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Effect of aging on ultrasonic vocalizations and laryngeal sensorimotor neurons in rats

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Abstract

While decline in vocal quality is prevalent in an aging population, the underlying neurobiological mechanisms contributing to age-related dysphonia are unknown and difficult to study in humans. Development of an animal model appears critical for investigating this issue. Using an established aging rat model, we evaluated if 50-kHz ultrasonic vocalizations in 10, 32-month-old (old) Fischer 344/Brown Norway rats differed from those in 10, 9-month-old (young adult) rats. The retrograde tracer, Cholera Toxin β , was injected to the thyroarytenoid muscle to determine if motoneuron loss in the nucleus ambiguus was associated with age. Results indicated that older rats had vocalizations with diminished acoustic complexity as demonstrated by reduced bandwidth, intensity, and peak frequency, and these changes were dependent on the type of 50-kHz vocalization. Simple calls of old rats had reduced bandwidth, peak frequency, and intensity while frequency-modulated calls of old rats had reduced bandwidth and intensity. Surprisingly, one call type, step calls, had increased duration in the aged rats. These findings reflect phonatory changes observed in older humans. We also found significant motoneuron loss in the nucleus ambiguus of aged rats, which suggests that motoneuron loss may be a contributing factor to decreased complexity and quality of ultrasonic vocalizations. These findings suggest that a rat ultrasonic phonation model may be useful for studying age-related changes in vocalization observed in humans.

Keywords

Aging; Voice; Nucleus ambiguus; Neurodegeneration; Ultrasonic vocalization; Rat

Introduction

Vocal function and voice quality change with age and are characterized by decreased vocal intensity and frequency range (Endres et al. 1971; Hagen et al. 1996; Linville 1987;

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McGlone and Hollien 1963; Roy et al. 2007). These changes are likely related to central and peripheral neurodegenerative processes. Age-related changes in vocal function are a concern because reductions in vocal capacity may adversely affect quality of life (Hagen et al. 1996; Linville 2004; Ramig et al. 2001; Roy et al. 2007; Shindo and Hanson 1990; Smith et al. 1996). While age-related changes in the voice are relatively prominent, affecting up to 60 % of elders, there has been little research examining neurobiological mechanisms underlying normal aging-associated vocal dysfunction (Roy et al. 2007).

The etiology of age-related vocal decline is unclear. The thyroarytenoid (TA) muscle is an adductor muscle in the larynx that has a primary role in vocal production and is known to undergo morphological changes with advancing age (Honjo and Isshiki 1980; Jürgens 2002; Kersing and Jennekens 2004; Ludlow 2005; Shindo and Hanson 1990; Thomas et al. 2008). The TA is innervated by the recurrent laryngeal nerve that emanates from the vagus nerve, with motoneuron nuclei located in the loose formation of the nucleus ambiguus (Altschuler et al. 1991; Bieger and Hopkins 1987; Ciriello et al. 2003; Davis and Nail 1984; Ezure et al. 1988; Fay and Norgren 1997; Friedland et al. 1995; Jürgens 2002; Jürgens and Hage 2007; Kalia and Sullivan 1982; Lobera et al. 1981; Ludlow 2005; Portillo and Pasaro 1988; Saxon et al. 1996; Van Daele and Cassell 2009; Wright and Spink 1959). In humans, many age-related changes in the laryngeal muscles have been found to be degenerative in nature and may involve neuronal changes at the level of the nucleus ambiguus or the neuromuscular junction (Bach et al. 1941; Segre 1971; Shindo and Hanson 1990; Thomas et al. 2008). Therefore, examination of motoneuron changes may elucidate a pattern of decline that underlies the changes observed in laryngeal function with age.

In the brainstem cranial nerve nuclei, morphological changes in motoneurons have been found with age. For instance, in the hypoglossal nucleus in aged rats, there was a decrease in the number of primary dendrites per neuron that may affect synaptic efficiency (Schwarz et al. 2009). Germane to the nucleus ambiguus, a decrease in the number of motoneurons was found in old mice, although the decrease was not uniform throughout the nucleus. Changes were primarily found in the compact formation, which innervates esophageal muscles; however, the other regions were not looked at separately due to the difficulty of delineating the other regions of the nucleus (Sturrock 1990). Due to the dearth of studies regarding motoneuron change in the nucleus ambiguus as a result of aging, it is unclear if previously reported changes in structure are consistently found among species. Furthermore, it is unknown if changes in motoneuron structure in the loose formation, which innervates laryngeal musculature, co-occur with alterations in vocal function. This study aims to answer this question.

