

Development of an Animal Model to Study Effects of Auricular Vagal Nerve Stimulation on Brain Plasticity and Auditory Learning of Complex Phoneme Discrimination – A Preliminary Study

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During the acquisition of spoken language, children learn to distinguish different acoustic stimuli, acquire the phonemes that form the building blocks of language, and associate the combinations of these sounds with meaning. Some of the basic underlying components of auditory discrimination and learning can be studied in animal models. For the first time, we developed a new behavioral paradigm and trained ferrets to discriminate Mandarin Chinese tonal phonemes (a total of eight “tonemes”). In order to discover whether auditory learning could be accelerated, we describe preliminary experimental results using vagal nerve stimulation (VNS) as a tool to explore the role of brain neuromodulators (released by VNS) in enhancing the process of learning complex auditory tasks, including discrimination of Mandarin Chinese tonal phonemes. Previous studies have shown that VNS activates the vagal nerve and that this activation causes the release of brain neuromodulators acetylcholine, norepinephrine and serotonin, all known to modulate attention and synaptic plasticity. We examined the effects of VNS from a dual perspective: both behavioral (task acquisition and performance) and neural (brain activity and plasticity). Our experiments specifically assessed whether stimulation of the external ear concha (auricular VNS or a-VNS), when paired with behavioral training on a complex auditory task, enhanced task acquisition and/or performance. Behavioral data on several auditory tasks in control ferrets (that did not receive a-VNS) provided a baseline for comparison with animals trained with a-VNS. Our initial, preliminary results indicate that the learning speed and performance level of ferrets in the control (no-VNS) group and the a-VNS group were not significantly different. However, although we did not find behavioral improvements from a-VNS, we did observe striking effects of a-VNS on neural responses in the primary auditory cortex (A1) of task-naïve ferrets, indicating that the neural representation of sound can be re-shaped, even by brief episodes of VNS.

Abbreviations: a-VNS – auricular VNS; c-VNS – cervical nerve cuff VNS; AC – Auditory Cortex; LC – Locus Coeruleus; NB – Nucleus Basalis; NTS – Nucleus Tractus Solitarius; Primary Auditory Cortex – A1; VNS – Vagal Nerve Stimulation

Keywords: Auditory Cortex; Auditory Plasticity; Auditory Learning; Language Acquisition; Mandarin Chinese; Neuroplasticity; Tonal Language; Vagal Nerve Stimulation

Introduction

There is growing interest in the possible value of vagal nerve stimulation (VNS) for therapeutic purposes and also to enhance brain plasticity. The twin goals of this study were to develop an animal model to explore the effects of VNS on acquisition of behavior (phonological learning) and on brain plasticity at the level of single cortical neurons.

The vagus nerve is the 10th cranial nerve, and is the longest nerve in the autonomic nervous system in the human body. It has widespread afferent and efferent projections and interfaces with parasympathetic control of numerous organs in the body including the heart, lungs and digestive system and also sympathetic function via peripheral chemoreceptors (Groves, 2005; Keaney et al. 2017). The afferents of the vagus nerve project to the Nucleus Tractus Solitarius (NTS) in the brainstem which in turn projects to the Locus Coeruleus (LC) and the Nucleus Basalis (NB) (see Figure 1) and the Dorsal Raphe nuclei, which have extensive projections throughout the brain and release the neuromodulators norepinephrine and acetylcholine and serotonin, respectively (Engineer et al., 2013; Hays et al., 2013; Hulsey et al., 2019). These two neuromodulators have been known to enhance neural plasticity in the thalamus and neocortex in all sensory and motor systems that have been studied. For example, VNS enhances motor cortical plasticity, but only in the presence of cholinergic innervation (Hulsey et al., 2016). Stimulation of NB leads to enhanced neural plasticity in the auditory cortex of rats (Metherate and Weinberger, 1990; Bakin and Weinberger, 1996; Kilgard and Merzenich, 1998; Shetake et al., 2012; Nichols et al., 2011; Hays et al., 2013; Lin et al., 2015) and acts to enhance transmission through the thalamic projections to the cortex, enhances cortical processing of stimuli and enhances auditory learning (Reed et al., 2008). NB stimulation also enhances visual perception in mice (Goard and Dan, 2009; Pinto et al., 2013; Sugihara et al., 2016). Stimulation of the LC also increases auditory responses in LC neurons and enhances responses in the auditory cortex (Martins and Froemke, 2015). In addition to its brain effects

via neuromodulator release, VNS causes a significant increase in pupillary dilation (Lai and David, 2019), a biomarker measure associated with enhanced cognitive focus and attention (Jodoin et al., 2015; Karatekin et al., 2004).

Although initially there were concerns about the safety of VNS in humans, there is now considerable clinical evidence for the therapeutic value of cervical vagal nerve stimulation (c-VNS) for treatment of epilepsy, depression and other neurological diseases in humans, leading to FDA approval for c-VNS treatment for epilepsy. Currently, over 70,000 patients in the USA have been treated with c-VNS for epilepsy and depression (Engineer et al., 2013; Lulic et al., 2009) with minimal side effects. Double blind, randomized, placebo-controlled studies demonstrate that c-VNS is effective for improving epileptic symptoms (Ben-Menachem, 2002). In these studies, patients treated with higher levels of VNS resulted in a more significant reduction in seizures in comparison to a low VNS patient group. Other clinical applications for c-VNS that have led to positive outcomes include treatment for PTSD (post-traumatic stress disorder), phantom limb pain, tinnitus (Hays et al., 2013), anxiety (George et al., 2008), and tissue inflammation (Borovikova et al., 2000; Zhang et al., 2009). While requiring invasive neck surgery, c-VNS is an FDA approved treatment for human patients, meaning the treatment is believed to be safe for human use and poses low or no risk to human health, and is likely to be beneficial for ameliorating or curing several diseases. Some recent evidence suggests that c-VNS may also improve cognitive function, although this claim is controversial (Vonck et al., 2014). It is in this context of widespread clinical use of VNS, but controversy over whether VNS could be used in healthy subjects to improve cognitive function, that animal studies become critically important in order to clarify the possible cognitive value of VNS.

