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Characterizing and Investigating the Electrophysiological Properties of the Plastic Cricket Auditory System in Response to Cooling

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An Honors Project for the Program of Neuroscience

By Hannah Tess Scotch

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ABSTRACT

The auditory system of the Mediterranean field cricket (*Gryllus bimaculatus*) is capable of profound compensatory plasticity. Following deafferentation due to the loss of an auditory organ, the dendrites of intermediate auditory neuron Ascending Neuron 2 (AN-2) grow across the midline and functionally connect to contralateral afferents. The loss of the auditory organ can be mimicked with reversible cold-deactivation, in which cooled Peltier elements silence the auditory organ and its afferents. Though this would presumably prevent AN-2 from firing, cooling instead induces a novel firing pattern called DOPE (delayed-onset, prolonged-excitation). In this study, intracellular physiological recordings were completed before, during, and after cooling in response to "chirp" and "pulse" sounds. Analysis was performed within and across crickets to characterize DOPE. Results revealed expected variability across individuals, as well as a wider spread of onset delay and a decrease in spike frequency and number of spikes per burst relative to baseline within individuals during cooling. Generally, subsequent warming only partially restored the neuronal responses to baseline as measured by all three parameters. This was particularly true in response to "pulse" stimuli. Future experiments will investigate if DOPE is caused by synaptic inputs or intrinsic properties of AN-2, as well as the role of inhibition in the circuit. Eventually, we hope to develop a complete model of the auditory circuit for future investigations of plasticity, with ramifications for treating human neuronal injury.

INTRODUCTION

Neuroplasticity has become an extensively studied area of neuroscience in recent decades, and although we have learned an enormous amount about the nervous systems of diverse creatures and how they can change in response to stimulus and injury, many details about the mechanisms driving such changes remain unknown. In humans, this is an issue of great importance, as the adult central nervous system is essentially not plastic, at least not on a large, morphological scale, and especially not in response to injuries. Electrophysiology provides an interesting avenue to study neural plasticity, as it allows us to determine how specifically a manipulation has affected a given circuit. In turn, this will allow us to explore that aspect of the circuit in greater detail to obtain a better sense of how these changes occur.

The G. bimaculatus Auditory System

An important strategy in furthering our understanding of compensatory plasticity in human injury scenarios, and doing so through the lens of electrophysiology, is to look to other species that are capable of profound change in response to injury. One such species is *Gryllus bimaculatus*, the Mediterranean field cricket. When these animals lose an auditory organ (a fairly common phenomenon given its placement on the animal's foreleg), the system undergoes dramatic reorganization, presumably to account for the loss of the auditory organ (Nolen and Hoy, 1984; Horch et al., 2017). Following deafferentation due to the auditory organ's removal, dendrites from the auditory neuron Ascending Neuron 2 (AN-2) sprout across the midline, a boundary they normally respect, and become connected to afferents from the contralateral auditory organ (Nolen and Hoy, 1984; Horch et al., 2017). This reorganization, which occurs in the animal's prothoracic ganglion (PTG), allows the cell to once again maximally respond to high frequency sounds produced by predatory bats (~18 kHz) and is presumably integral to the

cricket's continued mating and survival (Wohlers and Huber, 1982; Nolen and Hoy, 1984; Horch et al., 2017). Despite the obvious importance of AN-2's ability to compensate dramatically after injury, little is known about the characteristics of the electrophysiological changes of AN-2 and the mechanism that drives them following deafferentation. According to our current (and perhaps incomplete) understanding of the circuitry of the auditory system within the PTG, each side of the midline is a mirror image of the other, which is critical in allowing the cricket to localize sound (Wohlers and Huber, 1982). On each side, there are two ascending auditory neurons (AN-1 and AN-2) that project anteriorly to the brain (Wohlers and Huber, 1982; Pollack and Hedwig, 2017). AN-1 is tuned so that it responds maximally to the low frequency (~4.5 kHz) sounds produced by other crickets, and AN-2 is tuned so that it responds maximally to the high frequency (~18 kHz) sounds produced by predatory bats (Wohlers and Huber, 1982; Nolen and Hoy, 1984). An interneuron (Omega Neuron 1, or ON-1) on each side of the PTG, responds indiscriminately to low and high frequency sounds and provides inhibition to both of the relevant neurons on the other side of the midline, as well as reciprocal inhibition to its mirror image pair (Fig. 1) (Wohlers and Huber, 1982; Pollack and Hedwig, 2017). The specific inhibition from the ON-1 neurons helps bring about elevated bilateral contrast of auditory stimuli, aids in sound localization, helps with the recognition of auditory patterns, and contributes to the recognition of species-specific auditory stimuli (Wohlers and Huber, 1982; Zhang and Hedwig, 2019).

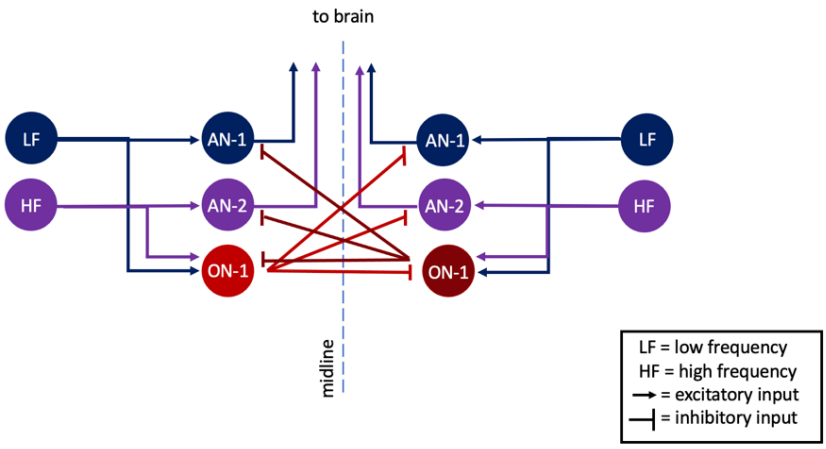

Figure 1: The current model of the circuitry of the cricket auditory system within the PTG, depicting the high frequency-tuned AN-2, low frequency-tuned AN-1, and broadly tuned ON-1. This model depicts the system prior to deafferentation (adapted from Pollack and Hedwig, 2017).

