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Current Neuropharmacology, **2015**, *13,* **211-219**

Changes in Rat 50-kHz Ultrasonic Vocalizations During Dopamine Denervation and Aging: Relevance to Neurodegeneration

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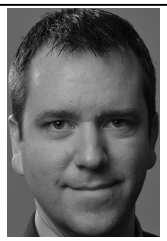

A.M. Johnson

Abstract: Vocal communication is negatively affected by neurodegenerative diseases, such as Parkinson disease, and by aging. The neurological and sensorimotor mechanisms underlying voice deficits in Parkinson disease and aging are not well-understood. Rat ultrasonic vocalizations provide a unique behavioral model for studying communication deficits and the mechanisms underlying these deficits in these conditions. The purpose of this review was to examine the existing literature for methods using rat ultrasonic vocalization with regard to the primary disease pathology of Parkinson disease, dopamine denervation, and aging. Although only a small amount of papers were found for each of these topics, results suggest that both shared and unique acoustic deficits in ultrasonic vocalizations exist across conditions and that these acoustic deficits are due to changes in either dopamine signaling or denervation and in aging models changes to the nucleus ambiguus, at the level of the neuromuscular junction, and the composition of the vocal folds in the larynx. We conclude that ultrasonic vocalizations are a useful tool for studying biologic mechanisms underlying vocal communication deficits in neurodegenerative diseases and aging.

Keywords: Aging, dopamine, rat, ultrasonic vocalization, voice, 6-OHDA.

INTRODUCTION

Disorders of voice (dysphonia) and speech production (dysarthria) can be related to developmental issues, neuro-degeneration, structural defects, and/or advanced aging. Neurodegeneration related to disease and aging causes significant negative changes to vocal production and thus affects communication ability and quality of life. For example, dysarthria is an early-onset sign in individuals with Parkinson disease typically starting with changes to vocal loudness and quality [1-6]. As the disease progresses vocal acoustic and auditory-perceptual features degrade and cause reduced amplitude (loudness), decreased variation of intonation (monopitch and monoloudness), and increased signal to noise ratio (breathiness/harshness in vocal quality), which significantly impair voice production and, therefore, speech intelligibility and communication efficacy [7-11]. Although the primary pathology of Parkinson disease is depletion of nigrostriatal dopamine, there are many signs and symptoms that do not fit with this model of the disease, including dysarthria. Unfortunately, pharmacologic and surgical inter-ventions aimed at modulating the nigrostriatal dopamine system/circuits (deep brain stimulation, levodopa) do not markedly improve and may even worsen voice production [12-20]. Further, it is now understood that Parkinson disease encompasses a widespread pathology [21], with changes to the autonomic nervous [22], peripheral nerves and muscles [23-25] and dysregulation of norepinephrine and degeneration of the locus coeruleus [26-29]. As such, how the complex pathology of Parkinson disease contributes to vocal dysfunction is unclear. To address this problem, a logical first step in understanding how Parkinson disease affects voice is to determine the effects of depleting nigrostriatal dopamine on vocal production, as this is the primary feature of disease pathology. Once this is defined, then systematically studying degeneration of other neural structures and neurotransmitters as they pertain to dysarthria will lead to a more complete picture of vocal dysfunction in Parkinson disease. For the purposes of this review, we will focus on work that has been done in depleting nigrostriatal dopamine.

Age-related voice disorders in older adults negatively impact quality of life, present both physical and mental health risks, and are common; the prevalence of voice disorders in older adults has been estimated to be between 20-30% [30, 31]. Laryngeal neuromuscular decline is associated with age-related voice problems and may also contribute to difficulty swallowing through compromised laryngeal closure [32-35]. Therefore, understanding the effects of advanced age on laryngeal neuromuscular mechanisms has important clinical implications.