Nerve fiber activation plays a critical role in achieving effects with VNS and clearly will be different in c-VNS (cervical VNS which can stimulate all afferent and efferent fibers in the vagus nerve at the cervical level) and a-VNS

(auricular VNS which likely stimulates only a small subset – perhaps only ~5% of the afferent vagus fibers since the vast majority of the afferent vagus nerves are visceral afferents). A study where unmyelinated C-fibers were destroyed in rats demonstrated that c-VNS was still effective at reducing drug-induced seizures, indicating that these effects are still possible by only stimulating afferent myelinated A and B fibers (Ben-Menachem, 2002). A and B-fibers have a lower threshold for activation, meaning that lower levels of current could be used to achieve the same effects (Howland, 2014). Other studies demonstrate an inverted U-shaped function for VNS current amplitude delivered and duration, indicating that there is an optimum level of VNS intensity, perhaps stimulating a subset of fibers, which optimally drives plasticity (Borland et al., 2016; Loerwald et al., 2018). The ferret vagal nerve has been studied at high resolution with electron microscopy, and has approximately 28,000 fibers, of which only 1000 are myelinated (Asala and Bower, 1986). We are initiating studies in the ferret to measure the compound nerve action potential during c-VNS to accurately determine the population of fibers that are activated, and which are critical for achieving neural and behavioral effects.

A non-invasive transdermal form of VNS is auricular VNS or a-VNS (see He et al. (2013) for a description of procedure), which is the transcutaneous stimulation of the auricular branch of the vagus nerve (ABVN); afferents from the ABVN also project to NTS, however nerve fiber populations may differ (Yuan, 2016; Safi, 2016). Adjusting the level of current administered by a-VNS to below pain threshold likely preferentially activates afferent myelinated A-fibers like those effective for seizure reduction in c-VNS (Yuan, 2016). Auricular VNS was approved in Europe to treat epilepsy and depression in 2010, followed by approval for pain treatment in 2012 (Yuan, 2016). If similar results to c-VNS can be attained with a-VNS across all domains of VNS research, comparable to the benefits of c-VNS, there would be no need for invasive cervical cuff implant surgery.

Experimental Approach and Hypotheses:

The overall goal of the present study was to determine whether a-VNS enhances neural

plasticity and/or also accelerates or increases behavioral learning, by reducing task acquisition time and/or by enhancing performance once the task is learned.

Since the ferrets have excellent hearing, and an audiogram similar to humans (Kelly et al., 1986, King and Nodal, 2014; Keating et al., 2014) the ferret is an ideal animal model to study the effects of VNS on auditory task acquisition. Although ferrets obviously cannot learn language, they can learn to discriminate different phonemes that form the basis for linguistic utterances. Adult humans sometimes struggle to learn to discriminate the phonemes in a foreign language which is a precondition for correct pronunciation. Thus, although ferrets are obviously not a good model for articulation or production, they can provide an excellent animal model for phoneme discrimination.

The prevalence of tonal languages and the desirability of second language acquisition provides the basis for choosing Mandarin Chinese as the language for the auditory tasks in our experiments. An estimated 60-70% of world languages are tonal, meaning that in these languages, pitch contours allow differentiation between words with the same phoneme sequence but different intonation (Yip, 2002). As indicated, we have chosen to use an animal model, the ferret, in our studies. Further evidence that the ferret is a suitable model for human hearing is shown by previous work indicating that only 50 cells in A1 (primary auditory cortex) of the ferret are sufficient for encoding all English phonemes (Mesgarani et al. 2008), suggesting that ferret auditory processing provides a truly excellent model to study neural encoding of speech.

We have chosen to train the ferrets on a Mandarin Chinese phoneme discrimination task (phonemes are the perceptually distinct units of sound in a given language). In a tonal language like Chinese, depending upon the pitch contour of the sound, the Chinese word “ma” can have four different meanings. It can have the meaning of mother (ma1 – or flat contour ma), hemp (ma2 – or rising contour ma), horse (ma3 – or falling then rising ma contour), or scold (ma4 – or falling contour ma) depending on the tonal contours of the sounds (see Figure 2 for the four tonal contours of the syllable “fa” and Figure 3 for tonal contours of syllable ‘ma’).

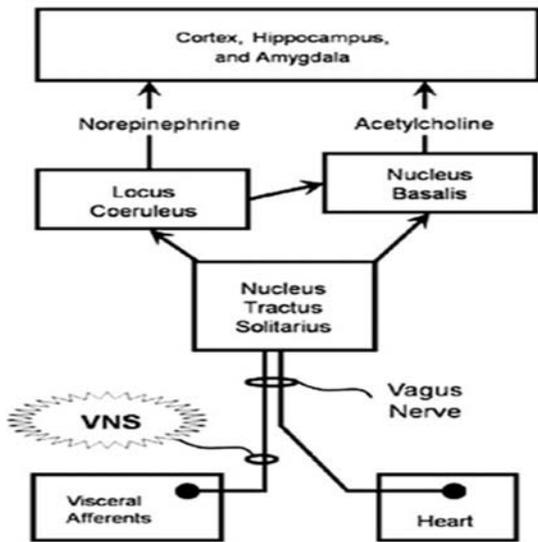


Figure 1. Vagal nerve stimulation of visceral afferents stimulates Nucleus Tractus Solitarius (NTS) which activates Locus Coeruleus (LC) and Nucleus Basalis (NB). LC releases norepinephrine and NB releases Acetylcholine to modulate brain activity. The brain regions affected include the cortex, hippocampus, and amygdala. Figure adapted from Engineer et al. (2013).