Fischer 344/Brown Norway rats are a useful animal model for human aging (Cartee et al. 1996), specifically laryngeal aging (Connor et al. 2002; Hodges et al. 2004; Suzuki et al. 2002). However, there has been limited study of the vocalization characteristics of these animals with respect to age (Wöhr et al. 2008; Wright et al. 2010). A rat model for examining vocalization characteristics as a function of age may be possible because rats are known to communicate through ultrasonic vocalizations (USVs), and the vocalizations are used in a variety of situations such as play behavior and germane to this study, mating (Barfield et al. 1979; Bialy et al. 2000; McGinnis and Vakulenko 2003). This study examined USVs in the 50-kHz range; these calls are often appetitive and produced in anticipation of a reward (Blumberg 1992; Burgdorf et al. 2000; Brudzynski and Pniak 2002; Portfors 2007). There is accumulating evidence that rats voluntarily modulate these vocalizations (Brudzynski 2005; Ciucci et al. 2009, 2007; Riede 2011; Roberts 1975a, b). Intensity and frequency bandwidth are affected by dopamine depletion in the basal ganglia (Ciucci et al. 2007), suggesting rat ultrasonic vocalizations are susceptible to neurodegenerative processes. Therefore, development of an aging rat vocalization model

may allow for the study of vocalization changes and their underlying anatomical substrates. To test the hypothesis that aging rats would manifest acoustic deficits in ultrasonic vocalizations as well as alterations in the loose formation of the nucleus ambiguus, specifically a reduction of motoneuron number, diameter, and number of primary dendrites as a function of age, we recorded ultrasonic vocalizations from 10 young adult and 10 old Fischer 344/Brown Norway rats and injected the neuroanatomical tracer, Cholera Toxin β , into the thyroarytenoid muscle to visualize cell bodies within the nucleus ambiguus.

Methods

Subjects

Twenty male Fischer 344/Brown Norway rats (NIA aging animal colony, Bethesda, MD) aged 9 months ($n = 10$) and 32 months ($n = 10$) were used in this study. In addition, 10 female Long Evans rats (Charles River, Raleigh, NC), aged 6 to 20 months, were used to elicit USVs from the male rats using a mating paradigm that has been used in several prior studies and has been shown to be a reliable method of eliciting 50-kilohertz (kHz) vocalizations (Ciucci et al. 2009, 2010), described below. Rats were housed in pairs in standard polycarbonate cages, with corn cob bedding, and maintained on a 12:12 reverse light cycle. All testing occurred during the dark period with partial red-light illumination. Food and water were provided ad libitum. Male rats were handled for 2 weeks and sexually experienced with female rats demonstrating sexual receptivity (darting, lordosis, ear wiggling) for 6 days prior to testing. All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee.

Ultrasonic vocalization data recording and analysis

Male USVs were recorded (Avisoft Recorder, Avisoft Bioacoustics, Germany) with an ultrasonic microphone (CM16, Avisoft, Germany) with a flat frequency response up to 150-kHz and a working frequency response range of 10–180-kHz. The microphone was placed 15 cm above the center of the male rat's homecage (isolated from cagemate). Calls were elicited by placing a sexually receptive female rat above the homecage until the male showed signs of interest (chasing, sniffing) and then the female was removed prior to recording. USVs were recorded for two consecutive days at a 16-bit resolution and sampled at 214,174 Hz for 70 s, for a total of 140 s. This recording duration has been determined to collect a sufficient number of calls as well as accommodate for day-to-day variability in calling. Two old rats were non-vocalizers and thus excluded from the USV analysis (Burgdorf et al. 2005).