Although considerable work has been done in humans, much of our understanding of these problems comes from animal models. Rat ultrasonic vocalizations (USVs) have been extensively studied with regard to basic neurophysiologic

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mechanisms underlying production as well as affect and reward/addiction [36-49]. USVs can also be useful to study sensorimotor control for actions of the larynx, such as vocalization, since USVs and human vocalizations share similar mechanisms of production; specifically, both are produced by rapidly constricting the laryngeal muscles to modulate an egressive airflow [50, 51]. The primary difference between human vocalizations and rat USVs is that human vocalizations are created by vibrations of the vocal folds, while USVs are thought to be produced using a whistle mechanism [50, 52, 53]. It has been shown that rat USVs share certain features with human speech communication in that they have semiotic value [54] serve to establish and maintain social contact [55], and consist of modular vocal behavior [56]. That is not to say that USVs are equivalent to human speech; rather, USVs, like speech, require fine sensorimotor control of the larynx, have meaning, and can change the behavior of the signal recipient. Thus, USVs can be an appropriate model for studying sensorimotor control in the context of communication. Recently, rodent ultrasonic vocalizations have been used to model aging and Parkinson disease. The purpose of this paper for the special issue is to review the literature related to rat 50-kHz USVs as it pertains to dopamine denervation as a model for Parkinson disease and aging.

DOPAMINE DENERVATION

Dopamine denervation has been modeled extensively in rats by infusing the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle or striatum, where it is taken up by the neuron causing a cascade of events leading to dopaminergic and noradrenergic cell death (for examples of literature out of thousands of papers see Ungerstedt, 1968 [57] and more recently Johnson *et al*., 1999 [58]). This has also been modeled less commonly with pesticides/herbicides such as rotenone and paraquat. Regardless of the mechanism, these methods attempt to model the cardinal features of Parkinson disease; tremor, postural instability, bradykinesia by causing striatal dopamine depletion (for a review see Blesa, Phani, Jackson-Lewis, Przedborski, 2012 [59]). However, the important clinical features of Parkinson disease have expanded to include other sensorimotor deficits such as freezing, dysarthria and dysphagia (disordered swallowing) (for a review see Ciucci *et al*., 2013 [60]) and non-motor deficits such as hyposmia, sleep and mood disturbances, cognition and autonomic dysfunction (see Natale *et al*., 2013 [22], and as an example in the 6-OHDA rat model see Tadaiesky, *et al*., 2008 [61]). Thus, efforts have been made to define which signs are attributed to dopamine depletion in nigrostriatal pathways or other more recently identified extranigral and non-dopaminergic mechanisms [21, 26, 62].

In our review of the literature, only two papers addressed the effects of unilateral dopamine denervation on 50-kHz vocalizations with 6-OHDA ([63, 64]); no studies were found using other neurotoxin or pesticide/herbicide models. The first study (Ciucci *et al*., 2007) [63] was preliminary, thus results from the 2009 study are reported here [64]. Using a mating paradigm, the authors elicited 50-kHz USVs in male Long-Evans rats by exposing sexually-experienced males to receptive females. The female rats were removed and recording of vocalizations were from individual males only. USV complexity (trill-like frequency modulated USVs vs. flat USVs) was decreased relative to controls without a decrease in the total number of USVs produced. Amplitude (acoustic intensity measured in decibels) and bandwidth (the frequency range of a vocalization, measured in Hertz as the difference between the highest and lowest frequency) were also significantly reduced compared to controls (for representative spectrogram, see Fig. **1** reprinted with permission from Ciucci, *et al*., 2008 [65]). Although it has been established in adult USVs that acoustic features, such as number of calls, intensity, and frequency range, carry communicative value, it is unknown how changes in these parameters change the semiotic content and/or the response of the recipient [66]. Based on recent advances in understanding the physiology of USV production [50, 56, 67], however, these acoustic changes suggest that rats, like humans, have decreased control at the level of the larynx with dopamine denervation. However, although denervation with unilateral 6-OHDA infusion alters the acoustic structure of rat USVs, certain features do not change or change to a small degree. We calculated effect sizes from the Ciucci 2009 paper and there was a large effect on amplitude (Cohen's d 8.0, r=0.97) but a smaller effect on maximum frequency (Cohen's d=2.6, r=0.79), while there was no statistically significant difference in USV duration.