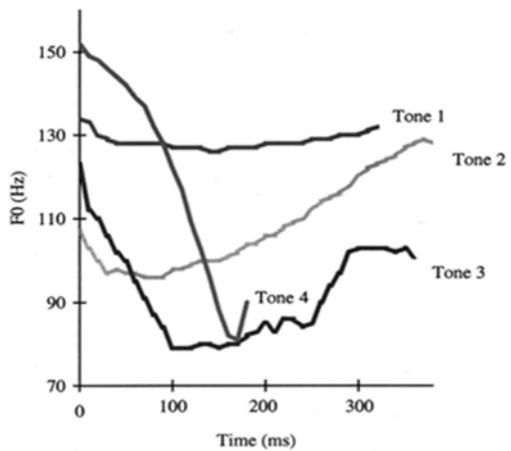


Figure 2. Mandarin syllable 'fa' tonal contours. The change in frequency of the pronunciation is indicated over time. In addition to different contours, note different starting frequencies for the different tonemes. Figure adapted from Wang (1999).

Four main tones in Chinese

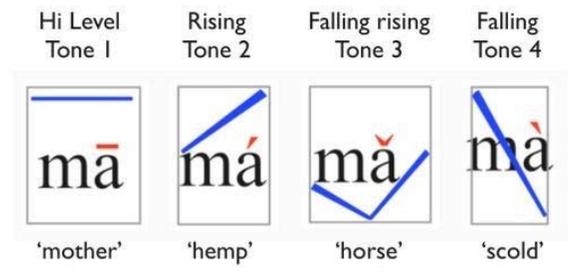


Figure 3. Mandarin syllable 'ma' tonal contours. The change in frequency of the pronunciation is indicated over time. Figure adapted from Eble (2016).

We predicted that the ferrets would be able to discriminate between FM (frequency-modulated) tonal sweeps (rising versus falling pitch) and Chinese phonemes (flat syllable vs. various contours) in light of previous experiments in which ferrets were already successfully trained to discriminate two tone contour sequences (Yin, 2014). We predicted that if the animals could detect changes in pitch in the FM direction discrimination task, then the ferrets should also be able to learn the more complex spectrotemporal Chinese phoneme discrimination task and also be able to learn this task with multiple speakers, including both male and female voices.

The initial hypotheses that we tested were that ferrets could be successfully trained to discriminate Mandarin Chinese phonemes, independent of syllable and independent of speaker voice. We also explored whether ferrets trained with a-VNS would learn significantly more quickly (on average) Chinese phoneme discrimination than ferrets trained without VNS and/or would learn the task to a higher level of performance. In order to test this hypothesis, we measured the time (in number of behavioral trials or training days) it took for the ferrets to achieve a criterion level of behavioral performance indicative of task-learning. We also monitored performance during and after task learning. The performance metric used was a measure of the discrimination rate (the product of safe rate and hit rate in the conditioned avoidance Go-NoGo behavioral paradigm (Heffner and Heffner, 1995; Fritz et al., 2003).

In addition to studying the behavioral effects of a-VNS on the ferret model for auditory

learning, we also investigated the effects of a-VNS on neural responses from extracellular recordings in the auditory cortex in ferrets. We will be used to quantify effects of a-VNS with respects to auditory learning and neuroplasticity. Training and performance on Chinese phoneme discrimination tasks will be assessed with VNS (a-VNS) or without VNS to determine if VNS can enhance behavioral performance in ferrets.

Hypothesis I: Ferrets can be trained to distinguish and behaviorally discriminate the four Mandarin tonal contours.

Hypothesis II: (A) Ferrets learning tonal contours for one Chinese Mandarin syllable will transfer (or generalize) to other Mandarin syllables with similar contours and they will learn to discriminate subsequent syllables more quickly than they learned the original syllable contours. (B) Ferrets can learn these tonal contours independent of speaker identity (for male and female speakers).

Hypothesis III: Auricular a-VNS, paired with target stimuli during task learning, will enhance the speed of behavioral learning (based on the number of trials needed to reach behavioral task criterion) and/or level of performance (based on discrimination rate (safe rate and hit rate)).

Hypothesis IV: a-VNS, paired with acoustic stimuli, will enhance auditory neural responses in A1 neurons in the ferret auditory cortex.

Material and Methods

Seven adult ferrets (spayed females, 1-3 years of age, purchased from Marshall BioResources, North Rose, New York) were trained on auditory tasks. Five ferrets were trained in the control group (no VNS) and two ferrets were trained with paired stimulation (a-VNS). The training paradigm used in the behavioral aspect of the study for all seven ferrets was based on prior experimental studies and utilized conditioned avoidance, an aversive conditioning paradigm that can be used for training animals on auditory discrimination or detection tasks (Fritz et al., 2003, 2005; Heffner and Heffner, 1995). The surgical, stimulation and behavioral protocols were reviewed and

approved by the Institutional Animal Care and Use Committee (IACUC) at University of Maryland (June 12, 2017) and appropriately met the NIH standards.

We used two setups for animal training: one in a free running arena and one using a holder that secured and stabilized the head position of head posted animals (see Fritz et al. (2003) for a description of headpost implant procedure). Training areas were placed within sound-attenuated booths IAC (Industrial Acoustics, Inc.). The sound attenuation was important to reduce ambient room noise so that the ferrets could focus on task-relevant acoustic stimuli (at 70-75 dB SPL) presented inside the training booths. In the free running condition, five of the ferrets were trained in a wire enclosure of dimensions 20 cm width, 38 cm depth, and 23 cm height in which they were free to move forward to lick the water spout and engage in the task. In contrast, in the head fixed setup, two ferrets were placed in a horizontal Lucite cylindrical restraint in which the head of the ferret was stabilized in place in a holder that secured a surgically implanted head post. This stability of the head was critical for long-term neurophysiological recordings and also for accurate pupillary measurements. In this condition, the ferret indicated behavioral choice in the Go-NoGo task by licking, or refraining from licking the spout (each tongue lick was measured when tongue protrusion broke a photobeam positioned near the spout).