Cold-deactivation and Anomalous Intracellular Responses

Though this simple model is appealing, past research has indicated that there is much that such a model does not account for. Primarily, intracellular recordings show that AN-1 and AN-2 both still respond to frequencies outside of their maximal range, and there is notable variability in the strength and characteristics of such responses, even in the same neuron (Wohlers and Huber, 1982). More recently, research involving the honing of a reversible technique for deafferentation using cooled Peltier elements and a method of recording AN-2 intracellularly in the PTG has contributed to this complication (Zhang and Hedwig, 2019; Brill-Weil, 2020). Given that recordings of AN-2 are generally performed extracellularly, which provides less detail than intracellular recordings, this intracellular technique is hugely impactful (Kostarakos and Hedwig, 2017). Additionally, reversible cooling decouples the sides of the auditory system (by functionally removing half of the circuit at a time) and reveals which changes are impermanent (as those that are permanent will be reversed by warming), providing a clearer understanding of how the system's physiology changes after injury. Unfortunately, there is still a dearth of data, which is made more important by the fact that Brill-Weil's single intracellular

recording confirmed that an unexpected, novel response he had previously observed extracellularly was a legitimate facet of the system and not noise (Brill-Weil, 2020).

The observed response was characterized by a delayed-onset and prolonged excitation, leading him to refer to it as DOPE (Brill-Weil, 2020). The presence of this response, even if only observed in a single intracellular recording, is surprising and warrants further research. Given that AN-2 in the deafferented (or cold-deactivated) condition receives input solely from contralateral auditory afferents and does not begin to show the impacts of the formation of new synaptic connections until 4-6 days after deafferentation (Brodfuehrer and Hoy, 1988), one would expect that cold-deactivation would immediately result in a silencing of AN-2. The fact that the DOPE response was found instead indicates that this circuit, as well as the plasticity seen therein, is potentially more complex than previously imagined.

Hypotheses about the Origin of DOPE

Given that ipsilateral cold-deactivation silences all but contralateral inputs that inhibit AN-2, it is possible that DOPE is the result of post-inhibitory rebound (PIR), in which sustained hyperpolarization of a neuron results in its immediate firing following the termination of the hyperpolarization. There is evidence that such a mechanism is, at least in part, responsible for maintaining the strict temporal regulation of auditory inputs in other organisms (Large and Crawford, 2002), so its involvement in the cricket auditory system is reasonable. Additionally, other examples of PIR have been recorded in the cricket nervous system, specifically as driven by the H current in the Giant Interneurons in the terminal ganglia (Kloppenburg and Hörner, 1998). However, such a phenomenon has not been explored or characterized in the auditory system, and its possible presence warrants further analysis.

Behavioral Correlates: Negative Phonotaxis

While searching for prey during nocturnal flight, bats emit high frequency ultrasonic pulses, and the ability of prey such as crickets and moths to hear and respond to these pulses is integral in securing their continual survival (Roeder, 1962; The Earl of Cranbook et al., 1965; Simmons et al., 1975; Sansom et al., 2009). The fact that AN-2 is attuned to respond maximally to these high frequency stimuli means that it contributes dramatically to the behavioral response that allows for such avoidance, which is called negative phonotacic behavior, or negative phonotaxis. In this behavior, the cricket, upon hearing the sound, turns its abdomen away from the source of the sound, thereby creating drag and changing its flight pattern away from the predator (Moiseff et al., 1978; Huber et al., 1989). The success of this behavior is contingent upon the cricket's ability to distinguish between the timing, frequencies, and patterns of different sounds, as these factors provide information as to the location of the predator (Moiseff et al., 1978). Given the specific frequency-sensing capabilities of AN-2 and the fact that contralateral inhibition from ON-1 contributes to sound localization, it is clear that the auditory system in the PTG is integral in this process. Additionally, because the behavior is both so simple and necessary for survival, it should match the physiological responses in the cell very well. If there were many steps in between the sensing of the sound by AN-2 and the cricket's response to it, the likelihood of survival would be decreased dramatically, so it seems unlikely that this would be the case.

In this way, we can use behavior as a correlate of physiology. If, for instance, a cricket responds well (quickly, effectively, and robustly) to sound, it would make sense that the response of AN-2 to sound stimuli would also be quick and robust. Correlational experiments between the two will not only provide a better sense of the role of AN-2 on a behavioral scale, but they will

also provide insight into the efficacy of correlational experiments generally. In a system as manipulable as the cricket, having the ability to make measurements on multiple different scales can generate deeper knowledge about the function of individual variability and the ways in which systems within a single organism work together to create more complex behaviors.

Study Overview

Ultimately, AN-2 is an integral player in the overall compensatory plasticity of the auditory system; however, the characteristics of its output and its role in the larger auditory circuit remain poorly understood. As such, in order to fully understand this plasticity, it is first important that we have a deeper understanding of the auditory circuit and of the characteristics of AN-2 itself. A main goal in this endeavor is determining whether the DOPE response is in fact a form of PIR and determining exactly what drives it.

However, prior to determining the specific factors that contribute to such aberrant responses as DOPE, it is important to first characterize AN-2 under normal circumstances. This I worked to do through the implementation of field potential and intracellular recordings of AN-2 in the PTG of control and cold-deactivated crickets. With these experiments, I characterized the delay time, spike number, and spike frequency of AN-2 in response to different sound stimuli, as well as how these parameters differ after cold-deactivation. I also performed correlational behavioral experiments that aimed to explore the relationship between physiological responses to high frequency sound and behavioral responses to the same aversive stimuli, with the goal of better understanding individual variability.

