labeling for α 3NKA was weaker by 18% (2900 ± 204 vs. $4476 \pm 263 \mu\text{m}^2$ and 66 ± 3 vs. 80 ± 3 pixel intensity units).

Based on the analysis of profile size and intensity of labeling, the group of α 3NKA-positive S1 DRG neurons could be further subdivided into subgroups of neurons having medium- and large-size profiles (**Fig. 3B**) and subgroups of moderately and intensively labeled cells (**Fig. 3C**). In both cases, a fit with sum of two Gaussian equations performed substantially better in terms of goodness of fit (R^2) than did a single Gaussian curve (fitting the size distribution of α 3NKA neurons of older subjects required the width of first component of the fitting curve to be set to a constant to prevent overestimate of the height of this component; **Table 2**). Both size and labeling intensity distributions were statistically significantly different between young and advanced-age subjects (X^2 (df = 5) = 31.83 (size) and X^2 (df = 5) = 34.88 (intensity of labeling); $p < 0.001$). Cut-off values, ordinates of the valleys between adjacent peaks in younger subjects neuronal profile size and plasma membrane labeling intensity distributions, were $5585 \mu\text{m}^2$ and 75 pixel intensity units; for older subjects these values were $2704 \mu\text{m}^2$ and 55 pixel intensity units, respectively (**Fig 3, B and C**; labeled vertical dashed lines)

Using young subject data and the set cut-off values, neuronal profiles could be classified as large size and moderately or strongly labeled (LM and LS, respectively) or medium size and moderately or strongly labeled (MM and MS; see **Fig 4A,B** for examples). Distribution of profiles between these four categories was 22%, 9%, 20% and 49% of the entire sample of

45 profiles, respectively **Fig. 4C**, open circles). Using the same criteria, no LM or LS profiles were present in advanced-age subjects' DRG sections (**Fig 4C**, closed circles).

Using the data from old subjects and the set cut-off values, 17%, 34%, 14% and 34% of the total of 29 profiles belonged to LM, LS, MM and MS profile groups, respectively (statistically different from distribution of younger subjects data; X^2 (df = 3) = 10.2; $p=0.017$; **Fig. 4D**, closed circles). To more directly compare the data from young and old subjects, we then adjusted the young subjects' data based on the assumption that aging is associated with an overall 35% atrophy of all $\alpha 3$ -NKA neurons and an 18% loss in expression of $\alpha 3$ NKA in these neurons (numbers above). Importantly, this adjustment resulted in a fractional distribution of LM, LS, MM and MS profiles that is not statistically different from that observed for the profiles of $\alpha 3$ NKA neurons of older subjects DRG: 22%:29%:13%:36% (X^2 (df = 3) = 1.127; $p=0.771$; **Fig. 4D**, opened circles).

4.0 Discussion

The major finding of this work is the demonstration of an age-dependent decline in percentage of human $\alpha 3$ NKA-positive tibial nerve myelinated axonal and S1 DRG neuronal profiles. The $\alpha 3$ NKA is a key ion transporter and marker of muscle spindle afferent neurons of vertebrates (Dobretsov et al., 2003; Dobretsov and Stimers, 2005; Romanovsky et al., 2005; Romanovsky et al., 2007). Our data therefore suggest that, in addition to other factors, an age-dependent decline of deep tendon reflexes may be associated with a functional and possibly structural deterioration of the afferent limb of the reflex arc.

4.1 Percentage of $\alpha 3$ NKA-labeled axonal and neuronal profiles

Our estimate of the density of large myelinated fibers in tibial nerve fascicles ($6,254 \pm 1,068$ per mm^2 of fascicular area) is in reasonable agreement with previous studies of formaldehyde-fixed human anterior tibial and sciatic nerves (a total of 10,000 to 13,000 myelinated fibers per mm^2 , with about half of those being of small size (see Swallow, 1966). The density of counted DRG neuron profiles, 10 to 24 per mm^2 of DRG neuropil, is also in the reported range of 10 to 27 profiles of neurons with nucleoli per mm^2 of neuropil in 10% formaldehyde-fixed human cervical and lumbar DRGs (Nagashima and Oota, 1974).