In another study, neurodegeneration was induced by unilaterally injecting recombinant adeno-associated virus (rAAV) serotype 2/5-expressing human wildtype α-synuclein, a protein implicated in the neuropathology of Parkinson disease, in the rat substantia nigra [68]. Functional impairment in measures of forelimb use were observed in rats with 8 week duration of α-synuclien (α-syn) expression and these deficits were highly correlated with striatal tyrosine hydroxylase loss. Rats in this cohort also demonstrated decreased USV amplitude and rate, but there were no significant differences in USV type, duration, bandwidth, peak frequency or latency to call between naïve control and rAAV2/5-α-syn rats [68]. This suggests that while dopamine denervation affects acoustic properties of the USV, it does not affect all aspects of the USV, pointing toward other mechanisms. This in part has been recently addressed in a mouse model over-expressing human wildtype α-syn. Mice showed a significant reduction in the duration and amplitude of USVs with an altered USV profile [69]. These acoustic deficits occur in the absence of nigrostriatal dopamine loss, although α-syn pathology was found in the periaqueductal gray, a region important for vocal production [70-72]. Thus, we can tentatively conclude that dopamine denervation as well as other pathological mechanisms associated with Parkinson disease contribute to vocal dysfunction. In other words, like humans, vocal communication in rats is susceptible to pathologies related to Parkinson disease, exhibit acoustic changes that mimic those seen in the human manifestation of the disease (reductions in frequency bandwidth and amplitude), and can serve as useful tool in understanding the behavioral and underlying neurological complexities of Parkinson disease.