METHODS

Animals

A colony of Mediterranean field crickets (*Gryllus bimaculatus*) was housed at Bowdoin College with 60-70% humidity at 26° C on a 12:12 hour light/dark cycle. Animals were fed cat chow and drinking water *ad libitum* (Horch et al., 2009). Both male and female crickets in their final molt were used in this study. Unless otherwise noted, crickets were isolated during the large wing-bud stage and were provided individual shelter, food, and water. They were monitored for growth, and 5-7 days after their final molt, they were used for experiments. The sex and morph type (black or brown) was noted for each insect.

Auditory Stimuli

White noise, alternating "chirp" and "pulse," and localizing stimuli were made using Audacity. The white noise stimulus consisted of repeating 200 msec long bursts of white noise separated by 1 sec. The localizing stimulus was a series of four 100 msec long tones separated by 250 msec of silence and followed by 100 msec of white noise. The tones increased in frequency over time, going from 5 kHz to 20 kHz and increasing by 5 kHz with each tone.

The combined chirp and pulse stimulus was made up of a combination of two distinct tonal stimuli. The pulse stimulus was a 200 msec-long tone, and the chirp was a series of four syllables, each 20 msec in duration and separated by 20 msec. The stimulus was presented at either 20 kHz (the frequency to which AN-2 maximally responds) or ~4.5 kHz. Although this lower frequency is below the optimal range for AN-2, it was used because it has previously been found to elicit DOPE better than other frequencies (Brill-Weil, 2020).

In physiological experiments, the chirp and pulse tones alternated throughout the duration of the recording, separated by 250 msec of silence each time (Fig. 2). The chirp stimulus was

meant to mimic a natural stimulus, whereas the pulse stimulus, with no silence, served as a stronger stimulus, aimed at amplifying the response of AN-2.

In behavioral experiments, stimulus presentation varied throughout the experiment both in terms of type of stimulus and direction of stimulus presentation, as there was a speaker located on each side of the cricket. The pulse stimulus was first presented in isolation (with 250 msec of silence between each pulse) from the left speaker for 3 minutes, followed by the same presentation 15 seconds later from the right speaker. After 15 seconds of silence, the same pattern was then repeated with the chirp/pulse combined stimulus, beginning with the left speaker. In both types of experiments, the stimuli were played from a speaker placed roughly 6 inches away from the animal, as in previous research (Brill-Weil, 2020).

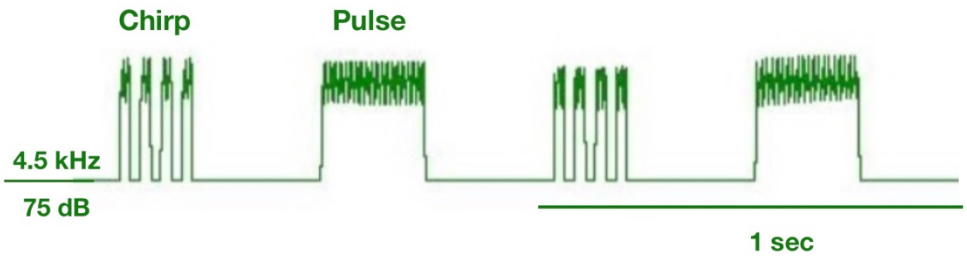

Figure 2: The "chirp" and "pulse" stimuli. The chirp stimulus consisted of four 20 msec syllables separated by 20 msec of silence. Each pulse stimulus was a single 200 msec-long tone. The silence between stimuli lasted for 250 msec, and the stimuli were presented at either ~ 4.5 kHz or 20 kHz.

Behavioral Experiments

5-7 days after the insect's final molt, each cricket underwent behavioral experiments ("flying"). In these experiments, the cricket was briefly anesthetized via cooling, the tips of the wings were clipped to expose the abdomen, and the thorax was then waxed, using a mixture of cello rosin and beeswax in a 50:50 ratio, to a plastic screw in the testing box. A standard house fan was placed opposite a hole in the back of the box covered by a plastic grate, so that a wind stream of about 1.4-1.6m/s was produced when the fan was active. Two speakers were mounted

on either side of the cricket at a distance of 11 inches to produce binaural stimuli. Crickets were observed to see when they assumed the flight position, with the hind legs extended and the two anterior sets of wings tucked close to the abdomen. Once this position had been assumed, a video recording was begun using a Panasonic HC-V700 video camera inside the box, and the sound file was played. Following the completion of the sound file, the video was stopped and saved for analysis. The cricket was then returned to its isolated container for later physiological testing.

Behavioral Data Analysis

Initially, the accuracy of the cricket's turning was measured. It was defined as whether the cricket correctly turned away from the stimulus at the beginning of the stimulus presentation (since the 20kHz stimulus should result in negative phonotaxis). Those crickets who turned in the appropriate direction were denoted with a "+1," those that did not respond were given a "0," and those that turned towards the sound instead of away were given a "-1." The average score across all four stimuli was then calculated for each cricket. Additionally, the latency to turn was measured, since this quality translates most directly to the physiological DOPE response. This was defined as the amount of time (in ms) between the onset of the stimulus and the initiation of the turn. Latency was calculated by counting the number of frames between the onset of the sound stimulus and the beginning of the turn, with the knowledge that the video was filmed at 64 frames per second.

Turning accuracy was measured once for each of the four stimuli, at the beginning of the stimulus presentation. Measurements of turning latency were made in response to the first stimulus, a stimulus in the middle of the presentation, and a stimulus at the end of the presentation in order to investigate the potential presence of habituation. As with the other parameters, these measurements were taken for both the chirp and pulse and pulse only stimuli.