Acoustic analysis was performed offline by an experienced rater masked to condition with Saslab Pro (Avisoft, Germany) (see Ciucci et al. 2010). The rater was determined to be sufficiently experienced when intra and inter-rater reliability, as measured by intraclass correlations were above 0.95. Spectrograms were generated under a 512 FFT length and 75 % overlap frame setup. The 50-kHz calls were categorized based on frequency properties into simple, step, and frequency-modulated (FM) (Ciucci et al. 2007, 2009) (see Fig. 1). Acoustic properties that are common in communication signals among various species, including rats and humans, were measured within each call category including: duration (offset of the signal minus the onset, in milliseconds), bandwidth (maximum minus minimum frequency in Hz), peak frequency (the frequency at the loudest part of the call, measured in Hz), and intensity in decibels (dB). Bandwidth was measured manually because the program's automatic parameters often under-estimated the highest frequency due to a reduction in intensity in the higher frequency ranges of calls. Call categorization was done by an experienced rater using visual and aural inspection (slowing down calls by a factor of 11), and at this time, all sounds determined to be extraneous noises and not USVs were

removed if they interfered with the USV measurements. All other acoustic parameters were measured automatically using Saslab Pro. Overall complexity was determined by the percentage of complex calls (step and frequency-modulated) of total calls and the proportion of the total duration of complex calls. Call rate, in calls per second, of 50-kHz USVs was also measured as an indicator of the appetitive value of the mating paradigm. For each animal, the average value for the sample and the maximal (absolute highest) values were measured for bandwidth, peak frequency, and intensity to gain insight into the animals' "average" and "best" performance, respectively. The individual animals' values for each variable were averaged to obtain group means for each variable.

Neuroanatomical tracer injections and tissue preparation

Rats were anesthetized with an intraperitoneal injection of ketamine (50–80 mg/kg) and xylazine (5–10 mg/kg) and placed on a heating pad to maintain body temperature throughout the procedure. The rat was secured on an operating platform in a near vertical position with the mouth fixed open, and a microlaryngoscope was inserted to provide a view of the larynx with an operating microscope, as seen in Fig. 2 (see Inagi et al. 1997). We injected 2 μ L of cholera toxin β (List Biological Laboratories, Campbell, CA, USA) into the thyroarytenoid muscle at the midportion of the left vocal fold (5- μ L syringe, 26-gauge, 50-mm needle, Hamilton, Reno, NV). After the injection, the animal was removed from the platform and given a subcutaneous injection of buprenorphine (0.01 mg/kg) for post-surgical pain and was placed back into the home cage upon recovery.

Three days after the laryngeal injections, the animals were euthanized with a lethal intraperitoneal dose (100 mg/kg) of pentobarbital and perfused transcardially with 250 mL of saline (0.9 %) followed by 500 mL of 4 % paraformaldehyde. Brain tissue was extracted and postfixed in 4 % paraformaldehyde for 1–4 h and then transferred to 0.1 M PBS/0.02 % NaN_3 for storage. Brains were transferred to a cutting cryoprotectant (20 % sucrose + 5 % glycerol in 0.1 M PBS) 48 h prior to slicing. The medulla was sliced into 40 μ m sections with a freezing microtome (Leica 2000R, Bannockburn, IL, USA).

Immunohistochemical analysis

Free floating sections were stained for reaction with cholera toxin β and the neuronal marker NeuN as a control. Slices were rinsed with TBS (tris-buffered saline) and then with TBST [TBS + Triton X-100 (Acros Organics, Pittsburgh, PA)] and placed in donkey blocking serum (Millipore, Temecula, CA, USA) for 2 h at room temperature. They were incubated in the primary antibodies with TBST and blocking serum overnight [1:20,000 anti-CT β raised in goat (List Biological Laboratories, Campbell, CA, USA) and 1:10,000 anti-NeuN, raised in mouse (MAB377: Millipore, Temecula, CA, USA)]. Sections were rinsed and incubated in secondary antibodies (1:500) for 2 h at room temperature [Alexafluor 568 mouse anti-goat and Alexafluor 488 donkey anti-goat (Molecular Probes, Invitrogen, Carlsbad, CA, USA)]. Sections were rinsed, mounted onto slides, and coverslipped using Prolong Gold media (Molecular Probes, Invitrogen, Carlsbad, CA, USA), and cured overnight. Figure 2 shows retro-labeled motoneurons, stained for both CT β and NeuN.

