Diabetic Complications Consortium

Application Title: Stretch reflex afferent neurons in human aging and diabetic neuropathy

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1. Project Accomplishments:

Problem: It is estimated that by 2030 number of people older than 65 years of age and number of people with diabetes and associated complications will nearly double (Wild *et al.*, 2004). Impaired coordination and gait and balance control is one of major reasons for high morbidity rate in population of elderly people and people with diabetic neuropathy. **The atrophy and degeneration of peripheral neurons innervating skeletal muscle stretch (spindle) receptors is a pathogenic mechanism that may lead to these deficiencies.** Until recently, however, the lack of specific markers limited direct histological evaluation of the state of stretch receptor afferent pathway in biopsy or autopsy samples of human nerves. Our finding showing that stretch-reflex afferents of vertebrates (including humans) can be specifically identified using immunohistochemical staining for α3 isoform of Na^+,K^+-ATPase, the ion transporter maintaining Na^+ and K^+ gradients on the cell membrane (α3 NKA) for the first time allowed to directly study the question above.

To evaluate the effect of aging and diabetes on stretch receptor afferent neurons we studied and compared density and morphological characteristics of a3 NKA-expressing neurons (muscle spindle afferents) in the samples of human upper sacral DRGs (S1; Achilles tendon reflex) obtained via the National Disease Research Interchange (NDRI) Program. Samples were collected post mortem from normal adult human subjects and from subjects with and without a history of diabetes and DPN. The a3 NKA-expressing neurons were identified in DRG sections using immunohistochemistry and NKA isoform-specific antibodies. Neuronal densities and size-distributions of small, large and a3 NKA-positive neurons were analyzed and compared between samples to address the specific aims of this study.

Our hypothesis stated that **normal human aging associates with a decrease in density of stretch-receptor primary afferent neurons (αNKA-positive neurons, Specific Aim 1) and diabetes and diabetic neuropathy accelerates this decline (Specific Aim 2)**. Unfortunately NDRI failed procurement of negotiated number of samples (6 ganglia per group, see **Table 1**). Furthermore some of procured and delivered samples were severely damaged or contained no neurons (**Table 1**). Nonetheless, despite these setbacks our study:

1) provided to date most comprehensive analysis of morphological characteristics α3NKA-positive (putatively stretch reflex) afferent neurons in human DRG).
2) confirmed our hypothesis on age-dependent decline in density of stretch-receptor afferent neurons in human DRG (**Specific Aim 1**) and identified BMI as another, possibly independent of age factor determining expression of α3NKA in these neurons, and
3) suggested that diabetes may associate with the increase (contrary to our original hypothesis) in density off α3NKA-expressing neurons in human DRG (**Specific Aim 2**)

2. Specific Aims:

This section provides summary of our approach, analysis and findings sorted by a Specific Aims. More elaborate description of methods, results and analysis, as well as supporting materials (tables and figures) can be found in the Section 4 of this report.

Standard methods were used in both Specific Aims. Briefly, collected postmortem and fixed in 10% formaldehyde S1 DRG autopsy samples from human subjects were received from NDRI along with Donor Information Forms providing the information on donor's age, weight, height, gender, race, time and cause of death, available medical history and medicine usage. Upon delivery samples were post-fixed in 4%

formaldehyde for about 1 week and then embedded into paraffin. Three pairs of 5 μm thick cross sections were cut from the center of the sample at a distance of about 150 μm between sequential pairs. One section from each pair was H&E stained while another one was immunostained to detect α3NKA. Neuronal profiles containing nuclei with nucleolus/nucleoli were counted and their area was measured in H&E stained sections to characterize total distribution of neurons by size. Similarly only α3NKA-immunolabeled profiles containing nucleus with nucleolus/nucleoli (referred to in this report as α3NKA neurons) were counted and measured in immune-stained sections (**Fig.1**). Density of α3NKA neurons was defined as ratio of counts of α3NKA-positive and H&E neuronal profiles. Using these three (per subject) determinations, mean (±SE) density of α3NKA neurons was calculated and used for further analysis. In addition, clock-scan protocol was used to measure integral cytoplasmic and membrane intensity of labeling of α3NKA neurons and some of found in the same section α3NKA-negative neurons. Based on the latter measurements, mean background clock-scan pixel intensity profile was calculated and subtracted from each individual α3NKA neuron intensity profile. These, corrected for background profiles of α3NKA neurons were then used to measure intensity of cytoplasmic and plasma membrane labeling for α3NKA (INT_{mem}). The latter was measured as an area of the membrane peak of a clock-scan intensity profile and therefore INT_{mem} value represents both thickness and intensity of membrane labeling (shades of grey*μm, **Fig.2**).

2.1. **Specific Aim 1:** to test the effect of normal human aging on density of α3NKA-labeled primary afferent neurons of S1 DRG

Subjects' characteristics: In total, thirteen S1 DRG autopsy samples from subjects with no known diabetes or diabetic neuropathy were received from NDRI, post-fixed in 4% formaldehyde and embedded into paraffin (**Table 1**). Two of these samples were severely damaged; the sample #2001-02 because of in situ degenerative process and sample # 2001-05 because mishandling during procurement (squeezed with forceps or other surgical instrument). Another two samples (# 2001-06 and 2001-07) contained no neurons but nerve fascicules and axonal profiles only. No signs of the former presence of the neurons, such as nodules of Nageotte were noted in these two samples either. Therefore these cases were probably the cases of misidentification of other parts of roots/spinal nerve as DRG during procurement. Furthermore, by unknown reason the staining for α3NKA failed to produce detectable labeling in any of sections cut from the sample #2001-01 and it was removed from the study.

Thus the final size of our control group was 8 ganglia (**Table 1**). Most of the subjects were Caucasians and male/female ratio was 1:1 in this group. Notably distributions of control subjects by age, weight, BMI and most importantly of density of α3NKA neurons passed the Shapiro-Wilk normality test, empowering using the analysis of Z-scores in the studies comparing control samples to those obtained from subjects with diabetes (Specific Aim 2). The only characteristic which distribution failed the normality test was subject's height. It was also the only biometric characteristic differing between male and female participants of our study (F/M: 63.5 ± 0.3 in / 70 ± 2 in, two-population t-test; p = 0.048). However as described below, neither height nor gender demonstrated significant association with neuronal labeling for α3NKA.

Density and characteristics of α3NKA-positive neurons: Density of α3NKA-positive neurons in control group DRGs varied from 11% to 23% (mean 17 ± 2%) which is just marginally higher than 10-17% in our previous studies of rat, mouse, pigeon, chicken, and guinea pig upper sacral-lower lumbar DRGs (Romanovsky *et al.*, 2005;Romanovsky *et al.*, 2007). In another agreement with our previous studies, all α3NKA-labeled neurons in this work belonged to a subpopulation of large DRG neurons. The population of DRG neurons is known to consist of subpopulations of small and large neurons, which are composed of mostly pain/temperature-perceptive and mechanoreceptive afferents, respectively (see (Perl, 1992)). Accordingly, the best-fit analysis in our experiments also required use of the sum of two Gaussian functions to describe the area distribution of human S1 DRG neurons (**Fig. 3A**, open bars). Areas of small neuronal profiles were centered at 1416 ± 65 μm^2 and those of large neurons at 2945 ± 882 μm^2. The population of α3 NKA -positive neurons from the same ganglia could be satisfactorily fitted with a single Gaussian curve with the center at 3085 ± 77(**Fig. 3A**, greyed bars). However, more detailed evaluation of size distribution of α3NKA cells demonstrates also the presence

(about 10% of these neurons) of a tail of large neurons beyond the 95% UCI of the major peak (**Fig, 3B**, right panel).