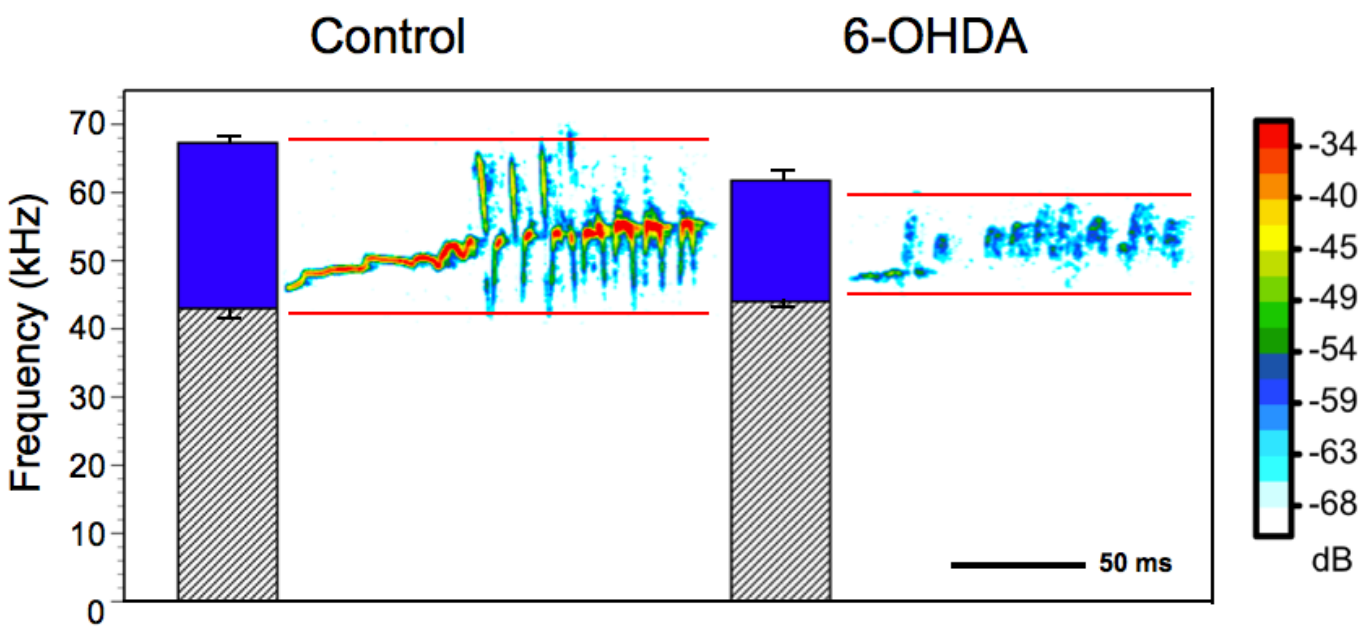

Fig. (1). Representative spectrograms of frequency modulated trill-type USVs demonstrating the reduced amplitude and bandwidth in unilateral 6-OHDA infusion (dopamine denervation model of Parkinson disease) male rats compared with control. Averages and standard errors of the maximum and minimum peak frequency are represented by the bar graphs. Solid color between the maximum and minimum peak frequency indicates the bandwidth of the call. Reprinted with permission from Ciucci, *et al*., 2008 [65].

PHARMACOLOGICAL MANIPULATIONS OF DOPAMINE

Although it is beyond the scope of the current paper to review all effects of pharmacologic manipulation on USVs, a considerable amount of work has been done to characterize dopaminergic mechanisms in the production of USVs [42, 73-75]. Specifically, manipulations of dopamine with drugs such as amphetamine [40, 43, 48, 76, 77], cocaine [78, 79], apomorphine [79], and methylphenidate [80] result in an increase in the quantity of 50-kHz USVs. Likewise, agonizing or antagonizing dopaminergic receptors affects both the quantity and quality of 50-kHz vocalizations [81-86]. For example, selective antagonism of the dopamine D_2 receptor sub-type with haloperidol results in decreased bandwidth, amplitude (loudness), and complexity of USVs, similar to what is observed following unilateral infusions of 6-OHDA [63, 64]. Likewise, selective D_1, D_2, or combined D_1 + D_2 antagonism results in not only decreased call rate and altered call profile [81, 82], but also reductions in latency to call, duration, intensity, bandwidth and peak frequency [82]. Given the role of dopamine in rewarding and appetitive behaviors, there is also a body of literature devoted to characterizing both direct manipulations of the nucleus accumbens [40, 43, 46, 76, 87] and the 50-kHz USV as an indicator of positive affect (for examples, see [54, 88, 89]). While not as well characterized as dopaminergic mechanisms in USVs, manipulations of other neurotransmitters such as norepinephrine [75, 90-96] and serotonin [97] have also been shown to play a role in the production of 50-kHz USVs. Although pharmacological manipulations do not recapitulate dopaminergic denervation, as it relates to Parkinson disease, these studies offer valuable insight into the relationship between specific dopaminergic mechanisms and qualitative aspects of USVs. This, in turn, may help to better understand how and why voice deficits manifest in Parkinson disease and inform future treatment approaches.

EFFECT OF ADVANCED AGE ON 50-kHz VOCALIZATIONS

Only a handful of studies have looked at the effects of advanced age on rat USVs [98-100]. Overall, age-related acoustic changes in 50-kHz USVs are similar to those found with unilateral dopamine denervation with 6-OHDA, with aged rats demonstrating decreases in measures of frequency (smaller bandwidth and decreased maximum and peak frequency) and decreased amplitude of vocalizations relative to young adult rats [98-100]. Different than the findings in the 6-OHDA model, however, vocalization complexity and rate do not appear to be influenced by advanced age. Furthermore, aging does appear to impact the duration of complex vocalizations, with longer mean durations of step [98] and frequency modulated [99] USVs in old adult rats compared to USVs of young adult rats. Peterson *et al*. (2013) [100] did not report results on duration. Although few in number, these studies indicate aging does have an impact on the acoustics of rat 50-kHz USVs, and that the acoustic deficits have some similarities with those found with dopamine denervation.