Prothoracic Ganglion Exposure

No more than 24 hours after behavioral testing, crickets were removed from their cages and placed on ice for 1 hour for anesthetic purposes. The animals were then mounted ventral-side-up on a clay surface attached to a ball-joint platform. Cut paper clips and staples were used to secure the neck, the thorax just above the middle set of legs, the posterior end of the abdomen, the middle and hind limbs at the second joint, and the forelimbs at the shoulder joint. The forelimbs were then waxed (with the same wax as in the behavioral experiments) to Peltier elements at a 45° angle on either side of the insect (Zhang and Hedwig, 2019). Special care was taken to ensure that the tympanic membranes were not covered or harmed during this process. A small pair of spring scissors was then used to cut open the thorax and expose the PTG (Fig. 3). Dental foam and Kimwipes were used to remove excess fat from the area. A small spatula, to which the silver reference electrode was secured, was then maneuvered between the two posterior connectives and underneath the PTG; the spatula was ultimately used to elevate the PTG without exerting excess pressure on the connectives. The reference electrode was placed inside the cavity of the thorax, and a Vaseline well was built around the perimeter of the cavity and filled with saline.

AN-2 Intracellular Recording

A micropipette puller (Sutter Instruments P-97) was used to pull microelectrodes that were filled with Leech Cytoplasmic Fill (in mM: 7.6 NaCl, 1.4 Na_2SO_4, 10.0 HEPES, 112.0 potassium gluconate, 0.2 $MgCl_2$) to obtain a resistance of 30-50MΩ, as in previous research (Hooper et al., 2015; Zhang and Hedwig, 2019; Brill-Weil, 2020). The electrodes were lowered into the PTG, which was stimulated externally with pulses of white noise. Once the microelectrode had entered AN-2's main dendrite or was directly above it, as indicated by a

response to the sound stimulus, the chirp and pulse stimulus was played (Fig. 3) (Brill-Weil, 2020). Recordings were amplified with a computer-controlled microelectrode amplifier (Axoclamp 900A, Axon Instruments, Sunnyvale, CA), and data were collected with a CED 1401 data acquisition device (Micro1401 mkII, Cambridge Electronic Design, Cambridge, UK) controlled by Spike2 (Version 7) at a 20kHz sampling rate.

Cold-Deactivation

After AN-2 had been located and the electrode was placed inside the cell, baseline (precooled) recordings were collected for 30 minutes at room temperature. At this point, the TackLife MCD01 power supplier connected to the Peltier element ipsilateral to the recorded AN-2 was turned on. This supplied 2.3 A of current to the Peltier element, cooling it (Fig. 3). Thermistors were used to simultaneously monitor the surface temperature of both Peltier elements as they were gradually cooled to 7.2° C, consistent with previous research (Brill-Weil, 2020). The temperature of the surface of the Peltier elements during the cooling process was measured using single-channel temperature controllers from Warner Instruments which were attached to the thermistors. Once this temperature had been reached, the recording was held for 30 minutes (fully cooled), after which power was gradually reduced until the Peltier element reached its initial temperature (this was once again room temperature). A final recording was taken for 30 minutes at this temperature (postcool).

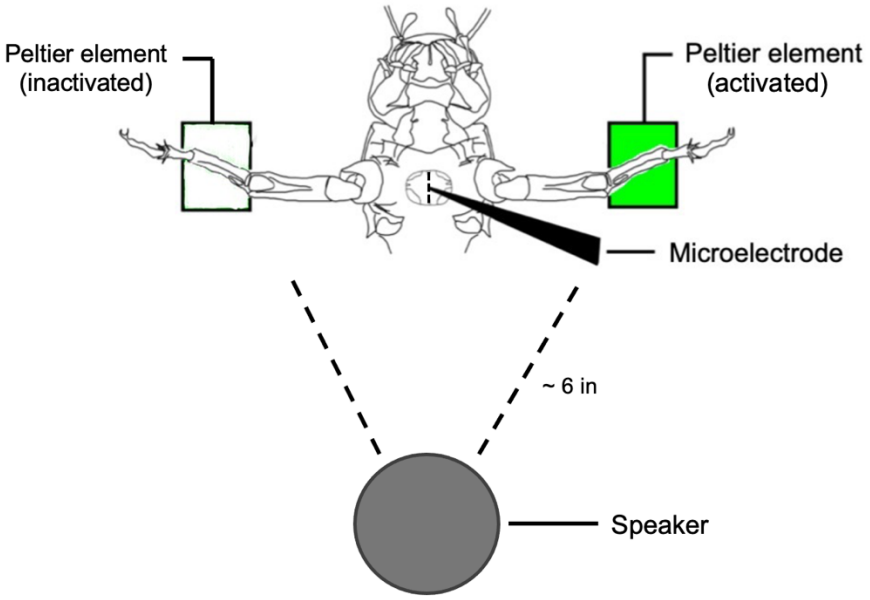

Figure 3: A schematic detailing the experimental setup. The cricket was placed on a clay surface ~ 6 in away from the speaker. Following exposure of the PTG and isolation of AN-2 via a microelectrode, the Peltier element ipsilateral to the AN-2 that was being recorded was activated. Figure adapted from Zhang and Hedwig, 2019

Data Analysis

All recordings were analyzed post hoc in Spike2 using scripts designed by Dirk Bucher. The "mkbursts" script was used separately on both the recording of AN-2 and the recording of the sound stimulus to differentiate each spiking group (aligned with a sound stimulus) as its own burst and each chirp sequence (not syllable) or pulse as a distinct burst. The "phaseburst" script was subsequently used to compare the bursts of sound to the spike bursts.

The analysis of the successful recordings explored three parameters: delay time, number of spikes, and spike frequency (which was defined as burst duration divided by number of spikes) (Fig. 4). Given that DOPE is characterized by an increase in the time between the stimulus and the initiation of AN-2's response, delay time was the primary focus of this analysis. However, such a response would also presumably bring about a related decrease in both number

of spikes and spike frequency relative to baseline values. These trends were expected to be consistent both within and across individuals, with some variability across crickets.