Integral intensity of membrane labeling, of α3NKA-positive neurons varied from very week to extremely strong. Sum of at least three Gaussian functions required to describe this distribution, suggesting existence of three distinct by level of expression of membrane α3NKA sub-populations of neurons (**Fig. 3B,** left panel). Importantly in the most cases neurons with weak, moderate and strong plasma membrane labeling could be found within the same section arguing that existence of subpopulations in the distribution based on the pooled data is not an artefact of the section-to-section or experiment-to-experiment staining variability.

Although statistically significant, overall correlation between the neuronal body size and intensity of plasma membrane labeling was weak (linear regression slope = 9 ± 4 units of labeling per 1000 μm^2 increase in the cell area; Adj.R^2 = 0.0219, p =0.048; not shown). However, using the 95% UCI approach three size/intensity populations of α3NKA neurons could be tentatively identified: 1) moderate size-moderate labeling, 2) large size - moderate labeling and 3) moderate size- intense labeling (83%, 12% and 4.4% of 138 studied α3NKA neurons). Of these cells, only one neuron had both a large size and intensely labeled membrane (**Fig. 3C**).

Determinants of density of α3NKA neurons: As it was mentioned above, within-group analysis has not revealed any significant association between the density of S1 DRG α3NKA neurons and control subject's either gender or height (**Fig. 4A and B**). Similarly, no significant correlation was detected between density of α3NKA neurons and subject's body weight (Adj. R^2 = 0.260, p = 0.112; data not shown).

However, strong and statistically significant reverse relationships were found to exist between age and density of α3NKA neurons (**Fig.4C**). Linear model predicted 3.7 ± 0.7% decrease in α3NKA neurons density per each decade of life over the 35 – 70 years age span (Adj. R^2 = 0.811, F(1,7) = 31.1, p = 0.001). Further studies are needed to verify the actual shape of age-α3NKA neuronal density relationships, which with expansion of studied age diapason will surely deviate from a simple linear course. Thus both linear (Adj. R^2 = 0.668) and non-linear (sigmoidal function; Adj. R^2=0.646) models were equally good in describing combined data on age-dependence of densities of human α3NKA-positive DRG neurons and α3NKA-positive large myelinated tibial nerve axons (**Fig. 4D**, our current and previous, unpublished observations). Accordingly to this latter (sigmoidal curve) estimate, the decline in density of α3NKA neurons starts around 50 years of age and passes 50% of normal density level in 70 years-old subjects. Regardless of the issue above however, our data provide an unquestionable support to the suggestion of age as critical determinant of density of α3NKA-positive neurons in spinal ganglia and peripheral nerves.

Unexpectedly, our analysis revealed BMI as another although opposite to age in direction determinant of density of α3NKA-positive DRG neurons (**Fig 4E**). Multiple regression model using BMI and age as independent predictors was able to account for 97% of the between-subject variance in density of α3NKA neurons, F(2, 7) = 80.16, p < .001, Adj. R^2 = 0.97). Similarly there is a strong linear relationship between combined BMI/age index and relative density of α3 NKA DRG neurons (Adj. R^2 = 0.911, **Fig4. F**). Furthermore, linear regression analysis demonstrated a substantially weaker association between age and BMI (Adj. R2 = 0.498, p =0.03) than between either of these characteristics and density of α3NKA neurons (Adj. R^2 > 0.8, p < 0.05; see **Fig.4C, E**). Thus collinearity is unlikely a factor in the multiple regression analysis above. However, since all three obese subjects were substantially younger than four overweight and one normal subject in our control group (BMI > 35, age 44 ± 5 years; BMI 25 – 35, age 59 ± 3 years and BMI < 25, age 66 years) further research on the effect of BMI on density of α3NKA S1 DRG neurons is warranted.

Comparison of subjects with different BMI/age indexes: To gain further insight into possible mechanisms of the age/BMI related differences in the densities of α3NKA-labeled neurons in control subjects we have compared neuronal size distributions and membrane labeling intensities of the group of obese and younger (n=3, BMI/Age = 0.72 ± 0.03) vs. overweight and older subjects (n=4; BMI/Age = 0.50 ± 0.01). Mean age, BMI and α3NKA neuron density in the sub-group of obese subjects were 44 ± 5, 35.0 ± 0.8 and 0.215 ± 0.005, respectively. These characteristics for overweight subjects were 59 ± 3, 27.5 ± 0.6 and 0.15 ± 0.01 (all means are statistically significantly different by two-population t-test at p values of 0.04, 0.0006 and 0.001; **Table 2**). Mean values and frequency distributions of neurons by size or INT_{mem} did not differ between these groups of subjects (p values >0.05; **Fig. 5A, C-E and D-F**). However, analysis of α3NKA neurons' size – INT_{mem} relationships (**Fig. 5D**) suggests that lower density of α3NKA neurons in the advanced age-low BMI

subjects may be associated with the relative paucity of large and moderately-labeled, and medium-sized and intensively-labeled neurons in DRGs of these subjects **(Fig. 5B-F**). Using derived from the best fit parameters the intensity (second peak + 1.96SD = 280 shades of grey*μm) and neuronal area (first peak + 1.96SD = 5740 μm^2) 95%UCI as cut-off values (**Fig. 5**, dashed lines "a" and "b," respectively), the contingency table may be formed which analysis confirms statistical significance of the differences above (X^2 = 7.32, n=123, p=0.0068).

Further studies are needed to determine the role of a decrease in expression of α3NKA or neuronal atrophy or degeneration in the differences detected. Whatever is the mechanism however it must be α3NKA neuronal sub-population specific. Thus, likely reflecting atrophy of small and medium-sized α3NKA neurons, the major peak in neuron size distribution is shifted to the left in older and having lower BMI subjects (from 3701 μm^2 to 2850 μm^2 **Fig. 5D,F**). This 23% change could probably account for disappearance of sub-population of large and moderately labeled neurons from the size distribution. It however cannot explain disappearance of medium-sized strongly labeled cells. On the other hand, across the board decrease in intensity of membrane labeling (10-15% as can be calculated from leftward shifts of the first and second peaks in INT_{mem} distributions) is not sufficient to account for age/BMI associated differences in counts of large and moderately α3NKA-expressing cells in human DRG **(Fig.5**).

<u>Conclusion:</u> Thus our data are consistent with the hypothesis suggesting that advanced age associates with the loss of α3NKA-positive (putative stretch-reflex) primary afferent neurons. Both a decrease in expression α3NKA and atrophy/degeneration of α3NKA-expressing cells is likely to account for this change. However, different sub-populations of α3NKA neurons are likely to be affected differentially. The questions about BMI, as independent of age determinant of a3NKA size and survival as well as the reason for age-dependent decline in α3NKA neuronal density require further studies.

2.2. **Specific Aim 2:** To test the hypothesis that the number of muscle spindle afferent neurons is severely affected in subjects with DPN we proposed comparing densities and size distributions of α3 NKA-positive neurons in DRG of subjects diagnosed with DPN with respective characteristics of DRGs of age-matched control subjects studied in Aim 1

<u>Subject's characteristics:</u> After it become clear that NDRI cannot guarantee procurement of samples from subjects with diagnosed DPN, this requirement was changed to requirement of diagnosed diabetes. Ganglia procured from two subjects with diabetes (type 1 DM of 20+ years of duration and type 2 DM of unknown duration; **Tables 1 and 3**) were processed in accord with a standard for this study protocol (see above). Considering the paucity of samples we had no choice but use the Z-score approach to determine if these individual samples are different in any of characteristics of interest from control (no diabetes) sample population. Z-score was calculated as follows: $Z = (Value_{subject} – Mean_{controls})/SD_{controls}$). The Z value outside of ±1.96 range indicates that the null hypothesis (studied parameter value is not different from control) can be rejected with 95% confidence (see (Rolke *et al.*, 2006;Maier *et al.*, 2010).