These acoustic changes in USVs may be due to age-related changes in laryngeal structure and/or central and peripheral denervation-like neuromuscular adaptations. In the aging rat larynx, senescence is associated with neuromuscular deficits, including decreased laryngeal kinematics during resting breathing, reduced capillary surface area and branch points in the thyroarytenoid muscle (the primary muscle of the vocal fold), and a shift to slower

contracting muscle fiber types in the thyroarytenoid muscle [101-103]. In both the human and rat larynx, aging impacts laryngeal neuromuscular junction (NMJ) morphology in a manner similar to denervation, but in ways unique from NMJs found in the limb musculature [32, 104-106]. Peterson *et al*. (2013) [100] reported age-related acoustic deficits of USVs were correlated with structural changes in the microarchitecture of the vocal folds, including an increase in connective tissue and collagen and a decline in elastin and hyaluronic acid, all of which may result in increased stiffness of the vocal folds. Peripheral neuromuscular changes, such as dispersion of the motor endplate within the NMJ [99], and central changes, such as a decreased number of motoneurons in the nucleus ambiguus [98], have also been associated with acoustic deficits in 50-kHz USVs. Furthermore, other unexplored mechanisms underlying USV production may be impacted by advanced age and cause changes in acoustic parameters. For example, frequency modulation of USVs is related to respiratory patterns [67]; therefore, age-related changes in respiratory capacity and function may also play a role in acoustic deficits. No direct studies have been performed, however, investigating USV production and respiratory function in an aging model. Overall, these results suggest advanced age negatively impacts the physiology of the rat laryngeal neuromuscular system, and therefore, the acoustic signal of 50-kHz USVs.

Age-related acoustic deficits in USVs correspond to those found in the aging human voice. Typical age-related acoustic changes in the human voice include alterations in average fundamental frequency, decreased fundamental frequency range, decreased vocal stability (increased perturbation), and decreased volume (amplitude) (for review, see Baken (2005) [107]). As with the rat, these acoustic changes in the human voice are likely due to underlying age-related changes in vocal fold microarchitecture and biomechanics [108-110] and neuromuscular deficits [111]. Therefore, study of age-related changes in rat USVs and laryngeal neuromuscular system can provide insight into age-related changes in the human voice.

DISCUSSION

This paper reviewed the currently published literature on male rat USVs as it pertains to dopamine denervation and aging. There were only 2 papers published on dopamine denervation with the neurotoxin 6-OHDA and one paper using viral transfection of α-syn to deplete dopamine in the substantia nigra; no other neurotoxin or herbicide/pesticide studies examining USVs were found. Findings from these papers show an altered USV profile (less complex and more simple USVs) along with reduced bandwidth and amplitude of USVs. Other acoustic parameters, such as duration, were not affected. Thus, unilateral denervation of nigrostriatal fibers in the medial forebrain bundle or substantia nigra is associated with acoustic deficits in male rat USVs. However, as vocal deficits appear early in humans and are refractory to treatments aimed at nigrostriatal dopamine depletion, other mechanisms in the complex pathology of Parkinson disease are indicated with potential association to dopamine pathways. Midbrain dopamine cells that die in the disease appear particularly susceptible to abnormal alpha-synuclein protein deposits [26]. Transgenic mouse models that over-express human wild type α-syn under the Thy-1 promoter demonstrate early and progressive USV deficits accompanied by α-syn pathology in the periaqueductal gray (PAG) which happens prior to substantial dopamine cell loss [69].

As described in the seminal works by Braak [21, 26, 62], dopamine neurons appear to be vulnerable to degeneration and alpha-synuclein aggregation and the association between alpha-synuclein and dopamine signaling pathways may be mediated by molecular intermediaries. The forkhead family of transcription factors including Foxp1 and Foxp2 are likely candidates. Knockout mice with α-syn deficiency and altered USVs show downregulation of *Foxp1* mRNA in the striatum during developmental and adult periods [112]. Recent evidence from the zebra finch songbird suggests that lentiviral-mediated knockdown of the speech-related gene FoxP2 in the basal ganglia song nucleus, Area X, affects dopamine modulation in cortico-striatal circuitry and modification of the song [113]. In addition to these FoxP molecules, a whole cascade of other behaviorally-driven genes in zebra finches associate with dopamine-modulated pathways in the basal ganglia [114, 115]. These molecular interactions may be key mediators of vocal plasticity necessary for song learning and on-going maintenance. These examples from multiple species highlight the need to leverage the advantages of genetic tools in mice and rats with the well-characterized neural circuitry of songbirds in order to move forward in the investigation of the onset, progression, and pathology of vocalization deficits in motor disorders, particularly ones associated with dopamine denervation.