Tissues were imaged with a fluorescent microscope (Olympus, BX60). Images of the nucleus ambiguus were analyzed with ImageJ (Image Processing and Analysis in Java, NIH). All tissue sections that were determined anatomically to be in the region of the nucleus ambiguus and that showed co-localization of CT β and NeuN reactivity were analyzed. Each sample was analyzed for the number, size (area of the 2-D image, measured automatically in ImageJ), and the number of primary dendrites, using only motoneurons that were in the horizontal plane with visible fluorescent staining. The five slices with the most motoneurons were averaged for each animal to determine average motoneuron number per

slice. From these samples, five neurons per animal were chosen randomly and their number of primary dendrites were analyzed. Motoneuron area was similarly averaged from a random sample of 5 neurons per animal. Due to inadequate tissue visualization, two young and two old rats were excluded from this analysis.

Statistical analyses

Statistical analyses for the USV and motoneuron variables were performed with simple one-tailed *T* tests (Satterthwaite correction for non-normal distribution), since there was a directional hypothesis based on previous work in the nucleus ambiguus (Sturrock 1990), the degeneration of the TA with age (Bach et al. 1941; Shindo and Hanson 1990; Segre 1971), and with neurodegenerative diseases and USVs (Ciucci et al. 2009, 2007). The critical level for determining statistical significance was set a priori at 0.05.

Results

Means and standard error of the mean (SEM) for all acoustic and neuronal parameters are displayed in Table 1.

Complexity and call rate

There was no significant difference in the percentage of complex calls between young adult and aged rats ($t(16) = 0.43$, $p = 0.34$). There was also no difference in the proportion of the duration between simple and complex calls in young adult and aged animals ($t(16) = 0.86$, $p = 0.20$). There was no significant difference between call rate of young and old rats ($t(16) = 0.076$, $p = 0.94007$).

Duration

There was no difference in the duration of simple calls produced by young adult and aged animals ($t(7.29) = -1.38$, $p = 0.10$). Contrary to our hypothesis, there was a significant increase in the duration of step calls of aged animals as compared to young adult, as shown in Fig. 3 ($t(8.02) = -5.11$, $p = 0.0005$). There were no differences in duration of FM calls ($t(15) = 0.14$, $p = 0.45$).

Bandwidth

For aged animals, there was a significant decrease in the average bandwidth of simple calls ($t(16) = 2.68$, $p = 0.008$). However, a significant decrease was not seen in step calls ($t(16) = 1.44$, $p = 0.17$). FM calls also had a significant decrease in average bandwidth in old rats ($t(6.76) = 2.56$, $p = 0.019$) (Fig. 4). There were significant reductions in the maximal bandwidth of USVs from the aged rats compared with young rats for simple calls ($t(8.55) = 2.43$, $p = 0.02$). Step calls showed no difference in maximal bandwidth ($t(8.41) = 1.37$, $p = 0.21$). FM calls of old rats also showed a significant reduction in maximal bandwidth ($t(15) = 2.11$, $p = 0.026$) (Fig. 4).

Peak frequency

There was a significant decrease in the average peak frequency of aged animals as compared with young adult for simple calls ($t(16) = 2.68$, $p = 0.008$) (Fig. 5). There were no significant findings for the average peak frequency of step and FM calls (step: $t(16) = 1.15$, $p = 0.13$; FM: $t(15) = 1.71$, $p = 0.05$). For maximal peak frequency, no differences were seen between young and old animals for simple and step calls (simple: $t(16) = 1.52$, $p = 0.07$; step: $t(16) = 1.59$, $p = 0.07$). A significant decrease was seen in the maximal peak frequency of FM calls of aged rats ($t(15) = 2.32$, $p = 0.018$) (Fig. 5).

Intensity

There was a significant reduction in the average intensity of simple calls of aged rats ($t(16) = 2.11, p = 0.025$); however, this change was not seen in step calls ($t(9.17) = 0.65, p = 0.27$). This significant reduction of average intensity was also seen in FM calls of aged rats ($t(15) = 1.92, p = 0.037$) (Fig. 6). A significant decrease in maximal intensity was only seen in simple calls (simple: $t(8.22) = 2.33, p = 0.024$; step: $t(9.39) = 1.55, p = 0.08$; FM: $t(15) = 1.59, p = 0.04$) (Fig. 6).