For the youngest subjects, the mean percentage of $\alpha 3$ NKA-positive axonal/neuronal profiles counted in this study of human tibial nerve was $38 \pm 2\%$ of large myelinated fibers in tibial nerve, and $22 \pm 1\%$ of all neurons in S1 DRG. In spinal ganglia $\alpha 3$ NKA is a selective marker of muscle spindle afferents (Dobretsov et al., 2003; Edwards et al., 2013; Parekh et al., 2010) and our data suggest that human S1 DRG has a somewhat greater population of these neurons as compared to mouse, rat, pigeon (8%-17% of all neurons; (Dobretsov et al., 2003; Edwards et al., 2013; Romanovsky et al., 2007)) or human (11% of all neurons in one subject (Romanovsky et al., 2007)) lumbar DRG. It must be noted, however, that in addition to species dependence (Romanovsky et al., 2007) there is a strong spinal level-dependence for the percentage of stretch receptor afferents. Thus, as much as 73% of all neurons in mouse C4 DRG are reported to belong to this group of cells, which may correspond with the high muscle spindle density of the cervical myotome muscles (Edwards et al., 2013).

The fraction of $\alpha 3$ NKA-positive large myelinated fibers in human tibial nerve determined in this study ($38 \pm 2\%$, youngest subjects) is very similar to that in mouse tibial nerve ($36 \pm 2\%$ of large myelinated fibers; 3 month-old animals (Romanovsky et al., 2007)). At the level of mixed peripheral nerve, $\alpha 3$ NKA labels processes of all neurons innervating muscle spindles: that is type Ia and II afferent neurons, β -motor neurons (synapsing on both extrafusal and intrafusal muscle fibers) and γ -motor neurons (Romanovsky et al., 2007). Small myelinated fibers (γ -motor neurons) were not counted in our study. As to skeletofusimotor (β) innervation, its prevalence in human muscles and specifically soleus and gastrocnemius muscles has never been determined. We found no $\alpha 3$ -NKA-positive large myelinated fibers in human L4-L5 ventral root, suggesting a paucity of β -motor neurons at these spinal levels (Romanovsky et al., 2007). Nonetheless, 39% and 62% of spindles in human peroneus brevis muscle and extensor capri radialis muscles are reported as receiving β innervation, respectively (Kakuda et al., 1998; Murthy et al., 1982). Therefore, the presence of some processes of β -motor neurons among $\alpha 3$ NKA-positive tibial nerve fibers in our samples cannot be excluded. Nonetheless, as discussed below, our finding of essentially the same time course of decrease in percentages of $\alpha 3$ -NKA positive S1 DRG neuron and tibial nerve fiber profiles suggests that either the fraction of β -motor axons in tibial nerve is very small or that muscle spindle afferents and skeletofusimotor fibers are affected equally by age.

4.2. Percentage of $\alpha 3$ NKA axonal and neuronal profiles and the effect of age

Of the subjects' characteristics examined in this study (gender, race, age and cause of death/clinical history), age emerged as the single factor explaining most (84-87%; **Fig. 2**) of the variability in relative counts of $\alpha 3$ NKA-positive tibial nerve fibers and S1 DRG neuron cross-sectional profiles. Of note, previous studies have also reported no gender or race effects on age-related deficits in counts of the largest axonal profiles in human sural and anterior tibial nerves (O'Sullivan and Swallow, 1968; Swallow, 1966), or loss of tendon reflexes (Bowditch et al., 1996; Mold et al., 2004)).

To compare time courses of deterioration with age of our two measures with each other, we normalized our tibial nerve and S1 DRG data to the youngest within each group subject and plotted them together (**Fig. 5**; open and closed circles respectively). In addition, Figure 5 also contains clinical data from the literature (again normalized to the youngest subjects' results) on the bilateral loss of ankle reflex in a population of 'normal' human subjects ((Bowditch et al., 1996); **Fig 5**, crosses).