In order to motivate the ferrets to perform the tasks, for which they received liquid reward, they had restricted access to water from Sunday afternoon through Friday, receiving water for correct response during five daily task training sessions per week (Monday-Friday). Hydration and weight were monitored daily to ensure animal health. On weekends, all ferrets received *ad libitum* water.

Ferrets were trained in each daily session, using the Conditioned Avoidance Go-NoGo behavioral paradigm (Heffner and Heffner, 1995; Fritz et al., 2003). In this paradigm, water flowed continuously from a water spout in the sound booth. The animals learned to lick freely during “safe” reference sounds (Go sounds which were *not* associated with shock) and learned to refrain from licking

the spout for 400 milliseconds (the post-stimulus shock period) *after* the presentation of “danger” (i.e. “warning” NoGo) target stimuli in order to avoid receiving a mild shock. Mild shock (<1 mAmp) was delivered to the tongue through the water spout for freely moving animals and to the tail for head-fixed animals. A random number of presentations of the reference sound (from 1 to 6 presentations) preceded the presentation of the target stimulus. There were catch trials (no target) in ~20% of the trials. Each correct response to a target stimulus (brief cessation of licking after the target) was recorded as a hit. In each daily session, the ferrets were able to perform as many trials as they wished until they reached liquid satiation, typically between 80-120 trials per day. If the animal did not receive adequate water from training, additional water was supplemented up to 20-25 ml/kilogram/day. Behavior was continuously monitored by the human trainer observing the ferrets via a video camera located inside the training booth. Behavioral task performance and total session water consumption was recorded with a customized MATLAB program.

The tasks the animals learned progressively increased in complexity from the simplest task (pure Tone vs broadband (5-octave) Noise discrimination), to FM (Frequency-Modulated) Sweep Direction discrimination, to the most complex task of Mandarin Chinese phoneme discrimination (see Figures 2, 3). Ferrets learned to discriminate multiple Chinese phonemes beginning with the two syllables “Ma” and “Di” (recordings and sound files generously provided by Dr. Bharath Chandrasekaran). Five control ferrets were trained without VNS on these tasks. Two ferrets were trained with a-VNS presented at the time of the target stimulus on each trial. Intensity cues for the target were given to assist task acquisition. Initially the target was amplified 20dB relative to the reference stimuli, and then progressively reduced in intensity cue to 10dB and 5dB above the reference stimuli. Eventually, the cue was no longer necessary, and animals were able to discriminate the stimuli at isointensity (when the intensity cue was 0dB, this meant that target and reference were both at equal volume). Criteria for successful task acquisition were met when at 0dB cue intensity the ferret behavioral task performance showed two or more

consecutive trial blocks with a discrimination rate (DR) of 50 or higher (i.e. if the overall DR was greater than 50 after 2 trial blocks = 20 trials). We note that DR is a product of the hit rate and the safe rate (Heffner and Heffner, 1995). Behavior was assessed through performance on the task (see Figure 4).

Experimental ferrets received a-VNS (by stimulation of the external ear concha – which is innervated by the auricular branch of the vagus). The procedure for positioning the two stimulating leads for a-VNS has been previously described (He et al., 2013). We compared behavioral acquisition and performance of the task in animals stimulated with a-VNS during learning vs control animals (receiving no VNS) in order to test whether VNS is capable of accelerating learning of a complex auditory task. Stimulation parameters for a-VNS are given in Table 1; the amplitude of current was determined each day by observing the threshold for ferret behavioral response to stimulation. We reduced the current far below the threshold (to ½ current threshold) for eliciting any visible behavioral responses such as vocalization, jaw opening, pinna movement or facial wincing. Stimulation was paired only with the target stimuli, starting 250ms before the target and lasting the full duration of the target. Test sessions with no task intensity cues or VNS stimulation were given at the end of each week for a-VNS animals in order to test whether true learning had occurred, or whether animals were using a VNS cue, rather than learning to recognize the acoustic target stimuli.

In parallel to behavioral studies, we examined the effects of a-VNS on brain activity in awake ferret A1. We recorded neural activity with extracellular tungsten electrodes (with four independently moveable Alpha Omega electrodes or using chronic Neurolynx 16-electrode arrays). The tungsten electrodes (FHC) had a resistance of 2-8 MΩ. The two ferrets undergoing a-VNS during active behavioral training had implanted chronic Neurolynx arrays, with 16 channel neuronal recordings of A1 responses acquired daily (for details of neurophysiological methods see Fritz et al. (2003). Additionally, recordings were obtained during passive recording conditions (i.e. not during behavioral training or performance) of neural responses to tones and Chinese phonemes.

These passive recordings were made under two conditions: with or without paired a-VNS stimulation (see Figures 9, 10).

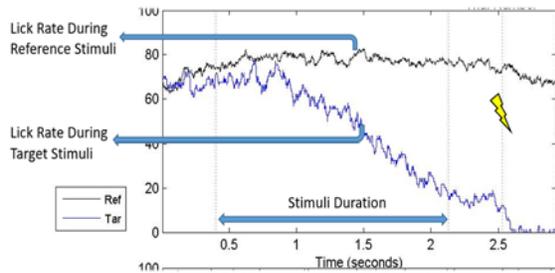


Figure 4. Sample behavioral training data. The small shock onset is indicated by the lightning bolt at 2.5 s (seconds). The safe rate, the lick rate during safe or reference sounds, is shown by the black line and remains relatively level throughout. The lick rate during target stimuli (shown by the blue line) decreases as the shock approaches, showing the animal reduces its licking when it detects the target, and pulls away from the spout.

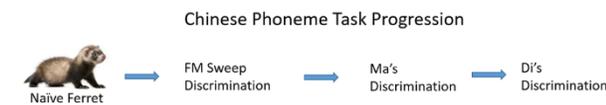


Figure 5. Chinese phoneme task sequence. “Ma” and “Di” are two distinctive Mandarin syllables, each containing four distinct tonal phonemes used in this study. Naïve ferrets are often trained first on the Noise vs Tone task before advancing to the FM (frequency modulation) Sweep Direction Discrimination task and then the Phoneme task.