<u>Effect of diabetes on subjects' characteristics and α3NKA DRG neurons:</u> By this approach the subject with T1DM was identified as having lower than control subjects BMI, and decreased total and α3NKA-positive DRG neurons area. No such differences were detected for T2DM subject. Interestingly however that DRG samples from both, T1DM and T2DM subjects were found to have higher than control density of α3NKA-labeled neurons (**Table 3**, in red). For the T1DM sample this difference could be accounted for by subject's age, but not by BMI or by BMI/age index. For the T2DM sample none of variables above could account for higher than in control density of α3NKA neurons (**Fig.4C,E,F**). Both of subjects with diabetes were Black, while most of the control subjects in our study were Caucasian. It is however unlikely that race plays any role in detected differences in density of α3NKA neurons. Thus, in our previous studies of density of α3NKApositive axons in human tibial nerve, this parameter did not differ between subjects of different races (30 ±8% and 33 ± 4% of all myelinated fibers in nerves of black (n=4) and white (n = 6) subjects, respectively; t(8) = 0.307, p = 0.766 by two-sample t-test; unpublished).

<u>Intensity and size distributions of α3NKA neurons in DRG of subjects with diabetes</u>:

Compared to control (five subjects with similar to T1DM subject BMD/age ratios) neuronal size and INT_{mem} distributions of T1DM subject demonstrated leftward shifts and severe loss of largest and intensively labeled cells (**Fig. 6**), suggesting selective atrophy and degeneration of these neurons. It is surprising therefore that relative density of α3NKA neurons in this sample was preserved (if age differences are taken into account) or even higher than in control DRG samples. The most likely explanation for the latter is that α3NKA-expressing cells may have better than other DRG neurons ability to survive diabetic conditions. The α3NKA was reported to have higher than other isoforms of NKA affinity to intracellular ATP, suggesting that cell equipped with this transporter isoform could be able to survive hypoxic conditions for a longer time than other neurons (see references in (Dobretsov & Stimers, 2005)). Further studies however are needed to test this hypothesis vs. alternatives including possibility of compensatory up-regulation of expression of all isoforms of NKA including α3NKA in all neurons, regardless of whether or not they were expressing α3NKA under normal conditions.

In the comparison of neuronal size and labeling intensity distributions of T2DM subject and subjects with matched BMI/age ratio (3 obese subjects), no statistically significant differences in total size distributions were detected (**Fig. 7A**). Suggesting atrophic changes, area distribution of α3NKA neurons of diabetic subject was shifted by about 30% to the left relative to the distribution of these neurons in control subjects **(Fig. 7B and D).** Membrane labeling intensity distributions also differed between neurons from control group ganglia and ganglion of subject with T2DM. The complexity of these changes (**Fig. 7C**) prevents their simple interpretation. The only outstanding difference consisted in the relatively high proportion of neurons with very strong membrane labeling (third peak in distribution) in the DRG sample from T2DM subject.

<u>Conclusion:</u> These data are consistent with the idea that diabetic conditions affect population of human α3NKA DRG neurons. They however contradict the original hypothesis that the effect of diabetes is going to be equivalent to accelerated aging effect. Higher, not lower density of α3NKA neurons was observed in samples from subjects with both, type 1 and type 2 diabetes. Further studies are needed to confirm these observations and to determine most affected subpopulations of neurons and the role of compensatory changes in expression of α3NKA expression as an undelaying mechanism.

3. <u>Publications:</u>

Manuscript is in preparation

4. <u>Literature Sited:</u>

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5. **Supplemental information, Tables and Figures:**

Table 1: Characteristics of DRG samples procured for and actually studied (in red) in this study

NDRI protocol number / group description	Sample code / side of procurement	Procurement date, mm/dd/yy	Subject / Sample information								
			Age, years	Race	Sex	Height, inches	Weight, kg	BMI, kg/m^2	BMI/Age index, $kg/m^2/yr$	Fractional density of α3NKA neurons	Medical History / Cause of death
2001 / Control, 20-50 yrs. old											
	2001-01-L	12/28/2013	29	C	M	70	82	25.9	No staining		Bipolar, Drug Use / Anoxia
	2001-02-R	1/25/2014	45	C	M	68	79	26.4	damaged		Depression, anxiety / Gunshot - head
	2001-03-L	1/27/2014	47	C	F	63	93	36.3	0.72	0.21 ± 0.05	Not remarkable / Head trauma
	2001-04-R	1/27/2014	35	C	F	64	89	33.5	0.77	0.23 ± 0.07	Kidney stones, IBS / CVA, stroke
	2001-05-L	3/11/2014	47	C	M	68	95	31.7	damaged		Stroke, dysphasia / Cardiac arrest
	2001-06-R	4/9/2014	33	C	F	69	65	21.2	No neurons		Not remarkable / Anoxia
	2001-07-L	5/6/2014	40	C	M	68	93	31.2	No neurons		HTN, CAD, drug use / Anoxia
2002 / Control, 51-80 yrs.old											
	2002-01-R	12/20/2013	67	B	F	63	68	26.6	0.46	0.13 ± 0.02	ICD, CRF, chronic ulcer r. leg / CVA
	2002-02-L	2/20/2014	56	C	M	74	100	28.3	0.52	0.15 ± 0.02	COPD, HTN, Asthma / Cardiac arrest
	2002-03-R	2/24/2014	53	C	F	64	70	26.4	0.49	0.17 ± 0.05	HTN / CVA
	2002-04-R	3/24/2014	66	C	M	72	77	23.1	0.41	0.16 ± 0.01	CAD, PTSD, dementia / Head trauma
	2002-05-R	4/1/2014	59	C	M	63	73	28.7	0.51	0.21 ± 0.02	Asbestos exposure / Stroke
	2002-06-R	6/19/2014	51	C	M	69	108	35.1	0.67	0.17 ± 0.02	Not remarkable / Head trauma
Mean Control			54 ± 4	1/7 (B/C)	4/4 (F/M)	67 ± 2	85 ± 5	30 ± 2	0.56 ± 0.05	0.17 ± 0.02	
Shapiro-Wilk normality test			pass	N/A	N/A	fail	pass	pass	pass	pass	
2003 Control, 80+ years of age											
2004 Diabetes, 20-50 yrs. old											
	2004-01-L	6/20/2014	42	B	M	74	73	20.7	0.39	0.26 ± 0.05	IDDM (20+ years), ESRD, gastritis / Anoxia
2005 Diabetes, 51-80 yrs. old											
	2005-01-R	6/9/2014	58	B	M	66	97	34.5	0.62	0.46 ± 0.11	NIDDM (duration is not known), CHR, ESRD, HTN / Anoxia
2006 Diabetes, 80+ years of age											

Table 2: Characteristics of subjects and α3NKA neurons from subjects with high and low BMI/age indexes (statistically significant between-group differences are shown in red)