This comparison shows that relative counts of both $\alpha 3$ NKA-positive tibial nerve fibers and $\alpha 3$ NKA-positive S1 DRG neuronal profiles are maintained at a stable, 'normal' level throughout the first 4 decades of human life, and that these counts then start to decline, approaching 50% of the original levels in 65-year-old subjects. Notably, up to this 65-year time point, the time course of decline for the $\alpha 3$ NKA neuronal/axonal percentages are very similar to that of clinical decline in prevalence of normal ankle reflexes. Overall however, a decline in percentage of $\alpha 3$ NKA-positive fibers/neuron profiles appear to precedes the loss of reflexes by about 10 years (**Fig. 5**, fit curves). Both our study and previous human ankle reflex studies (Bowditch et al., 1996) are cross-sectional and therefore strongly dependent upon exclusion of subjects with neuropathy or other clinical conditions that may add to

sensory-motor deficits associated with normal aging. Compared to our work, such selection was much more rigorous in the human reflex study. Thus it is possible that most of the people having abnormal reflexes in their fifth decade had by their 6th-7th decades developed clinically diagnosed disease and therefore were excluded from the reflex analysis. The advanced age subjects (>60 years) in our autopsy study included individuals who died with diagnoses of renal disease (subjects “e” and “d”; **Fig. 5** and **Table 1**), peripheral vascular disease (subject “f”), or cancer and chemotherapy (subject “a”). Therefore there is the possibility that these comorbid conditions may carry an increased chance of disease- or treatment-accelerated loss of ankle reflexes (Mold et al., 2004; Ropper, 1993). Once again, however, our analysis suggests that age and, as possible confounder, disease/treatment duration -- but not the particular disease state -- as the major determinant of variability in relative counts of α 3NKA neuronal and nerve fiber profiles in our samples. Thus, both the 67- and 81-year-old subjects “e” and “d” (**Fig. 5** and **Table 1**) had diagnoses of renal disease, but relative loss of α 3NKA profiles was about 2.5 times more advanced in the 81-year-old subject as compared to the 67-year-old subject.

4.3. Subpopulations of S1 DRG neuron profiles and effect of age

A comparison of total and α 3NKA-labeled neuronal profile size distributions from older and younger subjects in our study suggests that the aging effect is largely selective for α 3NKA-positive neurons. Thus, the leftward shifts of peaks of subpopulations of small and large DRG neuronal profiles, and the decline in the fraction of large profiles, in the total distribution amounts to only 6%-9% (**Fig 3A, Table 2**). At the same time, the observed leftward shifts and decreases in areas of the peaks of the size distribution of α 3-NKA-positive neuronal profiles range between 29% and 47% (**Fig. 3B, Table 2**). This pattern of changes is very different from the non-specific effect of a complete sciatic nerve transection on sub-populations of rat L5 DRG neuronal profiles, where 15 days after surgery the peak and area of size-distributions of all small, large and α 3NKA-positive neurons demonstrate about the same 20%-40% declines (Romanovsky et al., 2005). The finding of a selective vulnerability of α 3NKA-expressing afferent neurons (muscle spindle afferents) during aging is also in a good agreement with clinical observations that, among aged subjects with bilateral sensory deficits, absence of ankle reflexes is most pronounced (83% of cases), followed by loss of touch, vibration and joint position senses (31%, 15%, 7% of cases, respectively (neural functions mediated by other than muscle spindle large mechanoreceptive afferents) (Mold et al., 2004)).

4.4. Subpopulations of α 3NKA-positive DRG neurons and the effect of age

There are two major types of muscle spindle afferents known: large diameter, rapidly conducting, primary type Ia afferents innervating all bag1, bag2 and nuclear chain intrafusal muscle fibers within the muscle spindle; and the smaller and slower conducting, type II fibers establishing secondary nerve endings on bag2 and nuclear chain intrafusal fibers (Baker, 1974; Hunt, 1974). The reported ratio of type I to type II muscle spindle afferents in nerves supplying human finger extensor or baboon soleus muscle is in the 2:1 – 3:1 range (Cheney and Preston, 1976; Edin and Vallbo, 1990). Furthermore, we previously have shown that some rat DRG neurons express both α 1 and α 3 NKA; while others express α 3 NKA only; and that the ratio of these two categories of neuronal profiles was 2:1, suggesting

the interesting possibility that the pattern of expression of the α isoform of NKA may differ between different types of muscle spindle afferents (Dobretsov et al., 1999).

In this study we have identified 4 groups of neuronal profiles -- LM, LS, MM and MS -- with relative a fractional distribution of 22%, 9%, 20% and 49% in young and 17%, 29%, 13% and 36% in old subjects (**Fig. 4C**). Importantly, neuronal profiles with moderate and strong α 3NKA labeling could be found within a given section and sometimes in the same field (see **Fig. 4B**, right image containing both LM and MS cells), arguing that observed profile-to-profile differences in plasma membrane labeling intensity represent true differences in α 3NKA expression, and are not a consequence of section-to-section or staining-to-staining variability of the signal. However, considering that we have no data on α 1 NKA expression, and given the limited sample sizes and the further limitations of our study (see next section), the relationship between MM, MS, LM and LS neurons and pattern of α isoforms of NKA expression and type of muscle spindle afferents remain to be addressed by future studies.