Motoneuron analysis

Between young adult and old animals, there appeared to be no significant changes in the number of primary dendrites per motoneuron ($t(15) = 0.47, p = 0.65$). This was also true of the size (measured as area) of the motoneurons ($t(10) = -0.34, p = 0.74$). There was a decrease in the number of motoneurons in the nucleus ambiguus in aged animals compared with young adult animals ($t(14) = 1.84, p = 0.045$), as shown in Fig. 7.

Discussion

The hypothesis of this study was that aging rats would manifest acoustic deficits in USVs as well as alterations within the nucleus ambiguus, including reduced motoneuron number, diameter, and number of primary dendrites. Our findings allowed us to accept these hypotheses in part. The 50-kHz USVs of aged rats were diminished with respect to acoustic quality compared with vocalizations recorded in young adult rats. Two of the call types analyzed (simple and frequency-modulated) evidenced statistically significant reductions in the acoustic parameters of bandwidth, peak frequency, and intensity. In addition, the number of motoneurons within the nucleus ambiguus was reduced in old rats.

Contrary to our expectations, older rats displayed a longer duration in step calls than young adult rats. It may be that the longer duration of step calls was a compensatory strategy to increase the quality of the overall reduced vocalizations. More so, step calls remained relatively unaffected with respect to other parameters. This call type was associated with the most amount of variability within and between subjects, which likely accounts for the lack of statistical significance. Interestingly, call complexity was unaffected by age, while complexity is negatively affected in the neurotoxin models of parkinsonism (Ciucci et al. 2009, 2007). A possible explanation for this difference is that the changes seen with aging are more subtle than the rapid and severe lesion induced in the basal ganglia in the parkinsonian model.

With respect to average and maximum values, there were acoustic parameters with significant findings in just the average measure, just the maximum measure, and in both the average and maximum measure. However, there appeared to be no overrepresentation of significant findings in average parameters compared to maximum parameters, or vice versa. In general, data trend toward a reduction in acoustic qualities for all findings where significance was found in either average or maximal values, but not the other. Perhaps, a larger sample size would yield significance in these variables. It could also be interpreted in cases, where only the maximum findings were significant, that older animals still perform as well on average, but they are unable to reach those levels that younger animals can, from a sensorimotor performance standpoint. In the other case, where there were insignificant maximal findings but significant average findings, it could be that while possible, it is rare for older rats to reach the same acoustic measures as young rats in typical communication, perhaps due to the difficulty and sensorimotor skill required to produce them. Altered laryngeal sensorimotor control may account for findings of reduced bandwidth, intensity, and peak frequency in the old rats and may be analogous to hypophonia and asthenia

observed in humans with presbyphonia. With age, quantitative measures of voicing, such as vocal range and intensity, often change (Endres et al. 1971; Hagen et al. 1996; Higgins and Saxman 1991; Honjo and Isshiki 1980; Linville 1987, 2004; McGlone and Hollien 1963; Mueller et al. 1984; Mysak 1959; Ptacek et al. 1966; Ramig and Robert 1983; Ramig et al. 2001; Roy et al. 2007; Sataloff et al. 1997; Shindo and Hanson 1990; Smith et al. 1996; Torre and Barlow 2009). Overall, the parallel findings of diminished bandwidth and intensity suggest that USVs of rats are a potential translational model for examining the underlying mechanisms of age-related voice changes in humans.

Our data show that motoneuron loss occurs in the loose formation of the nucleus ambiguus, which innervates the laryngeal muscles (Altschuler et al. 1991; Bieger and Hopkins 1987; Saxon et al. 1996; Van Daele and Cassell 2009) and this loss co-occurred with a functional deficit in USVs. Our results suggest that motoneuron loss in the nucleus ambiguus is a potential factor that is associated with laryngeal neuronal degeneration. These findings are significant because there are mixed reports of motoneuron loss at the level of the brainstem nuclei as a function of the normal aging processes (Monagle and Brody 1974; Peng and Lee 1979; Schwarz et al. 2009; Soltanpour and Santer 1997; Sturrock 1990). Because the thyroarytenoid muscle is also involved in respiration and swallowing, it may be that these other vital life functions are affected by this degeneration in the elderly as well (Achem and DeVault 2005; Baum and Bodner 1983; Ekberg and Feinberg 1991; Hagen et al. 1996; Krumpe et al. 1985; Linville 1987, 2004; Lundy et al. 1998; McKee et al. 1998; Mueller et al. 1984; Mysak 1959; Nicosia et al. 2000; Ptacek et al. 1966; Ramig and Robert 1983; Ramig et al. 2001; Robbins et al. 1992; Roy et al. 2007; Sataloff et al. 1997; Torre and Barlow 2009; Tracy et al. 1989). It would be interesting to examine the potential link between selective neuronal degeneration in brainstem nuclei and functional deficits in speech, respiration, and swallowing that are seen with aging.