The data resulting from analysis of these three parameters was imported into Excel, and the chirp and pulse files were differentiated and analyzed separately. The average spike frequency, number of spikes, and delay of spiking onset was calculated for each burst of the AN-2 recording, as were the standard error values for each parameter. Since the chirps were analyzed holistically (as opposed to measuring each syllable individually), the measures of spike frequency collected here are not true measures of the frequency of firing for a given period of time. Instead, this value is artificially deflated by the presence of silence in the stimulus. However, this measure is a relative value that is useful for comparisons of responses to the same stimulus across conditions.

GraphPad Prism (Version 9) was used to depict these results, and the software was also used for statistical analysis. For the across-cricket analysis, a one-way ANOVA was performed on each parameter, so that the average from each condition (precool, cool, postcool) was compared with the average of every other condition for each parameter (delay time, number of spikes, and spike frequency).

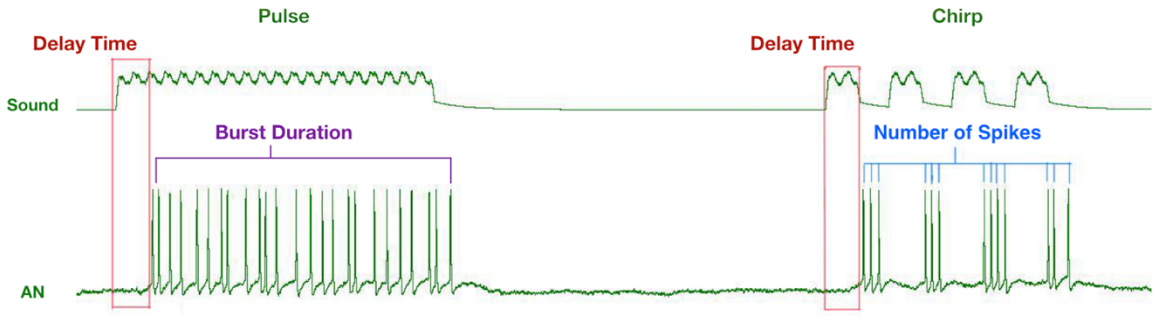

Figure 4: Illustration of the parameters that were measured in the analysis of recording from AN-2. All three parameters were measured in response to both chirp and pulse stimuli prior to, during, and after cooling. Delay time (in red) was a measure of the time difference between the beginning of the sound stimulus and the beginning of AN-2 firing. Number of spikes (in blue) counted the number of action potentials in each burst of AN-2 firing (with each burst being defined as the firing associated with a sound stimulus), and spike frequency, which is burst duration (in purple) divided by number of spikes, measured the distance between the action potentials. Spike frequency was averaged across the entire stimulus, and each entire chirp was considered a single stimulus (rather than each syllable).

RESULTS

Physiological Results: Cooling Efficacy

The response of ten AN-2's to the chirp and pulse stimuli were analyzed; four of these were subjected to the ipsilateral cooling protocol. In all of these cases, the firing paired well to the sound stimuli in normal conditions (Fig. 5) In four cases, cooling was effective, and the neuronal response to the sound stimuli did not change dramatically as the temperature of the Peltier element dropped (Fig. 6). Unexpectedly, in the remaining six cases, cooling abolished the sound-based response entirely, and it was not recoverable after warming. Though it was initially unclear why this occurred, evidence from recordings support the conclusion that the cooling itself caused the cell to die. This may either be because the experience of cooling caused the cricket to move slightly, changing the location of the electrode, and possibly killing AN-2, or that the cooling itself resulted in a contraction of the muscles that similarly changed the location of the electrode and killed AN-2. Regardless of exactly why it occurred, the movement of the electrode seems to have punctured the cell membrane, resulting in an injury discharge (as

characterized by frequent, random firing). This was clearly seen in one recording, where such discharge began just after cooling was initiated and eventually became less and less frequent until the cell stopped firing entirely and did not return, indicating that it had died (Fig. 7).

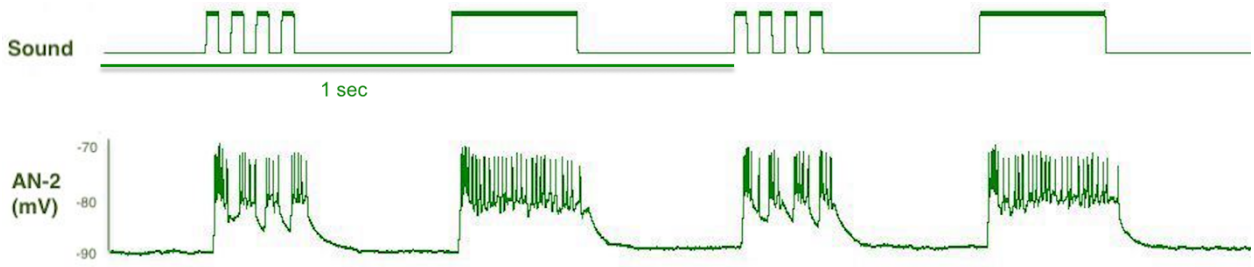

Figure 5: A characteristic intracellular response of AN-2 to chirp and pulse stimuli prior to cooling. The firing (at bottom) is clearly well-timed to the sound stimulus (at top), and the depolarizations that underlie the firing are large. This cell is notably hyperpolarized, but its function is not changed by this fact.