Parameter	Group 1: Young/obese (n=3)	Group 2: Older/overweight (n=4)	Group2 / Group1	Student's test statistics (df = 5)
Age, years	44 ± 4	59 ± 3	134%	t=2.68, p=0.044
BMI, kg/m^2	35.0 = 0.8	27.5 ± 0.6	79%	t=7.7, p=0.0006
BMI/Age, , kg/m^2/year	0.72 ± 0.03	0.50 ± 0.01	69%	t=7.6. p=0.0006
Area of α3NKA neurons, μm^2	4115 ± 288	3185 ± 295	77%	t=2.191, p = 0.08, NS
Cytosol labeling, shg	9.1 ± 1.3	2.7 ± 5.1	29%	t=1.069, p = 0.334, NS
IMI, shg*μm	178 ± 24	142 ± 19	80%	t=1.212, p = 0.280, NS
Fractional density of α3NKA neurons	0.215 ± 0.005	0.151 ± 0.01	70%	t = 6.48, p = 0.0013

Table 3: Comparison of subjects with diabetes vs. control group (statistically significant increase or decrease relative to control are shown in color, red and blue, respectively)

Parameter	Control group (mean ± SD; n=8)	T1DM, 20+ years		T2DM, duration unknown	
		Value ± SE	Z-score	Value ± SE	Z-score
Age, years	54.3 ± 10.4	42	-1.17	58	0.36
Race, gender	7C/1B, 4M/4F	B,M	NA	B,M	NA
Height, inches	66.5 ± 4.5	74	NA	66	NA
Weight, kg	84.8 ± 14.9	73	-0.79	97	0.82
BMI kg/m^2	29.8 ± 4.7	**20.7**	**-1.93**	34.5	1.01
BMI/Age, kg/m^2/yr	0.57 ± 0.05	0.39	-1.32	0.62	0.366
α3NKA n density	0.17 ± 0.02	**0.26 ± 0.05**	**2.19**	**0.46 ± 0.11**	**6.98**
Total n. area	2290 ± 283	**1450 ± 69**	**-2.97**	2388 ± 358	0.35
α3 NKA n area	3521 ± 681	**1952 ± 199**	**-2.3**	3010 ± 198	-0.75
α3 NKA n cytInt	6.4 ± 7.8	0.32 ± 4.4	-0.77	3.9 ± 2.9	-0.31
α3 NKA n IMI	154 ± 38	104 ± 18	-1.32	171 ± 14	0.44

NA – categorical data or control population data are not normally distributed

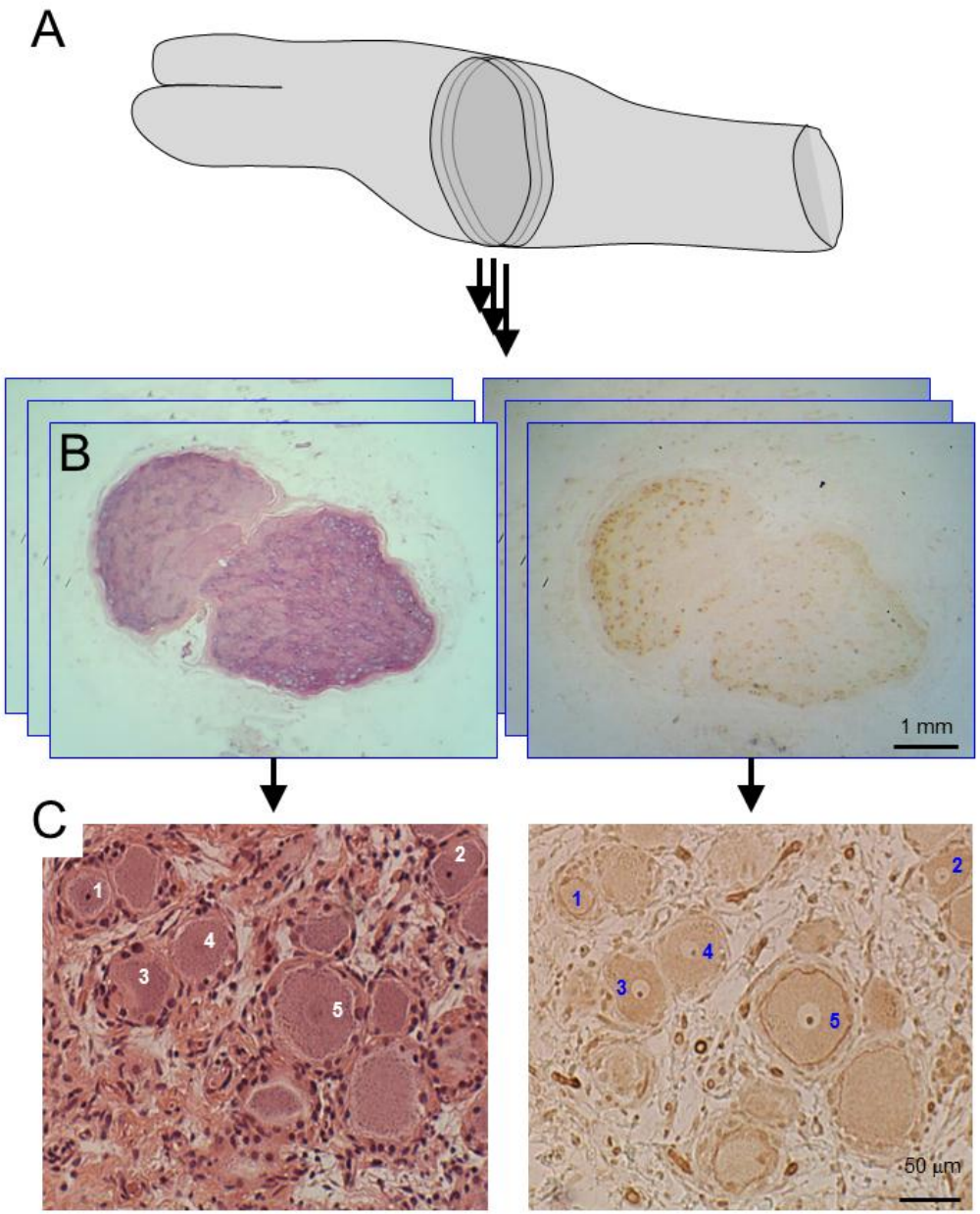


Figure 1: Tissue samples processing and staining

Isolated within 12 hours post-mortem and shipped to UAMS in fixative solution (10% formaldehyde in phosphate-buffered physiological saline; pH = 7.2) within next 2 days autopsy tissue samples were cleaned upon arrival, coded based on NDRI unique code and placed in fresh 4% PF solution for another 6-8 days. Then samples were dehydrated, cleared, cross-cut in half in the middle (widest part) and positioned in the block (Paraplast Extra) for the head-on, cross-sectional cutting. One of these blocks was used in experiments and another stored in the lab's tissue sample depository. Three pairs of five micron-thick sections were cut from the center of each ganglion at a 100-200 µm distance (**A, B**). One section from each pair was stained with H&E (**B, left**) while another was processed to identify α3NKA-expressing neurons **(B, right**). Immunostaining of tissue sections for the α_3 NKA was conducted as described (Dobretsov *et al.*, 1999;Dobretsov *et al.*, 2003). Briefly, after microwave unmasking, endogenous peroxidase and non-specific antibody binding was blocked by H_2O_2 and heat-inactivated 10% FBS (Sigma), respectively. Following overnight incubation at 4°C with primary antibody or purified IgG diluted (1:1500 in PBS/1% bovine serum albumin) sections were incubated with anti-rabbit secondary biotinilated antibody, peroxidase-conjugated streptavidin label, and finally, with 3,3'-diaminobenzidine chromogen solution. Antigen-specificity of anti-rat α3 NKA rabbit polyclonal IgG (Upstate Biotechnology) antibodies has been tested and validated previously (Blaustein & Juhaszova, 1997;Shyjan & Levenson, 1989;Dobretsov *et al.*, 1999). The sections were studied using upright BX51WI Olympus microscope. Images were captured using 10x or 20x (water-immersion) objective, a Coolsnap Cf Photometrix digital camera and analyzed with Image-Pro 5 (Media Cybernetics). In each section the neuronal profiles containing nucleus with nucleoli were counted and measured only. Thus in the fields shown in **C**, neurons 1 and 2, and neurons 3, 4 and 5 were measured in H&E and immune-stained sections, left and right panels, respectively.