This study did not investigate the causality of motoneuron loss with diminished USVs. There are several potential underlying mechanisms for the vocalization changes that we observed. Degeneration may be occurring in the loose formation of the nucleus ambiguus, causing the peripheral effect of thyroarytenoid deterioration that has been shown with aging (Bach et al. 1941; Honjo and Isshiki 1980; Shindo and Hanson 1990; Thomas et al. 2008). There may also be neuronal loss in the motor cortex accounting for the loss of sensorimotor control of the vocal folds that may be affecting the quality of the USVs, and the motoneuron loss in the nucleus ambiguus may not be related to these fine sensorimotor deficits. Future studies should include examining the acoustic parameters of aging rats and their functional relevance, as well as their relationships to things such as neurotrophic factors, vocal fold physiology, muscle morphology, and the complete motoneuron number of the loose formation of the nucleus ambiguus. These data could be provided by a longitudinal study.

A potential limitation in our measurements may be based on the fact that the loose formation of the nucleus ambiguus was difficult to visualize with the NeuN marker for mature neuronal bodies because of its formation, or lack thereof. Thus, it was necessary to rely primarily on cholera toxin- β reactivity for the quantification of motoneurons. Therefore, the motoneuron count only included motoneurons that were labeled by cholera toxin- β injected into the thyroarytenoid muscle. The next step would be to improve quantization of neurons in the nucleus ambiguus and to determine if motoneuron loss is occurring in all of the laryngeal muscles supplied by motoneurons within the nucleus ambiguus.

Research on the mechanisms of age-related vocal decline is necessary, as the elderly population continues to grow, and the findings suggest the need for preventative therapies. Our data support the use of a rat model to study putative age-related mechanisms, such as neuronal degeneration as the level of the brainstem, in future studies.

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Fig. 1.

Ultrasonic vocalization call types. Representative calls from young (9 month) and old (32 month) rats are shown. The three call-type categories include simple (constant frequency), step (varying frequencies, generally slow to change between frequencies), and frequency-modulated, or FM (trill-like). Relative intensity is represented with color, as depicted in the relative intensity scale shown

Fig. 2. Immunohistochemistry of retro-labeled motoneurons in the compact formation of the nucleus ambiguus. **a** Motoneurons stained for immunoreactivity to the retrograde tracer, Cholera Toxin β . **b** Mature neurons stained for immunoreactivity to the mature neuronal marker, NeuN. **c** An overlay of the two antibodies. **d** View of thyroarytenoid muscles of a F344/Brown Norway rat provided by the microlaryngoscope and microscope (magnification of $\times 32$ with a $\times 1.6$ lenspiece)

Fig. 3. Duration of calls. Means and standard error of the mean (SEM) of the average duration of simple, step, and frequency-modulated calls of young ($n = 10$) and old animals ($n = 8$). $*p < 0.05$

Fig. 4.
a Average Bandwidth. Means and standard error of the mean (SEM) of the average bandwidth of simple, step, and frequency-modulated calls of young ($n = 10$) and old animals ($n = 8$). **b** Maximum Bandwidth. Means and standard error of the mean (SEM) of the maximum peak frequency of simple, step, and frequency-modulated calls of young ($n = 10$) and old animals ($n = 8$). * $p < 0.05$

Fig. 5.

a Average Peak Frequency. Means and standard error of the mean (SEM) of the average peak frequency of simple, step, and frequency-modulated calls of young ($n = 10$) and old animals ($n = 8$). **b** Maximum Peak Frequency. Means and standard error of the mean (SEM) of the maximum peak frequency of simple, step, and frequency-modulated calls of young ($n = 10$) and old animals ($n = 8$). * $p < 0.05$

Fig. 6.