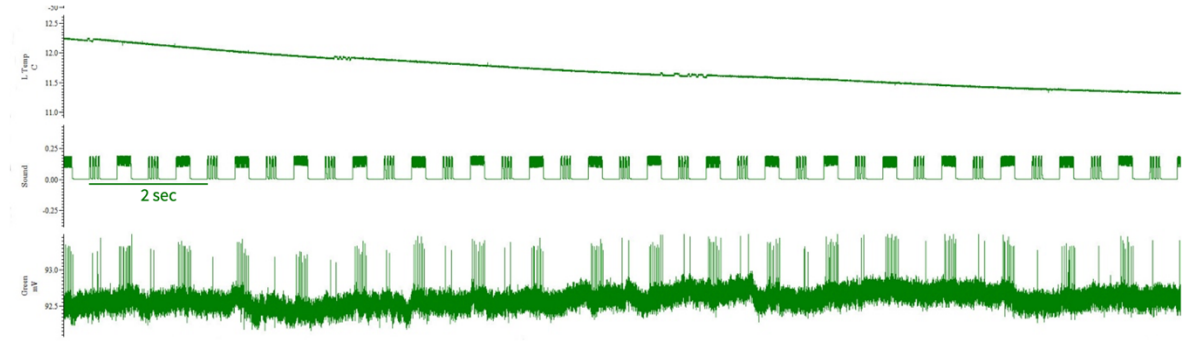

Figure 6: A characteristic intracellular response of AN-2 to the chirp and pulse stimuli during cooling. Here, it is clear that as the temperature of the Peltier element (shown in °C in the top panel) decreases, the firing of AN-2 (in the bottom panel) remains well paired to the sound stimulus (in the middle panel), and no injury discharge occurs.

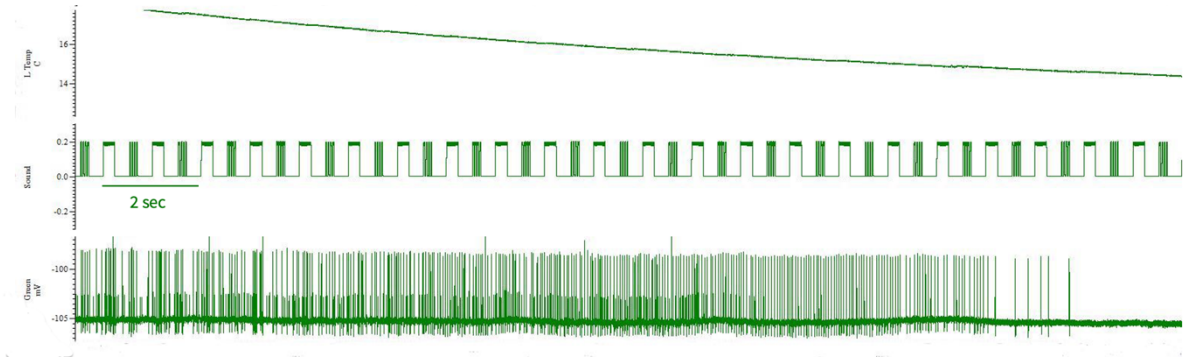

Figure 7: Injury discharge in response to the initiation of gradual cooling, when no such discharge was present prior to cooling. This eventually results in cell death (as shown through the lack of firing as time progresses). The temperature of the Peltier element (in °C) is shown in the top panel, the sound stimulus in the middle, and the firing of the cell in the bottom panel. The normal response for this specific cell is depicted in Figure 5.

Physiological Results: Across-cricket Analysis

Due to the dramatic variability in response to sound, the across-cricket analysis revealed few notable trends. It was expected that cooling would increase the delay time but would decrease the number of spikes and spike frequency. After the removal of an outlier, it was revealed that this was in fact the case, as cooling slightly increased the average delay time from 0.023 seconds (n=9) to 0.025 seconds (n=4) in response to chirp and pulse stimuli combined. Subsequent warming in the same animals had little effect on restoring this change, with the average remaining at 0.025 seconds (n=4) (Fig. 8). However, this effect was not significant (p=0.934). When the stimuli were separated out, similar trends were observed. In response to chirps alone, cooling increased the average delay time from 0.024 seconds (n=9) to 0.025 seconds (n=4), and subsequent warming brought the average delay time to 0.027 seconds (n=4), which was also not significant (p=0.921) (Fig. 8). In response to pulses alone, the average delay time prior to cooling was 0.022 seconds (n=9), the average when cooled was 0.024 seconds (n=4), and the average after warming was 0.024 seconds (n=4), which was not a significant change (p=0.870) (Fig. 8). For spike frequency and number of spikes, changes across conditions were also very minor. In all conditions, spike number and frequency were higher in response to

pulses than chirps, which was expected because of the fact that each chirp includes silence (Fig. 9, 10). With regard to spike number, cooling decreased the average number of spikes from 8.776 (n=10) to 7.572 (n=4) in response to chirps and from 17.09 (n=10) to 15.27 (n=4) in response to pulses. Here, warming had little effect on the average spike number in response to chirps, with the average number of spikes increasing slightly to 7.842 (n=4). However, warming once again brought the average spike number in response to pulses to a higher level than what was found at baseline, with an average of 18.09 spikes per burst in the post-cool condition (n=4) (Fig. 9) However, none of these changes reached significance (for chips p=0.906, for pulses p=0.932). Cooling similarly decreased the average spike frequency from 55.68 (n=10) to 51.04 (n=4) in response to chirps and from 84.97 (n=10) to 76.38 (n=4) in response to pulses. Warming changed the average frequency to 54.23 (n=4) and 90.64 (n=4), respectively, indicating a restoration of initial levels in response to both stimuli, and an over-restoration in response to pulses (Fig. 10). Again, none of these changes were significant (for chirps p=0.930, for pulses p=0.930).