With regard to the question of the effect of aging, comparison of profile size-intensity scatter plots of young and old subject data does create an impression that subpopulations of the largest and most strongly labeled cells are those that are specifically targeted (**Fig. 4C**). However, we were able to mathematically reproduce the age-associated change in distribution of neuronal profiles between MM, MS, LM and LS sub-categories by adjusting the values of young subjects for an 18% decrease in α 3NKA expression and a 35% neuronal atrophy (**Fig. 4D**). This suggests that there is a uniform effect of age for all neuronal profiles, and further suggests that the impression of age-associated down-regulation of α 3NKA expression and atrophy of groups of muscle spindle afferents may be simply the result of the fact that these effects of age are proportionally stronger in the larger and more strongly expressing α 3NKA cells.

4.5 Limitations, potential mechanisms and future studies

Throughout this work we characterized differences in relative counts of axonal and neuronal profiles using a technique that is sufficiently sensitive to characterize the prevalence of, and to detect relative loss of neuronal/axonal profiles as identified by a specific marker. The absolute loss of α 3-neuronal and fiber profiles may, however, be underestimated in our study if there is a widespread loss of non- α 3NKA-positive large myelinated fibers or DRG neurons with age. We also need to acknowledge the possibility of a progressive undercounting of α 3-NKA-positive tibial nerve fibers with age (since we used a standard 4-5 μ m diameter cut-off value to exclude small myelinated fibers throughout the study) and an over-counting of large DRG neuron profiles that have larger and more distinct nucleoli than compared to small cells (see (Coggeshall, 1992; Tandrup, 1993)). However, the similarity of time courses of decline in counts of α 3NKA fiber and neuronal profiles observed in this work (**Fig. 5**) suggests that the effect of these last two factors is probably small relative to the actual effect of aging.

Nonetheless, stereological analysis including use of a physical disector method and absolute cell counts would be necessary to determine to what degree aging results simply in down-regulation of expression of α 3NKA (loss of marker) as opposed to physical degeneration

and loss of α 3NKA afferent neurons and their fibers. Such analysis would also be needed to identify specific roles of neuronal atrophy vs. degeneration in age-associated differences in size distributions of α 3NKA neurons.

Tissue and cell shrinkage is an intrinsic artifact of formaldehyde fixation and paraffin embedding (see (Lawson and Waddell, 1991)). However, this should equally affect all large, as opposed to preferentially α 3NKA-positive, neuronal profiles. Therefore, selective atrophy of α 3NKA neurons remains the best explanation for our observations.

Finally, our study is cross-sectional, and hence may not suggest any causal relationships. Thus the observed association between disappearance of α 3NKA fibers and DRG neuron profiles, and clinical age-associated loss of reflexes (**Fig. 5**) should be verified in larger scale studies. Good clinical histories appear to be of critical significance as separation of ankle reflex loss associated with ‘normal aging’ from that resulting from aging-associated neuropathic conditions is a major challenge in such studies (Mold et al., 2004). Furthermore if atrophy and/or degeneration of α 3NKA neurons is confirmed, the pathogenesis of these events will also need to be evaluated in animal models of aging and disease. Age-dependent neuronal atrophy is a well-recognized phenomenon that is commonly attributed to a gradual decline in muscle neurotrophin production and neuronal sensitivity to these neurotrophins (see (Bergman and Ulfhake, 1998)). The same mechanism could be applicable to our findings as expression of α 3NKA in DRG neurons is known to be target-dependent (Romanovsky et al., 2005). For muscle spindles in aged human muscles, degenerative changes and loss of intrafusal nuclear chain fibers have been described (Liu et al., 2005; Swash and Fox, 1972). Thus, a decrease in expression of α 3NKA with subsequent atrophy and degeneration of muscle spindle afferents might also be attributable to diminished availability of target-derived neurotrophins with neurotrophin-3 as one of the leading candidates (Gorokhova et al., 2009).