a Average Intensity. Means and standard error of the mean (SEM) of the average intensity of simple, step, and frequency-modulated calls of young ($n = 10$) and old rats ($n = 8$). **b** Maximum intensity. Means and standard error of the mean (SEM) of the maximum intensity of simple, step, and frequency-modulated calls of young ($n = 10$) and old rats ($n = 8$). Please note that intensity, measured in decibels (dB), is a negative value, and a less negative value reflects a louder vocalization. * $p < 0.05$

Fig. 7. Motoneuron Number. Mean and standard error of the mean (SEM) of the number of motoneurons retro-traced with CT β from the thyroarytenoid muscle in the larynx to the nucleus ambiguus of young ($n = 8$) and old animals ($n = 8$). * $p < 0.05$

Table 1

Means and standard error of the mean (SEM) for all acoustic and neuronal parameters measured

Parameter	Young adult (Average $\pm$ SEM)	Old (Average $\pm$ SEM)
Number of motoneurons	19.33 $\pm$ 1.87	14.45 $\pm$ 2.10 *
Size (in area, μm^2), of average motoneuron	904.48 $\pm$ 133.33	965.32 $\pm$ 119.32
Number of primary dendrites per motoneuron	3.44 $\pm$ 0.17	3.33 $\pm$ 0.19
Call complexity-percentage of complex calls	59.2 $\pm$ 4.0	56.3 $\pm$ 6.0
Call complexity-proportion of duration of complex calls	0.6993 $\pm$ 0.045	0.623 $\pm$ 0.083
Call rate (calls per second)	0.79 $\pm$ 0.154	0.77 $\pm$ 0.216
Duration-simple (ms)	19.5 $\pm$ 1.9	37.6 $\pm$ 13.0
Duration-step (ms)	26.4 $\pm$ 0.9	43.2 $\pm$ 3.2 *
Duration-FM (ms)	40.9 $\pm$ 6.6	39.7 $\pm$ 4.5

	Average $\pm$ SEM	Maximal $\pm$ SEM	Average $\pm$ SEM	Maximal $\pm$ SEM
Bandwidth-simple (Hz)	7,616.4 $\pm$ 116.9	10,380 $\pm$ 128.9	6,920 $\pm$ 252.3 *	9,387.5 $\pm$ 388.0 *
Bandwidth-step (Hz)	22,131 $\pm$ 946.7	36,990 $\pm$ 1,394.6	19,146 $\pm$ 2,007.1	30,650 $\pm$ 4,402.9
Bandwidth-FM (Hz)	31,534 $\pm$ 555.9	49,300 $\pm$ 1,848.4	25,683 $\pm$ 2,218.5 *	40,414 $\pm$ 4,316.9 *
Peak frequency-simple (Hz)	53,626 $\pm$ 941.7	67,600 $\pm$ 2,116	49,370 $\pm$ 1,328.7 *	62,413 $\pm$ 2,738.3
Peak frequency-step (Hz)	58,551 $\pm$ 1,465.7	74,330 $\pm$ 2,268	55,815 $\pm$ 1,928	68,075 $\pm$ 3,368
Peak frequency-FM (Hz)	61,246 $\pm$ 1,545.2	79,310 $\pm$ 1,996.4	57,161 $\pm$ 1,808.4	71,343 $\pm$ 2,971.8 *
Intensity-simple (dB)	-37.1 $\pm$ 0.79	-21.5 $\pm$ 1.03	-40.8 $\pm$ 1.68 *	-30.1 $\pm$ 3.51 *
Intensity-step (dB)	-32.6 $\pm$ 0.93	-17.3 $\pm$ 1.40	-34.3 $\pm$ 2.37	-23.0 $\pm$ 3.39
Intensity-FM (dB)	-31.6 $\pm$ 0.84	-17.6 $\pm$ 1.05	-33.8 $\pm$ 0.71 *	-21.3 $\pm$ 2.42

Number of motoneurons represent an average of five 40 μm sections (per animal) through the loose formation of the nucleus ambiguus. Call complexity is defined by the percentage of complex calls (step and FM) from the total overall calls made by an animal and the proportion of the duration of complex calls from the total duration of all call types. Please note that intensity, measured in decibels (dB), is a negative value, and a less negative value reflects a louder vocalization.

* $p < 0.05$, between the same parameter for young and old animals