Table 1. Stimulation Parameters for a-VNS

Pulse Width	100 (μs) microseconds
Frequency	30 Hz (30 stimuli delivered per second)
Amplitude of Current	250-825 μA (usually 300 to 500 microamps)

Results

We were successfully able to train ferrets to discriminate between all four tonal contours in Mandarin Chinese for two syllables. All of the ferrets were able to learn the initial Chinese phoneme discrimination task (Ma1 vs Ma3) and some learned additional syllables and speakers over an average period of 3-6 months. However, we observed considerable variability in task acquisition between ferrets. As can be seen for the five no-VNS control ferrets (Figure 6), task

acquisition and learning speed also varied upon moving from the “Ma” to the “Di” syllable tonal discrimination. There was variability between ferrets and across contours and syllables. However, preliminary behavioral results indicate that learning time on the Chinese phoneme tasks were not statistically different (as evaluated by two sample t-tests) between the a-VNS group and the (no-VNS) control group for learning discrimination of Ma1 vs Ma3 (Figure 7) and Ma1 vs Ma2 (Figure 8) although we note, on average, that the a-VNS group was slightly quicker in learning Ma1 vs Ma3 and slower in learning Ma1 vs Ma2.

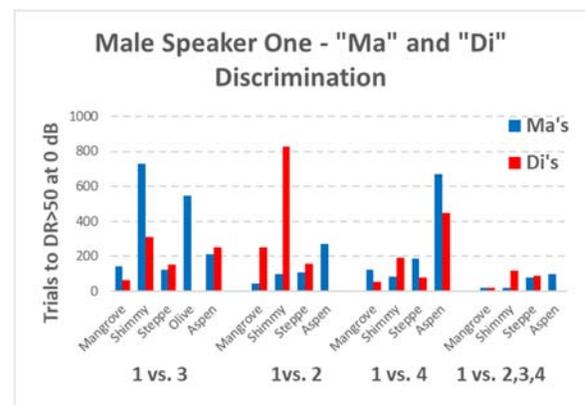


Figure 6. “Ma” phoneme discrimination task learning time (number trials to behavioral criterion) shown in blue bar plots for each animal; “Di” phoneme discrimination task learning time is given by the red bar plots. The abbreviation for each task is 1 vs. 3 (indicating Ma1 vs. Ma3 and Di1 vs. Di3) and so on. This figure demonstrates variability in learning speed for different ferrets on this task. Note: all animals in this figure were control (No-VNS) ferrets.

In contrast to the apparent lack of effect of a-VNS on behavioral learning, we observed statistically significant effects of a-VNS on neural activity in many A1 neurons (n=211). Multineuron recordings in A1 demonstrated enhancement in neural responses (measured in spikes/s) to pure tones during pairing of adjacent stimuli with a-VNS (note that in the pairing condition, 100 tones were presented, 50 of which were paired with a-VNS – the average responses shown were from the 50 non-paired stimuli to avoid stimulus artefacts). As shown in one example recording, pairing a-VNS with a 150 Hz tone lead to response enhancement at multiple time points (Figure 9). The peak response more

than tripled during the a-VNS paired stimulation condition. We have recorded 211 cells in A1 showing similar effects (Fritz et al., 2018).

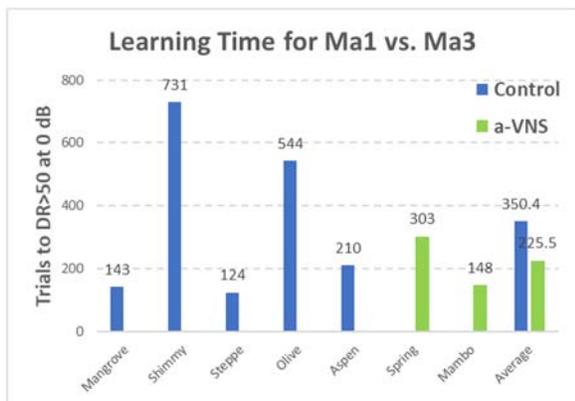


Figure 7. Trials to learn Ma1 vs. Ma3 for control ferrets (No-VNS) shown in blue, and ferrets trained with a-VNS (shown in orange). Shimmy and Mangrove (far left columns) learned a pure tone vs. Ma3 prior to Ma1 vs. Ma3, so the trials for the tone vs. Ma3 were added to their learning time in this graph, which increased their training time. Standard error is shown. We evaluated difference between learning in the two groups using a 2-sample t-test and found no significant difference ($p=0.574$ ($df=5$)).

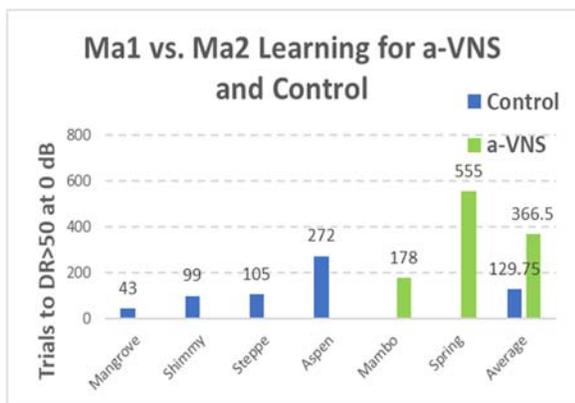


Figure 8. Learning time for Ma1 vs. Ma2 for both control ferrets (No-VNS) and ferrets trained with a-VNS. One control ferret (Olive) was excluded from this graph since she had not completed learning to discriminate Ma1 vs Ma2. No significant difference was found between the two groups (Two-sample t-test $p=0.160$ ($df=4$)).