4.4. Conclusions

In conclusion, our study provides further demonstration of the utility of α 3NKA immunostaining for the identification and evaluation of muscle spindle afferents in autopsy tissue samples (Dobretsov and Stimers, 2005). This work also is the first to provide direct morphological support for the hypothesis that deterioration of deep tendon reflexes in elderly people may be associated with an age-dependent weakening of the afferent limb of these reflexes. Further studies using human tissue samples, larger subject populations, a physical dissector technique, and markers for neurodegeneration are needed to substantiate this finding. In addition, animal models of aging are needed to determine the functional identity and mechanisms of vulnerability of subpopulations of stretch receptor afferent neurons to the effects of age.

Dedication and Acknowledgments

The authors wish to dedicate this publication to the memory of Dmitry Romanovsky, who tragically passed away in 2011. Dr. Romanovsky made immense contributions to our understanding of the neuronal function of α 3NKA, and pioneered the research which led to these findings.

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Abbreviations

α3NKA	α3 isoform of the sodium-potassium ATPase
DRG	dorsal root ganglion
MM	medium-sized, moderately α3NKA-labeled neurons
MS	medium-sized, strongly α3NKA-labeled neurons
LM	large, moderately α3NKA-labeled neurons
LS	large, strongly α3NKA-labeled neurons

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Highlights

- Muscle spindle afferents were identified in human autopsy nerve and spinal ganglia samples
- The percentage of muscle spindle afferents in humans decreases with age starting in the 5th decade
- Other large mechanoreceptive afferents appear to be less vulnerable to the effect of age

Figure 1. Representative examples of labeling for $\alpha 3$ NKA in samples of human tibial nerve and S1 DRG, and results of clock-scan analysis of neuronal membrane labeling

A: Tibial nerve cross sections were immuno-stained for $\alpha 3$ NKA (brown precipitate) and counterstained with hemotoxylin. Myelinated fiber profiles that show clearly distinguishable ring-like staining of the axon (white arrows) were counted as positive. Asterisks mark examples of $\alpha 3$ NKA-negative fibers; sharp black arrows mark fibers that were not counted either because of small size or because of structural damage making the persence of labeling vs. artifactual labeling difficult to ascertain. Question marks label axons to be classified at the discretion of the observer. Calibration bar: 12.5 μ m

B, C: Sequential pairs of 5- μ m-thick DRG sections were cut and stained: one with eosin and hematoxylin to obtain total neuronal profile counts and size distribution (B) and another with an α 3NKA-specific antibody to detect α 3NKA (C). Profiles of neurons seen in both sections are labeled 1 through 7. However, only profiles with nuclei and nucleoli were counted and measured in H&E and NKA-stained sections (profiles 1 and 2 in B and 3 and 4 in C). Calibration bars – 50 μ m.

D,E: Images of sections immunostained for α 3NKA DRG were captured at 20x magnification (D, calibration bar = 25 μ m) and membrane and intracellular distributions and intensity of labeling were analysed using a clock-scan protocol of image analysis (E, clock-scan profiles of some of number-labeled neurons in panels B – D are shown; see text for other details).

Figure 2. Effects of age (A-B), gender (C) and race (D) on percentage α3NKA-positive profiles of tibial nerve and S1 DRG myelinated fibers and neurons

A: Plot of relative counts of α3NKA-positive tibial nerve fiber profiles vs. subject's age.

Solid and dashed lines are linear regression and regression 95% CI lines.

B: Plot of relative counts of α3NKA-positive S1 DRG neuron profiles vs. subject's age.

Solid and dashed lines are as in panel A.

C. Relative counts of α3NKA-positive DRG neuronal and tibial nerve fibers: profiles were normalized to the respective group mean, pooled together and grouped by subjects' gender; means (horizontal bars) are not different by either two-sample t-test; $p = 0.22$ or Mann-Whitney test, $p = 0.257$.

D: Data were normalized and pooled as described in the panel C legend and then grouped by subjects' race; means (horizontal bars) are not different by either two-sample t-test; $p = 0.761$ or Mann-Whitney test, $p = 0.0821$.