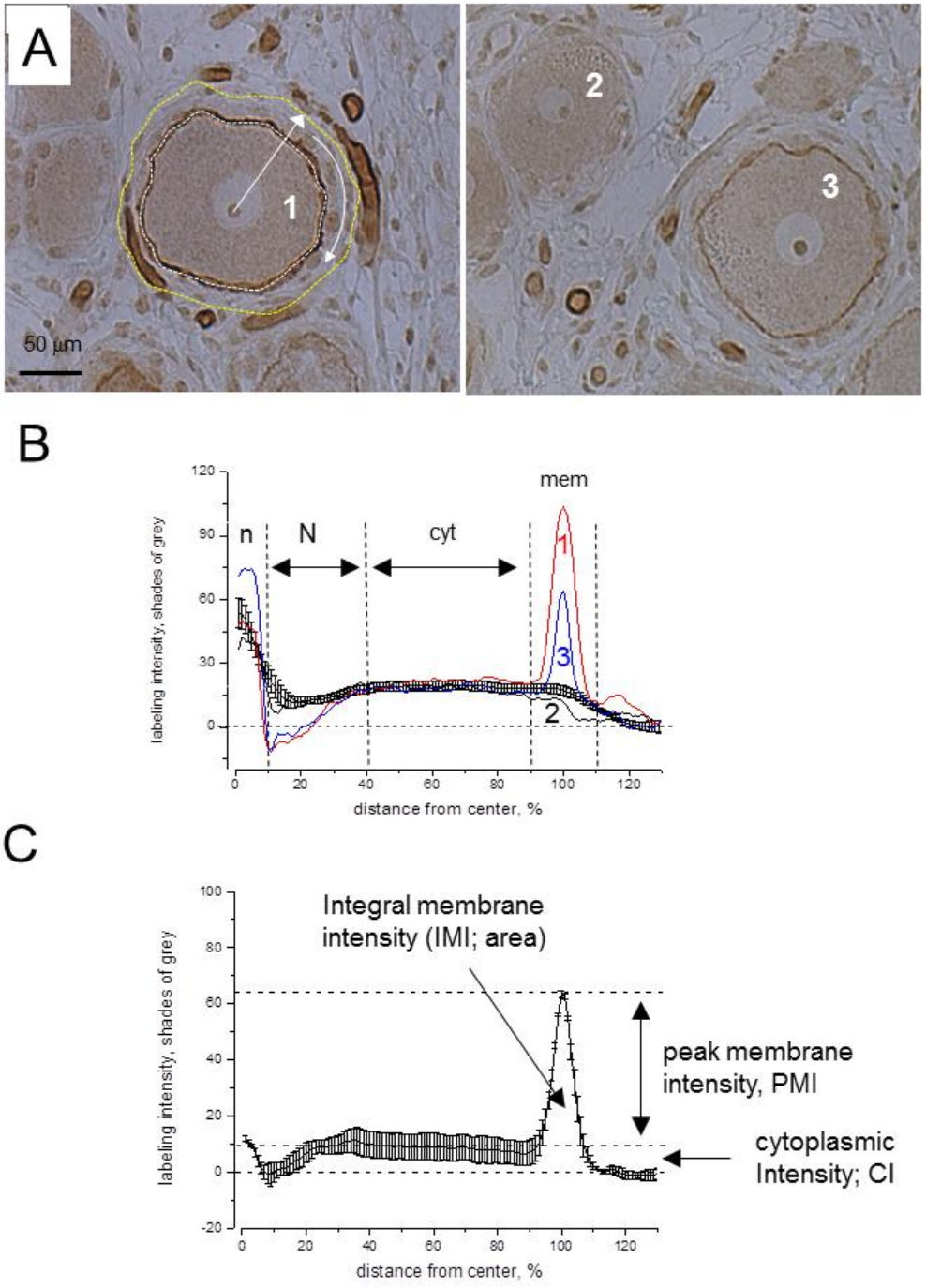
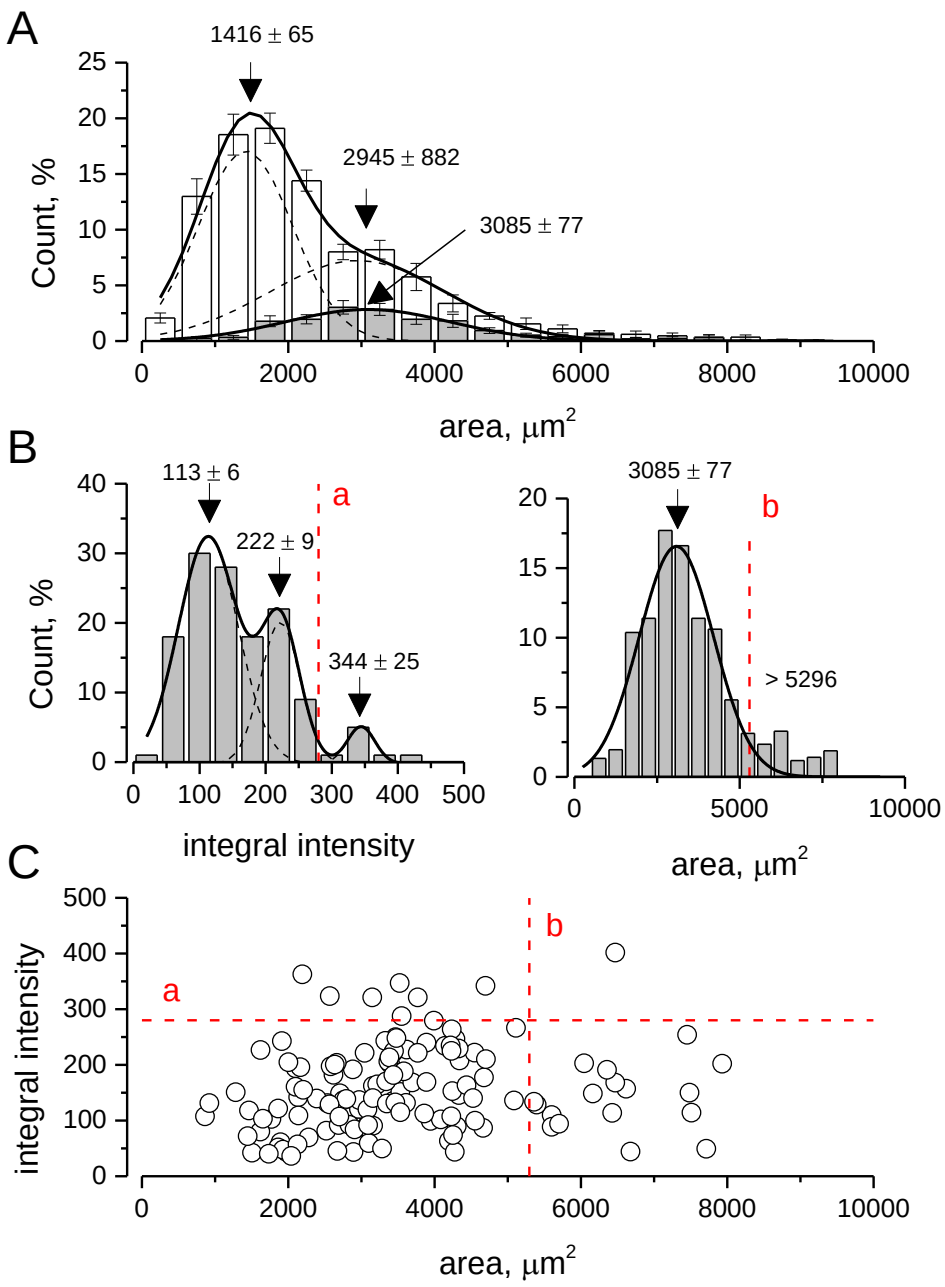