In some neurons, pairing a-VNS with Ma3 also lead to enhancement of peak response to phonetic stimuli. The peak response to Ma3 more than tripled during a-VNS paired stimulation (Figure 10–Panel 2). Across multiple stimuli (tones and phonemes), these enhanced effects were elicited during paired stimulation.

The increased firing rate elicited by the stimulus eventually returned to the original pre-VNS level in the post-VNS period, sometimes taking half an hour to return to its original response level (Figure 9–Panel 4; Figure 10–Panel 3).

These examples reveal a profound effect of VNS on neural activity. However, in contrast, our preliminary behavioral studies indicate VNS does not enhance learning to discriminate complex speech sounds, such as Chinese phonemes.

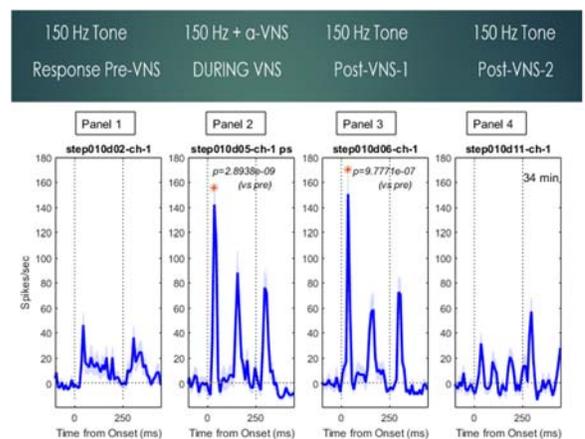


Figure 9. Post-stimulus-time histograms of a multiunit recording in A1 showing response to a 150 Hz tone. The vertical grey lines mark the onset and offset of the 250 ms tone. The four conditions shown are pre-VNS (panel 1), paired stimulation (panel 2), post-VNS-1 (panel 3), post-VNS-S-2. Each panel shows average response to 50 tone presentations. In panel 2, the paired stimulation condition (a-VNS), 100 tones were presented, of which 50 were randomly chosen to be paired with a-VNS, while the other 50 tones were not paired. This allowed measurement of the response to unpaired tones (thus avoiding the electrical stimulation artefact). Average neural response to the 50 unpaired stimuli in the context of a-VNS (panel 2) increased at peak onset and offset, as well as with emergence of a new neural response in the middle of the tone. These enhanced responses began to fade away in the two post-VNS conditions (panels 3,4). The increase in neuronal response (to the onset peak) during and after VNS is significant (Wilcoxon Rank Sum Test: $p<<0.001$).

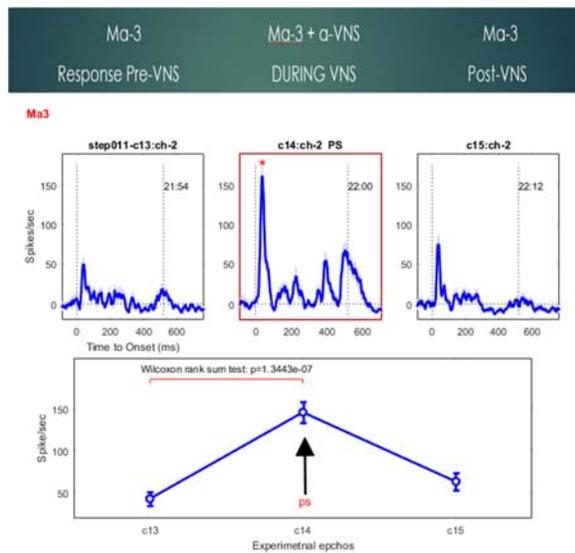


Figure 10. Post-stimulus time histograms of a multiunit recording in primary auditory cortex (A1) showing the response to the Chinese phoneme Ma3. Conditions shown (left to right) are pre-VNS, paired “during-VNS” stimulation, and post-VNS respectively. Each panel shows the response to 50 presentations of the Ma3 stimulus. In the paired condition (a-VNS) there were 100 presentations of Ma3, of which only 50 were randomly paired with a-VNS. The response to the 50 unpaired stimuli is shown in the middle panel, in which the star * indicates the peak onset response to Ma3. The change in peak response between pre-VNS and During-VNS was significant as assessed by a Wilcoxon Rank Sum Test: $p < 0.001$.

Discussion

In the behavioral experiments in this study, we successfully trained seven ferrets on a new and complex phoneme discrimination task, in which they learned to discriminate different Mandarin Chinese phonemes. This behavioral paradigm offers the opportunity to study the neural basis of phonological learning and memory. It also gives us an opportunity to test the efficacy of VNS to enhance phonological learning and/or performance in a behavioral animal model system.

Value of Animal Behavioral and Neurobiological Studies of Human Speech:

Evolutionarily, there are many parallels between animal communication and human speech, particularly when it comes to discrimination of voices of individual speakers

and discrimination of distinct phonemes (Schnupp et al., 2011). Using animals as a model for language allows thorough research at a single neuronal level that is not possible in healthy human subjects (although some language-related single unit neuronal data has recently been collected in epileptic patients implanted with ECoG) (Towle et al., 2008; Chan et al., 2014; Humphries et al., 2014). Additionally, in human studies, novices, with no prior experience of tonal languages, were trained on Mandarin Chinese phonemes successfully, with perceptual and sensory changes retained 8 weeks after training concluded (Reetzke et al., 2018), suggesting that this learning is long-lasting in humans, and likely to be long-term in trained ferrets as well.

Evaluating our Preliminary Results in Light of Our Original Hypotheses

Our preliminary results from the first year of a project exploring whether VNS affects brain activity and behavior in the ferret are not conclusive at this stage but raise important questions and point to some tentative answers to the hypotheses posed. We discuss our four hypotheses in light of our current, preliminary evidence:

Hypothesis I: Ferrets can learn to distinguish Mandarin tonal contours independent of speaker.