Figure 3. Size distributions of all (A) and of $\alpha 3$ NKA-labeled neuronal profiles (B) and intensity of plasma membrane labeling of $\alpha 3$ NKA-labeled neurons (C) obtained in studies of DRG neurons of young and advanced-age human subjects

A: Overlay of total size distributions of DRG neurons of three young (opened columns, 209 neurons) and three advanced age (greyed columns, 221 neurons) subjects: In this and in all other panels, the thick, smooth curves represent the result of best fit analysis of respective distributions with a sum of two Gaussian curves (solid curve – young subjects and dashed curve – old subjects). The thin, smooth, dashed lines represent individual components of the fits above. Respective best-fit parameters are listed in the **Table 2**.

B: Overlay of size distributions of $\alpha 3$ NKA-positive DRG neurons of young (opened columns, $n=45$) and old (grayed columns, $n=29$) subjects. Respective histogram between-peak valley values that were used as cut-off values (in μm^2) to classify neuronal profiles by size are shown by labeled vertical dashed lines.

C: Overlay of intensity of membrane labeling of $\alpha 3$ NKA-positive DRG neurons of young and advanced-age subjects (same datasets and column labeling as in B). Labeled vertical dashed lines represent between-peak valley values used as cut-off values (in pixel intensity units) to classify neuronal profiles by the plasma membrane labeling intensity.

Figure 4. Representative examples of various $\alpha 3\text{NKA S1}$ DRG neuronal profiles by size and intensity of membrane labeling (A, B) and relationships between neuronal profile size and intensity of membrane labeling in young (C) and aged subjects (D)

A, B: Examples of “MM” and “LS”, “LM” and “MS” sub-group neuronal profiles (left panel in A – older subject; other panels represent younger subjects). Numbers next to the neuronal profiles shown in A and B panels represent respective neuronal area/membrane intensity labeling values. All cells were classified using young subject size and intensity cut-off values; see panel C). A calibration bar (25 μm) common for all images is shown in B, left panel.

C: Scatterplots of “neuronal area - plasma membrane labeling intensity” for DRG neurons of young and old subjects (empty circles and filled circles, respectively). Vertical and horizontal dashed lines are area and staining intensity cut-off values defined based on young subjects' neuronal size and labeling intensity distributions between peak valley values as described in the **Fig.3** legend. Percentages of neuronal profiles of young subjects belonging to a specific sub-group of profiles are shown in the labels.

D. Data for old subjects (closed circles) were re-plotted and re-classified as MM, MS, LM and LS profiles in accord with the old-subject set of neuronal profile sizes and labeling cut-off values (**Fig. 3**). Percentages of these profiles belonging to a given sub-group of profiles are shown by the first number in the labels on the plot. The open circles in this plot represent young subjects' data corrected based on the suggestion that aging is associated with uniform 35% and 18% decreases in the cross-sectional profile area and plasma membrane labeling intensity, respectively, for all $\alpha 3$ NKA-expressing neurons. These “corrected for aging” neuronal profiles from young subjects were then classified using old subjects' profile area and intensity cut-off values (second number in the respective label).

Figure 5. Age dependence of relative counts of α 3NKA-positive tibial nerve and S1 DRG myelinated fibers and neurons: comparison with literature data on age dependence of ankle reflex loss

Data on relative counts of α 3NKA-positive tibial nerve fibers (empty circles) and α 3NKA-positive DRG neurons (filled circles) were normalized to the respective group's youngest subject counts and plotted vs. subjects' age. Solid line is the sigmoidal function line best fit to the pooled nerve and DRG data. The fit parameters are given in the lower left quadrant on the plot. Crosses with vertical lines are data ($\pm$ 95% CI) on the age-dependence of percentage of human subjects with bilaterally preserved ankle reflexes (based on the data of Bowditch et al., 1996 (in their Table 1)). Dashed smooth line is the sigmoidal function line best fit to the latter dataset (fit parameters are given above the curve).

Text labels next to the respective data points mark subjects with a history of chemotherapy treatment ("a"), chronic renal disease ("d" and "e") or military experience / agent orange exposure and peripheral vascular disease ("f", see also same notations in **Table 1**), pro-neuropathic conditions that may accelerate tendon reflex loss in elderly people (Mold et al., 2004).