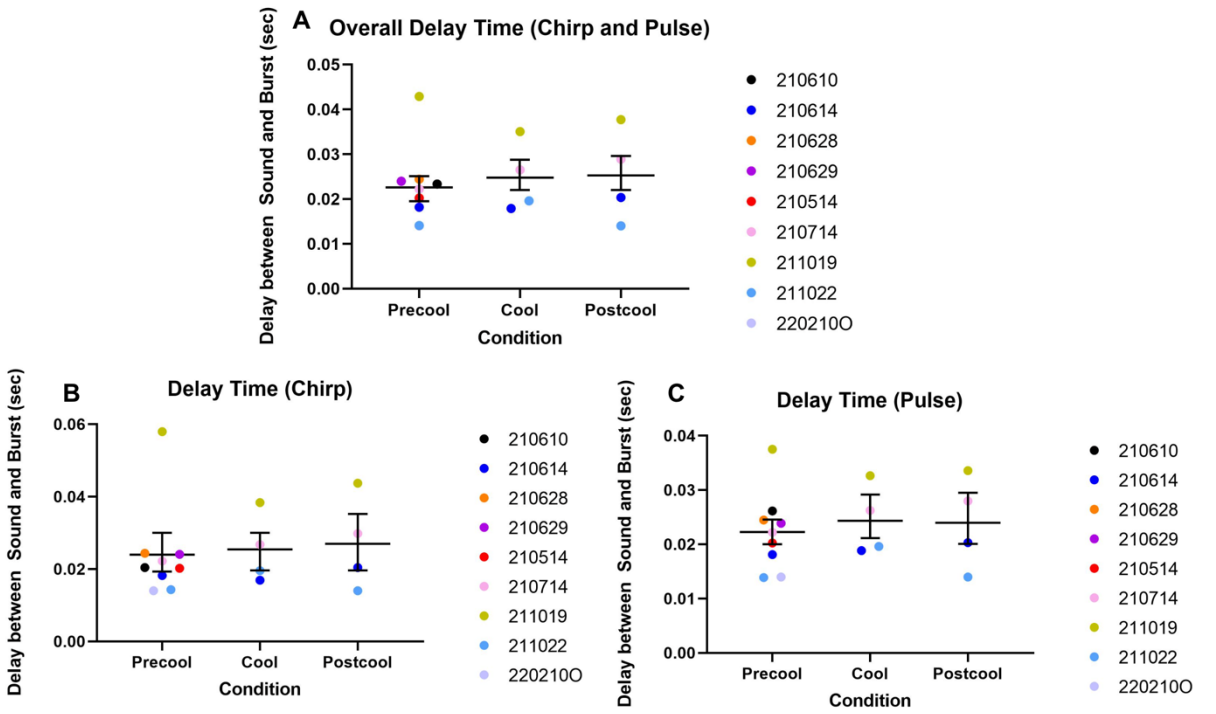

Figure 8: Comparison across crickets of the average delay time between stimulus presentation and firing onset for the combined stimuli (A), chirps (B), and pulses (C) prior to (n=9), during (n=4), and after (n=4) cooling. In all measures, the large standard error bars indicate a large amount of variability in response to sound, as is expected given previous research in the Horch Lab indicating great variability in the responses of individual crickets to sound. This makes it difficult to compare results across crickets. (A/B) On average, cooling increased the delay time in response to the combined stimuli and the chirps, neither of which were restored following warming. (C) In response to the pulse stimuli, cooling increased the delay time, and warming partially restored this result after warming. None of these changes were significant.

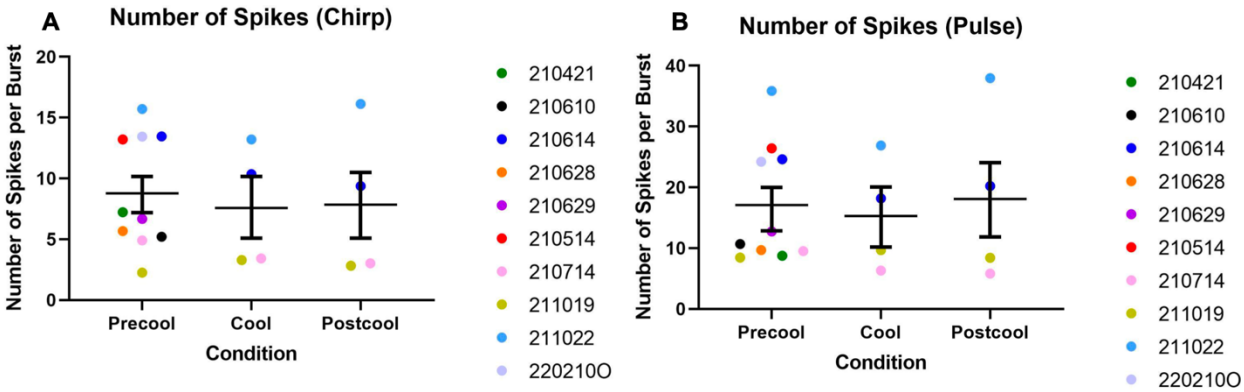

Figure 9: Comparison across crickets of the average number of spikes per burst in response to the chirp (A) and pulse (B) stimuli prior to (n=10), during (n=4), and after (n=4) cooling. In both cases, cooling decreased the average number of spikes. (A) In response to chirps, warming did little to reverse the effects of cooling, but in response to pulses (B), the trend reversed. There were no significant differences across any of the conditions.

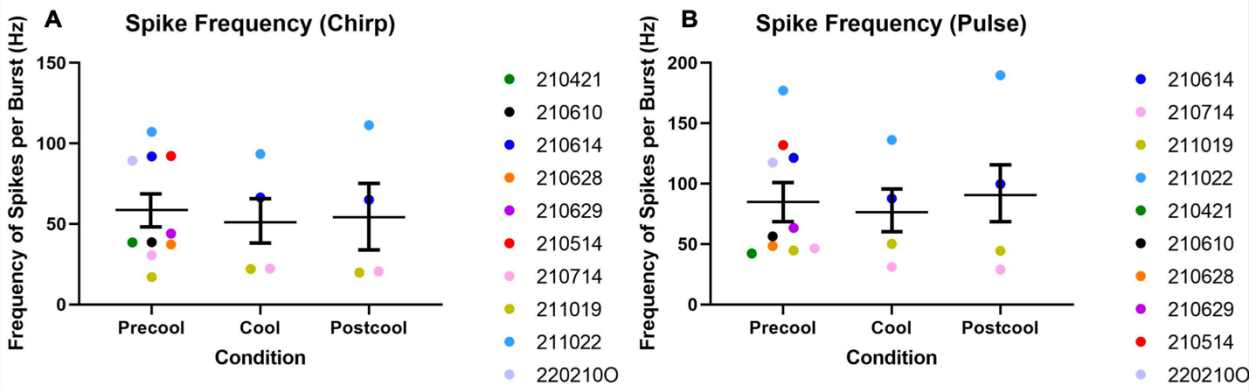

Figure 10: Comparison across crickets of the average spike frequency per burst in response to the chirp (A) and pulse (B) stimuli prior to (n=10), during (n=4), and after (n=4) cooling. In both cases, cooling decreased the average spike frequency, and subsequent warming restored the results to their initial values, though no changes were significant.