- Our behavioral study clearly demonstrate that ferrets can discriminate between the four different tonal contours in Mandarin Chinese phonemes for multiple syllables. This is an important result that lays the foundation for future studies of the neural basis for phonological learning of tonal languages.

Hypothesis II: (A) Ferrets learning tonal contours for one Chinese Mandarin syllable will transfer (or generalize) to other Mandarin syllables with the same four basic contours and hence ferrets will learn to discriminate subsequent syllables more readily and quickly than they learned the original syllable contours. (B) Ferrets can learn these tonal contours independent of speaker (category learning).

- Our results do not support Hypothesis II (A) because we did not observe transference of tonal phoneme

discrimination from one syllable to another (i.e. learning the four phonemes of one syllable set (Ma) did not appear to aid in subsequent learning of an additional Chinese syllable (Di)).

- However, ferrets *were* able to learn to distinguish phonemes and generalize across multiple speakers, including female speakers. This matched our predicted results from Hypothesis II (B).

Hypothesis III: a-VNS will enhance behavioral learning and performance.

- Our preliminary behavioral results do *not* support Hypothesis III, as a-VNS ferrets showed no significant difference from control (no-VNS) ferrets in learning speed or performance on Chinese phoneme discrimination tasks. However, we emphasize that these are preliminary results, and that we need to train additional ferrets with a-VNS, and obtain a larger sample size in order to fully evaluate and test this hypothesis.

Hypothesis IV: a-VNS will enhance auditory neural responses in A1 of the ferret auditory cortex.

- Our results aligned with this hypothesis: a-VNS demonstrated clear changes to many cells in A1 of ferret auditory cortex during paired stimulation with either tones or Chinese phonemes.

In summary, our experimental results demonstrate the ability of ferrets to learn to distinguish different Mandarin tonemes (Hypothesis I), independent of speaker (Hypothesis II-B). However, the ferrets do not apparently generalize their contour learning of one syllable to other syllables (Hypothesis II-A). It may be that they would need to learn multiple syllables in order to learn to generalize. We found that a-VNS does induce neural changes in ferrets (Hypothesis IV), but our preliminary data does not support the conjecture (Hypothesis III) that a-VNS would lead to enhanced learning since this was not observed in the behavioral learning data. While current results do not indicate a significant difference between learning in control (no-VNS) ferrets versus learning in a-VNS ferrets, we only

tested two ferrets with a-VNS (vs five control (no-VNS) ferrets), so our groups are not matched in size, and hence results are still tentative as to whether a-VNS can enhance learning. Additional animals need to be trained and tested in order to resolve this question.

In evaluating our preliminary behavioral results, there are many possible explanations for our failure to observe the predicted acceleration of learning (more rapid acquisition time) of complex auditory tasks for the two ferrets in the a-VNS condition.

The six most likely explanations are listed below: (1) One potential factor is that the a-VNS ferrets were all trained while being restrained in a holder and the control (no-VNS) ferrets were trained in a free moving booth. Additional experiments are needed, with non-VNS ferrets in the head-fixed holder set-up, to measure the time course for learning in the different training conditions – it may take longer for the ferrets to learn in the head-fixed condition than in the free running booth. (2) Additionally, the a-VNS ferrets had a stimulator placed unilaterally in their external ear concha, which exerted some pressure on the ear and may have been a distracting irritant for the ferrets, and may have interfered with task learning. In future experiments, we will re-design the aural stimulator to be more comfortable for the animal. (3) One other difference between the a-VNS ferrets and the non-VNS controls is that the a-VNS group was trained with a mildly aversive tail shock (leading to a tail flick) while the non-VNS control group was trained with a mild tongue shock (leading to tongue withdrawal) from the water spout.

It is unclear how influential these setup differences were in the learning experiences of the ferrets, but is something to be explored in future research. It is important to utilize similar training paradigms (with and without a-VNS) with similar reward, punishment and distractor conditions so that observed effects can be attributed to the presence or absence of VNS (which is the only variable between the two training conditions). It is also important to add another new control condition for non-VNS ferrets in which they receive an ear insert in their external ear concha, but do *not* receive VNS stimulation during training (i.e. sham ear

stimulation insert). Another control condition is to vary the timing of VNS relative to the acoustic stimuli being presented (rather than pairing simultaneously with sound). It is also crucial to test the effects of cervical cuff VNS (c-VNS) with implanted stimulating cuff electrodes positioned at the level of the cervical vagus and directly compare the effects of matched a-VNS with c-VNS.

As mentioned, our findings are preliminary for the three reasons mentioned above, and also (4) because there was a small, unmatched sample size in these preliminary behavioral studies ($n=2$ for VNS group, $n=5$ for the non-VNS control group), so that even if there were differences between groups, it would be difficult to prove statistically with such small and unmatched group sizes. Also, (5) optimal parameters for auricular a-VNS stimulation are not well defined, and there may be differences in optimal parameters for behavioral learning and for eliciting neural responses.

We note, in contrast to our preliminary results with a-VNS on an operant conditioning task, recent studies show that cervical VNS (c-VNS) can assist auditory learning in a classical conditioning paradigm in the ferret (Lai and David, 2019). Although a-VNS offers the attractive possibility of non-invasive vagal stimulation (c-VNS requires surgery to implant the cervical vagal cuff), in fact c-VNS may be advantageous for multiple reasons, including stability of the cuff, lack of irritation of the ear, and greater effect because a much higher proportion of vagal nerve fibers can be stimulated in c-VNS compared to a-VNS (as mentioned in the Introduction). Thus, another possible reason for an apparent lack of effect of a-VNS stimulation on behavioral acquisition or performance (in comparison with effects of v-VNS) may simply be a quantitative matter, since there are over 20 times more vagal nerve fibers in the cervical branch than in the auricular branch of the ferret vagus (Asala and Bower, 1986).