Table 1

Participant subjects' characteristics

Sample ID	Age, years	Sex	Race	Cause of death	Density of α 3NKA-positive profiles (% to total counts $\pm$ SE)
Tibial nerve					
04AU-55L	64	M	C	Cardiac arrhythmia (?)	16.2 $\pm$ 3.7
04AU-58L	68	M	C	Intracranial hemorrhage	23 $\pm$ 2.6
04AU-68L	65	M	C	Pseudomembranous colitis ^a	14.2 $\pm$ 1.5
04AU-85L	58	M	B	Pulmonary emboli ^b	30.2 $\pm$ 0.8
05AU-04L	48	M	B	Intracerebral hemorrhage	34.1 $\pm$ 3.3
05AU-19L	41	M	C	Congestive heart failure ^c	37.9 $\pm$ 5.5
05AU-23L	24	F	H	Sepsis/multiple organ failure	42.4 $\pm$ 4.4
05AU-61L	81	M	B	Endocarditis and pneumonia ^d	8.0 $\pm$ 2.5
Mean	56.1 $\pm$ 6.3	88% M	50% C		25.7 $\pm$ 4.4
S1 spinal ganglia					
2001-03L	47	F	C	Head trauma	20.8 $\pm$ 4.9
2001-04R	35	F	C	Cardiovascular disease/Stroke	22.5 $\pm$ 6.9
2002-01R	67	F	B	Cardiovascular disease ^e	12.9 $\pm$ 1.7
2002-02L	56	M	C	Cardiac arrest	15.2 $\pm$ 2.0
2002-03R	53	F	C	Cardiovascular disease	16.5 $\pm$ 5.0
2002-04R	66	M	C	Head trauma ^f	10.7 $\pm$ 0.8
2002-05R	59	M	C	Stroke	15.6 $\pm$ 1.0
2002-06R	51	M	C	Head trauma	21.3 $\pm$ 2.4
Mean	54.3 $\pm$ 3.7	50% M	88% C		16.9 $\pm$ 1.5

^a chemotherapy 6 rounds, most recently with Etoposide and Carboplatin 1 week prior to death^b died one day after surgical resection of glioblastoma^c heavy alcohol use^d end-stage renal disease^e chronic non-healing ulcer of right leg / chronic renal failure / acute cerebral ischemic changes^f agent orange exposure > 40 years ago, lithium toxicity 6 years ago, peripheral vascular disease, osteoarthritis

Table 2

Neuronal size and membrane labeling best fit parameters

Parameter	Young subjects	Old subjects	Old/Young fraction (% decrease with age)
Size distribution, all neurons			
Adjusted R ² of the fit	0.986	0.972	
Sub-population of small neurons			
Peak center, μm^2	1441 $\pm$ 54	1315 $\pm$ 37	0.938 (6%)
Peak area	23082 $\pm$ 7889	26104 $\pm$ 10559	
Subpopulation of large neurons			
Peak center, μm^2	2933 $\pm$ 413	2756 $\pm$ 700	0.940 (6%)
Peak area	25184 $\pm$ 8352	23457 $\pm$ 11710	
Large DRG neurons, % of all	52.2	47.3	0.906 (9%)
Size distribution, $\alpha 3\text{NKA}$ neurons			
Adjusted R ² of the fit	0.833	0.976	
Sub-population of medium-size $\alpha 3\text{NKA}$ neurons			
Peak center, μm^2	3665 $\pm$ 130	1930 $\pm$ 16	0.527 (47%)
Peak area	7912 $\pm$ 335	3255 $\pm$ 178	
Sub-population of large-size $\alpha 3\text{NKA}$ neurons			
Peak center, μm^2	6861 $\pm$ 145	3694 $\pm$ 80	0.538 (46%)
Peak area	3010 $\pm$ 619	3919 $\pm$ 317	
$\alpha 3\text{NKA}$ neurons, % of all $\alpha 3\text{NKA}$ neurons	22.6	14.5	0.642 (36%)
$\alpha 3\text{NKA}$ neurons, % of large DRG cells	43.4	30.6	0.705 (29%)
Membrane labeling intensity distribution, $\alpha 3\text{NKA}$ neurons			
Adjusted R ² of the fit	0.895	0.988	
Sub-population of medium-labeled $\alpha 3\text{NKA}$ neurons			
Peak center, μm^2	58 $\pm$ 3	42.0 $\pm$ 0.5	0.72 (28%)
Peak area	90 $\pm$ 20	41 $\pm$ 3	
Sub-population of strongly-labeled $\alpha 3\text{NKA}$ neurons			
Peak center, μm^2	96 $\pm$ 3	75.9 $\pm$ 0.4	0.79 (21%)
Peak area	123 $\pm$ 21	98 $\pm$ 3	