Figure 2: Clock-scan analysis of neuronal size and intensity cytoplasmic and plasma membrane labeling for α3NKA

"Clock-Scan" protocol software was used to measure neuronal size and intensity of labeling characteristics as described in (Dobretsov & Romanovsky, 2006). Briefly, the cell is first manually outlined in the displayed image by using the polygon drawing method (**A, neuron 1, white dashed line**). Automatically activated upon completing the outline software code calculates diameter, perimeter, area and the center of the selected cell. If desired, this parameter can be manually adjusted, and in our experiments it was always set to the center of the cell's nucleus. Finally, a fractional parameter ("scan limit") was set to 1.3, meaning that if the cell's radius in a given direction is rx, the radial scan length in this direction will be 1.3rx (or 130% of radius, **A yellow dashed line**). After the cell is outlined and its center is adjusted, the program code was activated to perform radial scanning of the object's pixel intensity. Each individual radial scan started at the object's center and is directed toward the object's border and beyond it, as determined above (**Fig.1A, straight arrow**). The direction of the first radial scan is the first point of the outline, the direction of the second scan is the second point of the outline, and so forth (clockwise), until the direction toward the last point of the outline has been scanned. Collected radial profiles are then aligned by scaling accordingly to the object radius in the direction of each individual scan (0 on the X-axis is the center and 100% is the border of the neuron) and integral cell radial intensity profile, point by point average of scaled radial profiles is calculated. Examples of clock-scan intensity profiles of neurons 1, 2, and 3 (**A**) are shown in **B** (left panel, number-labeled traces). Labels "n", "N", "cyt" and "mem" mark nucleoliar, nuclear, cytoplasmic and membrane regions of the profile, respectively. Trace with error bars represents average profile of several (in this case 24) α3NKA-negative neurons, such as neuron #2 to generate reference background staining profile. Panel **C**, shows average of profiles of α3NKA-positive neurons corrected for background by subtraction of the reference profile. Integral membrane intensity and cytoplasmic intensity were two major measures of neuronal labeling for α3NKA used in this work.

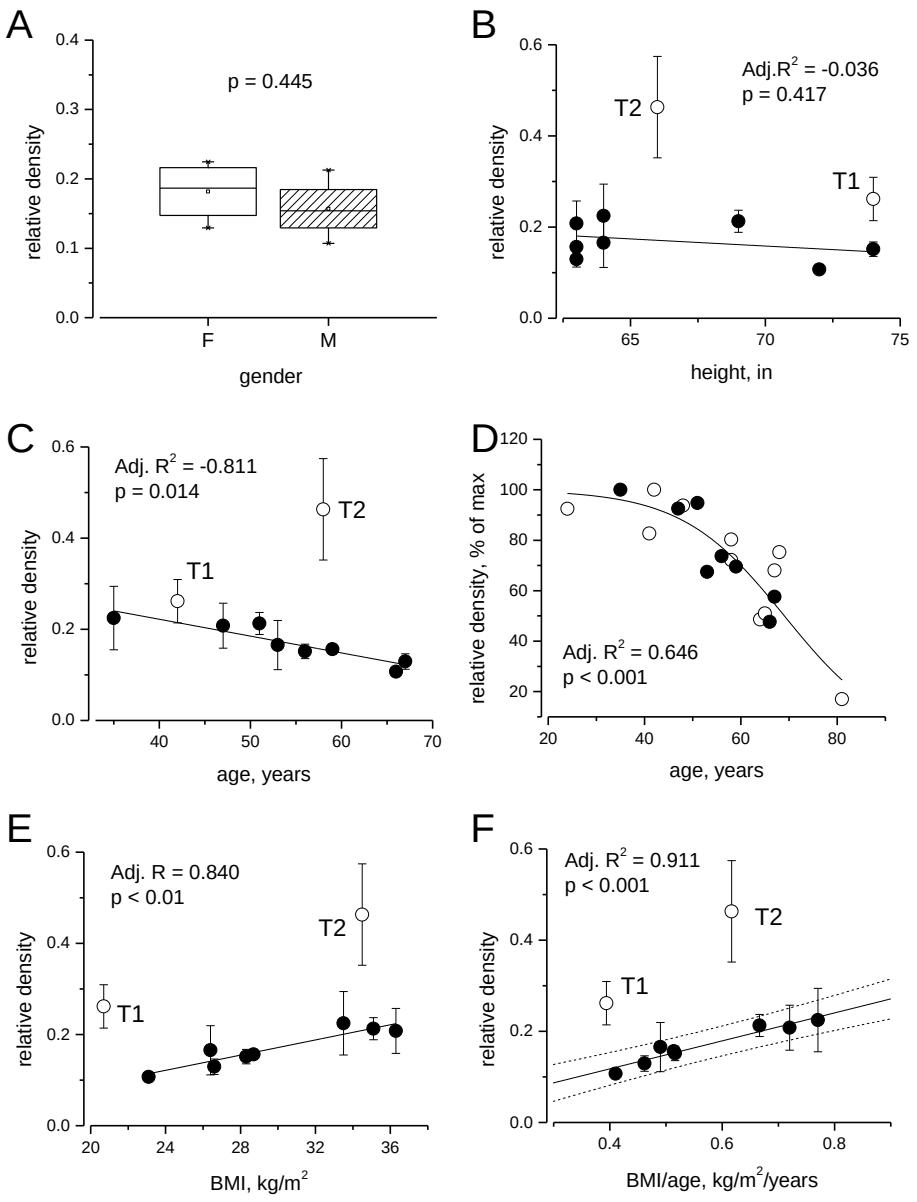


<u>Figure 3:</u> Neuronal size and intensity of membrane labeling of human S1 DRG neurons

A – Size distributions of all measured neurons (n = 807; opened bars) and α3NKA-immunolabeled neurons (n = 138; greyed bars). Solid lines are the best fit of one or two Gaussian function(s) to the data. Dashed lines represent individual Gaussian components of two-component fits. Numbers next to arrows indicate Gaussian peak values (±SE) predicted by the best fit procedures.

B – Integral intensity of membrane labeling (IML, left panel) and size (right panel) distribution of α3NKA-positive neurons. Smooth lines are as above, except that three Gaussian curves were required to describe IML distribution. Red dashed lines represent 95% upper confidence interval (UCI) for the second Gaussian component of IML (a) and major peak of area (b) distributions.

C – Neuronal area – IML relationships in population of α3NKA-positive neurons. Lines “a” and “b” are as in panels B. Most of the neurons, 83% of 138 studied are medium-sized cells having weak to moderate IML. However, 12% and 4.4% of all a3NKA neurons had very strong staining or very large cell bodies, respectively, and one neuron (0.7%) had both large body size and strongly membrane labeling.