Variability in Individual Behavioral Learning and Acquisition of Auditory Tasks:

Our behavioral results also reveal considerable individual variation in task acquisition. For example, the ferret Aspen took the longest to learn the Chinese phoneme

discrimination task but was the fastest to learn the FM Direction task. Our results show that the learning of all these tasks is feasible, since all five non-VNS control ferrets were able to meet criterion performance on the FM Direction task as well as the Chinese phoneme task. Two ferrets have completely learned all the tasks, which include Ma's and Di's, and were able to generalize phoneme identification and discrimination across multiple male and female speakers. The variability in task acquisition in ferrets is consistent with observed variability in auditory perceptual learning in humans (Fitzgerald and Wright, 2011).

Role of Neuromodulators in Mediating VNS Effects on Cortical Plasticity

In order to better understand and study VNS and its effects on auditory task acquisition and plasticity in auditory cortex, and design future experimental research, it is valuable to summarize what is currently known about vagal nerve stimulation from clinical and animal studies, and about the types of plasticity arising from VNS or enhanced neuromodulation. There are at least three major neuromodulators (acetylcholine, norepinephrine and serotonin) that are known to play role in cortical plasticity in the cortex as well as in attention and motivation (Gu, 2012; Hays et al., 2014). The direct application of acetylcholine (ACh) to auditory cortex (paired with sound) results in long term cortical plasticity (Metherate and Weinberger, 1989; Ma and Suga, 2005; Metherate et al., 2012; Gil and Metherate, 2018). Acetylcholine acts to affect the presynaptic release of neurotransmitters, control coordination of firing in clusters of neurons, and induce synaptic plasticity (Picciotto, 2012). Additionally, acetylcholine and norepinephrine cause enhanced neurotransmitter release from the cortical synapses of afferent fibers from NB and LC (Hasselmo, 1995). Norepinephrine induces glutamate uptake and increased glycolysis and glycogenolysis by astrocyte cells in preparation for increased neural activity. In response to arousal, aversive stimuli, and reward, the locus coeruleus (LC) modulates release of norepinephrine, which increases cortical neuron activity (O'Donnell, 2012; Martins and Froemke, 2015). There is also indication that a third

neuromodulator, serotonin, may play a key role in VNS-driven neuroplasticity (Hulsey et al., 2019). The main source of brain serotonin, the DRN (dorsal raphe nucleus) is also activated by VNS. Consistent with our results showing a-VNS can drive cortical plasticity in A1, earlier studies have also shown enhanced neural responses to tones following pairing with c-VNS (Borland et al., 2019). Future neuropharmacological measurements of enhanced neuromodulator release from VNS in parallel with studies of the effects of VNS on neuronal activity and behavior, would help unify the field of VNS research and deepen understanding of its effects.

Value of Animal Behavioral and Neurobiological Studies of Human Speech

Evolutionarily, there are many parallels between animal communication and human speech, particularly when it comes to discrimination of voices of individual speakers and discrimination of distinct phonemes (Schnupp et al., 2011). Using animals as a model for language allows thorough research at a single cell level that would not be possible in human subjects (although some language-related single unit neuronal data has been collected in epileptic patients implanted with ECoG) (Towle et al., 2008; Chan et al., 2014; Humphries et al., 2014). Additionally, human Chinese speakers and novices were trained on Mandarin Chinese phonemes successfully, with perceptual and sensory changes retained 8 weeks after training concluded (Reetzke et al., 2018).

Potential Clinical Applications and Promise of VNS:

Moving towards clinical studies involving VNS, there are indications that VNS may enhance auditory learning and neuroplasticity in the auditory cortex (Lai and David, 2018). VNS may be valuable as a method to enhance plasticity to help recovery from nerve or brain damage or neurological disorders (Hays et al., 2013; Meyers et al., 2019). As an example of targeted plasticity in the auditory cortex, vagal nerve stimulation repeatedly paired with tones in rats restored normal frequency tuning in the auditory cortex and alleviated or cured behavioral signs of noise-induced tinnitus in rats (Engineer et al., 2011). Although controversial, based on

these animal studies, VNS is currently being used in clinical trials to treat tinnitus in humans (Tyler et al., 2017). The properties of VNS modulated neuromodulator release indicate the therapeutic potential by enhancing cortical plasticity in the auditory cortex and the rest of the brain.

Future Directions:

Some planned avenues for future research include expanding these complex Chinese phoneme discrimination tasks to ferrets with cervical cuff implants for delivering stimulation directly to the vagal nerve (c-VNS), given the evidence for the effectiveness of c-VNS in other studies (Lai and David, 2019), training all ferrets in the head fixed holder to minimize training differences, and testing new stimulation settings (in freely moving animals) to optimize a-VNS.

While a-VNS has a significant effect on modulating auditory cortical responses and in many A1 neurons (examples in Figures 10,11), displays striking response enhancement (Fritz et al., 2018), however, whether such neural effects translate to enhanced learning is not yet clear. Although our preliminary behavioral data does not support the hypothesis that a-VNS accelerates auditory task learning, further careful studies with a-VNS, and additional research with c-VNS needs to be done in order to fully evaluate the possible beneficial neural and behavioral potential of VNS by providing a window of plasticity that could enhance a broad range of learning and cognitive abilities.

Although there have been some claims and hopes in the human literature that VNS can enhance learning of cognitive and skilled sensorimotor tasks, as varied as sharpshooting and second language acquisition, developing a well-defined and reliable animal model of VNS is key to a deeper understanding of the effects of VNS on behavior and cognition in animals and humans.

Acknowledgements

We would like to thank Dr. Bharath Chandrasekaran of the Department of Communication Science and Disorders,

University of Pittsburgh, for generously providing sound files for the Chinese phonemes used in this study. We also thank Dr. Michael Kilgard and colleagues at the Cortical Plasticity Laboratory in the Department of Behavioral and Brain Sciences at the University of Texas at Dallas for valuable and generous advice on VNS and for permission to adapt a figure from one of their earlier papers. Funding for this study was provided as part of the Targeted Neuroplasticity Training project by the Defense Advanced Research Projects Agency (DARPA).

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