The results of the aging studies may indicate an age-related decrease in fine motor control needed to accurately produce these relatively short (<50 ms), rapidly-modulated vocalizations. Loss of fine motor control may result from an increase in the size of the motor unit seen with aging [116]. Indeed, age-related acoustic changes in rats are associated with a decrease in the number of primary motoneurons in the nucleus ambiguus [98] and pre-synaptic remodeling of the NMJ [99], suggesting motor unit remodeling with age. This interpretation is supported by findings using electro-myography in the human larynx that show longer motor unit durations in older adults [117]. Additionally, age-related changes at the NMJ may be a primary cause of motor unit remodeling [118]. Evidence is limited, however, by the few studies on age-related changes in USVs and related changes in underlying neuromuscular mechanisms. Age-related changes both within the larynx and throughout the body likely impact USVs. For example, USVs are produced with an egressive airflow and, therefore, age-related changes in breathing and pulmonary function likely contribute to changes in USVs. Studies of human speech breathing have shown older adults use different respiratory mechanisms when producing speech, likely due to changes in the strength of respiratory musculature, compliance of the chest and lungs, and lung volume [119, 120]. Changes in rat pulmonary function with aging has been identified [121], although subglottal pressure during USV production has only been studied in young rats [50]. These multiple neurological and physiological mechanisms involved with USV production make USVs a unique behavioral

biomarker of aging, and offer a relatively untapped area of research.

One of the primary acoustic USV variables affected by both dopamine denervation and aging is USV amplitude. When measuring changes in amplitude, variations in distance from the sound source to the microphone will result in variations of amplitude. In the reviewed studies, rats were allowed to roam freely while vocalizations were recorded. Variability of individual rats was accounted for by taking an average measurement of many vocalizations for each rat. Therefore, the observed differences in USV amplitude were likely due to true differences between groups and not variations in mouth-to-microphone distance. A potential solution to control for this variation in microphone distance could be to use an array of microphones, allowing for localizing of the animal and subsequent calculation of a calibrated amplitude measure. Future investigations may consider this or another controlled method of measuring amplitude to provide further insight into USV amplitude changes.

Many of the reported changes in USV acoustics differed by vocalization subtype; however, there is no standard method for classifying USVs and, therefore, different methods of classification were used across studies. Although one study analyzed all 50-kHz USVs together without further classification [100], most of the studies discussed in this review manually classified 50-kHz USVs into at least 2 subtypes by frequency, either "flat" (constant frequency) or frequency modulated. The frequency modulated USVs are often further divided into 2, 4, or as many 12 different sub-classification [64, 77, 88]. Although automatic classification schemes have been proposed for both rat [122, 123] and mouse [124] USVs, none have been widely accepted. Evidence for a reliable, automated, meaningful classification scheme is critical for comparing results across studies.

Despite some methodological limitations, rat 50-kHz USVs have been a useful tool for studying changes to vocal production with regard to dopamine denervation and aging. Additionally, USVs also offer an opportunity to study behavioral interventions targeted at preventing and/or reversing these changes in vocal production and the underlying neurological and sensorimotor deficits [99, 125]. Although only a limited number of studies were identified by this review, it is clear rat 50-kHz USVs are an emerging tool to investigate many disorders of the nervous system and genetic conditions that cause communication deficits.

CONFLICT OF INTEREST STATEMENT

The authors have no financial or non-financial conflict of interest to declare.

ACKNOWLEDGEMENTS

The authors thank Cynthia A. Kelm-Nelson, PhD for assistance with figure preparation and Julie E Miller, PhD for critical comments regarding molecular intermediaries and birdsong models of vocalization. This work was supported by the National Institute on Deafness and Other Communication Disorders at the National Institutes of Health (T32 DC009401).

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Received: August 01, 2014 Revised: October 13, 2014 Accepted: October 16, 2014