<u>Figure 4:</u> Density of α3NKA-labeled S1 DRG neurons with respect to gender (A), height (B), age (C,D), BMI (E) and BMI/age index (F) of control and diabetic subjects

A - Males and females do not differ in density of S1 DRG α3NKA-positive neurons (two-population t-test, p = 0.445).
B – No statistically significant effect of a subject's height was detected by linear regression analysis in control group of ganglia (filled circles (±SE) and solid line). Empty circles represent measurements in type 1 (T1) and type 2 (T2) diabetic subjects' DRGs. These symbols' designations are also applicable for panels C, E and F in this figure, showing strong linear relationships between control group subjects' age, BMI or BMI/age index as independent and S1 DRG α3NKA neuron density as dependent variables (linear fit statistics are given in the respective panels' text labels)
D - Filled and empty circles represent normalized to respective maximum counts of α3 NKA-positive, respectively, S1 DRG neurons (this study) tibial nerve axons (our previous, unpublished experiments). Pooled together these data were best fit with a sigmoidal curve (smooth line) with a maximum and minimum density parameters set to 100% and 0% respectively. The results of this fit suggest that density of α3NKA-positive neurons and axons is maintained before the age of 50 years and then declines with age reaching 50% of initial level 69 ± 3 years-old subjects.
F - Dashed lines in this panel are 95% linear regression prediction bands.

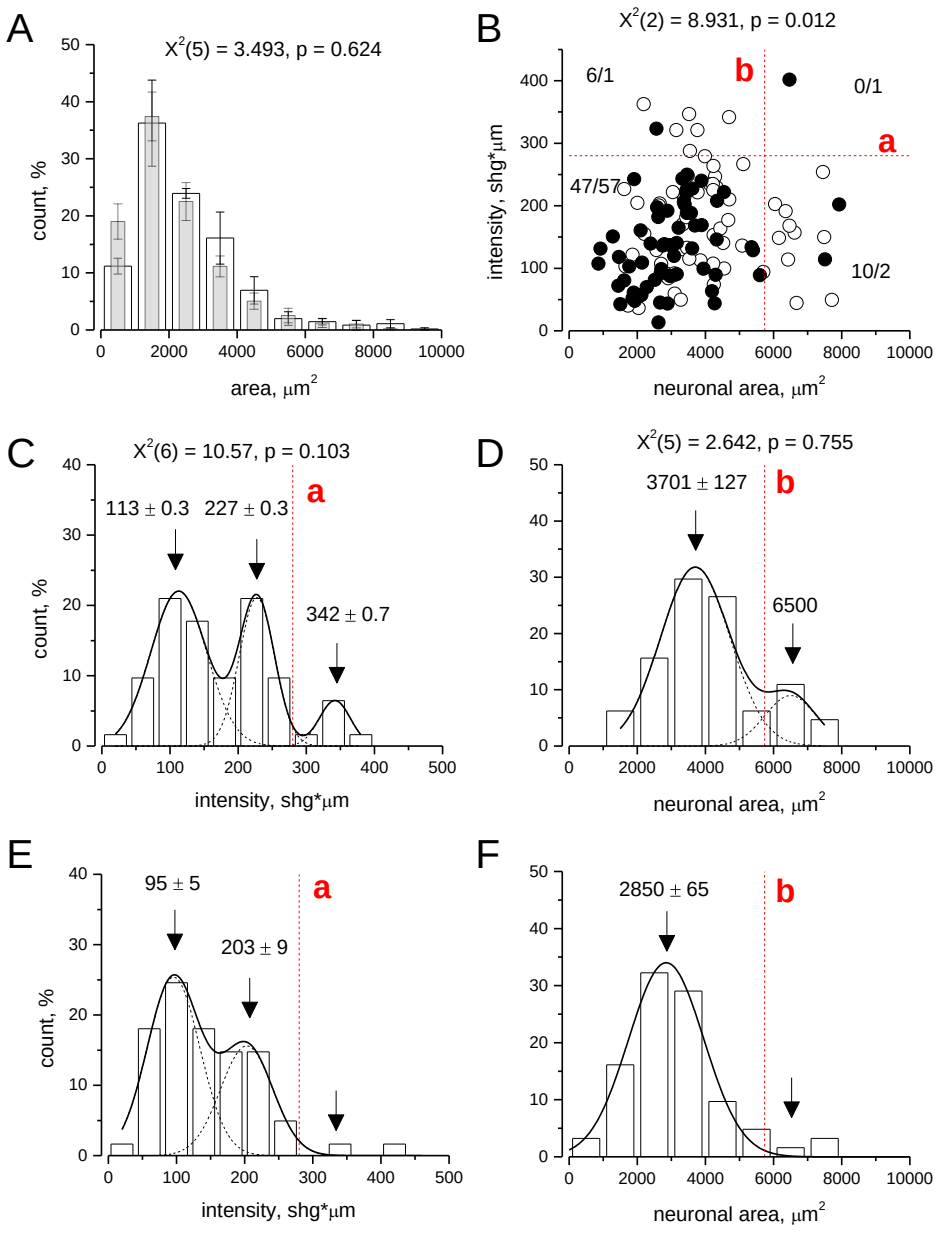


Figure 5: Comparison of size and plasma membrane labeling intensity of DRG neurons in populations of people with different BMI/age indexes (0.5 vs 0.72 kg/m^2/year)

A – size distributions of all counted neurons (empty and greyed columns represent populations with high and low BMI/age indexes)

B – relationship between neuronal size and plasma membrane labeling intensity of α3NKA-positive neurons (empty and filled circles are high and low BMI/age indexes)

C and D – neuronal plasma membrane intensity labeling and size distributions (subjects with a high BMI/age index)

E and F - neuronal plasma membrane intensity labeling and size distributions (subjects with low BMI/age index)

Red dashed lines “a” and “b” are defined as those in the Figure 4. Black smooth solid and dashed lines are the result of the best feet of three (C), two (D and E) or one (F) Gaussian functions to the data

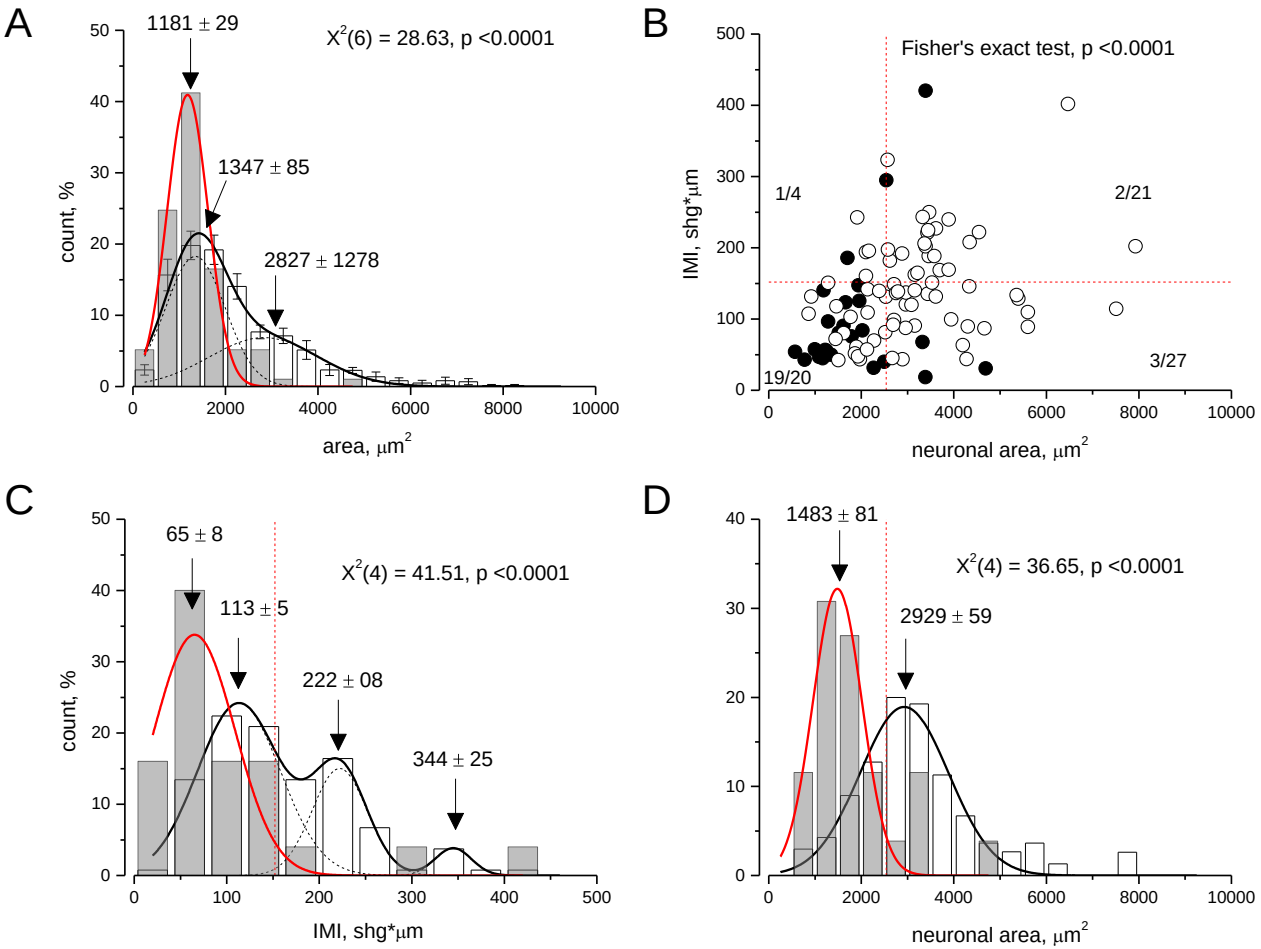


Figure 6: Comparison of size and plasma membrane labeling intensity of DRG neurons in populations of control (normal and overweight, empty bars and symbols) subjects and the subject with T1DM (greyed columns and filled circles)

A – Size distributions of all counted neurons

B – Relationship between neuronal size and plasma membrane labeling intensity of α3NKA-positive neurons

C – Frequency distributions of INT_{mem}, fit with a single Gaussian function (red smooth line, T1DM ganglion) or three Gaussian functions (dark smooth solid and dashed lines, ganglia from control subjects with low BMI/age ratios)

D – Size distributions of α3NKA-positive neurons from DRGs of T1DM and control subjects.

Dashed red lines in panels B-D are 95%UCIs calculated using parameters derived from the best fit to the T1DM neuronal INT_{mem} and area data

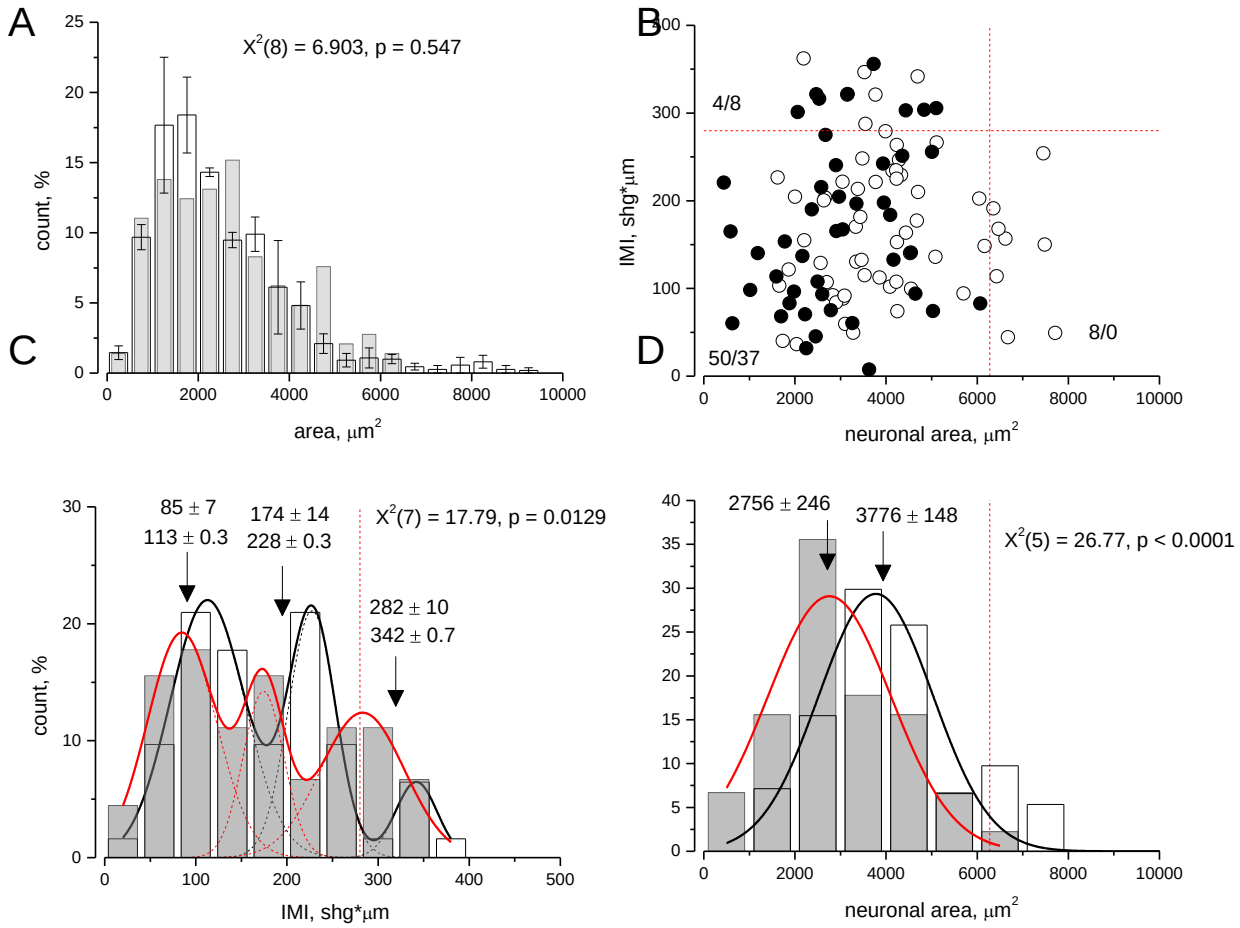


Figure 7: Comparison of size and plasma membrane labeling intensity of DRG neurons in populations of control obese people and the subject with T2DM (greyed columns and filled circles)

A – Size distributions of all counted neurons

B – Relationship between neuronal size and plasma membrane labeling intensity of α3NKA-positive neurons

C – Frequency distributions of INT_{mem}, fit with a three Gaussian functions (red smooth lines, T2DM ganglion and dark smooth solid and dashed lines, ganglia from control subjects with high BMI/age ratios)

D – Size distributions of α3NKA-positive neurons from DRGs of T2DM and control subjects.

Dashed red lines in panels B-D are 95%UCIs calculated using parameters derived from the best fit to the T2DM neuronal INT_{mem} (second peak) and area data