Physiological Results: Within-cricket Analysis

The within-individual analysis provided a much clearer picture of the ways in which cold-deactivation impacts the response of AN-2 to high frequency sound, though the results are still mixed. In four female crickets, regardless of baseline levels, the effects of cold-deactivation across all three parameters were highly variable. In two individuals, there was a pronounced difference, with cooling noticeably increasing delay time and decreasing both spike frequency and number of spikes (Fig. 11, 13). In one individual, cooling resulted in differential regulation of the three parameters (Fig. 12), and in one, cooling had a minimal effect across every measure (Fig. 14). Specifically with regard to delay time, the main parameter of interest for this study, cooling most often seemed to increase variability in the amount of time it took for the neuron to respond to the sound stimulus (Fig. 12, 13), but this was not always the case. In one individual, the effect on delay time was negligible (Fig. 14), but in another, it clearly increased the amount of time between stimulus presentation and neuronal response (Fig. 11).

The results are further complicated when observing the "postcool" condition. Given that cold-deactivation is regarded as reversible, it was expected that subsequent warming would

always result in a return to baseline levels in every parameter and in response to both stimuli types (Zhang and Hedwig, 2019). However, warming also had mixed results. In most instances, warming resulted in a partial restoration to baseline levels (Fig. 11, 12, 13), but this result was generally confined to the pulse stimulus, while responses to the chirp stimulus remained at cooled levels (Fig. 12, 13). That being said, there was additional variability in this regard. In one individual, warming had essentially no effect on any parameter (Fig. 14), but in another, warming restored all parameters to baseline levels, or even more beyond these levels (Fig. 11).

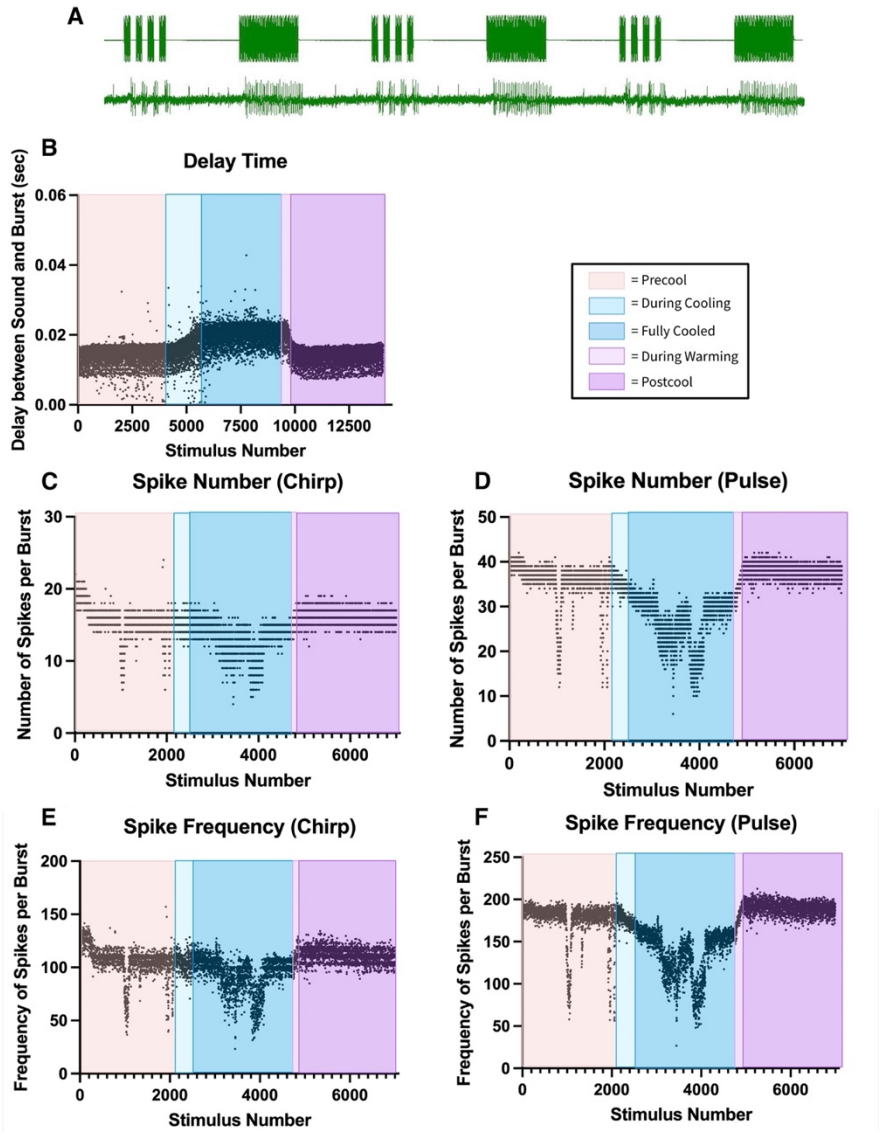

Figure 11: Field potential recordings (A) and analysis of the changes in delay time (B), spike number (C/D), and spike frequency (E/F) in cricket 211022, a female, black morph cricket, throughout the experimental process. (A) A raw trace from the cooled portion of the experiment. (B) Cold-deactivation increased the delay time, and warming returned it to baseline levels. (C/D) Cold-deactivation seems to have decreased the number of spikes per burst in response to both stimuli. Warming largely returned spike number to baseline in response in both instances. (E/F) Cold-deactivation also seems to have decreased the spike frequency in response to both stimuli, and warming restored this response equally in response to both.

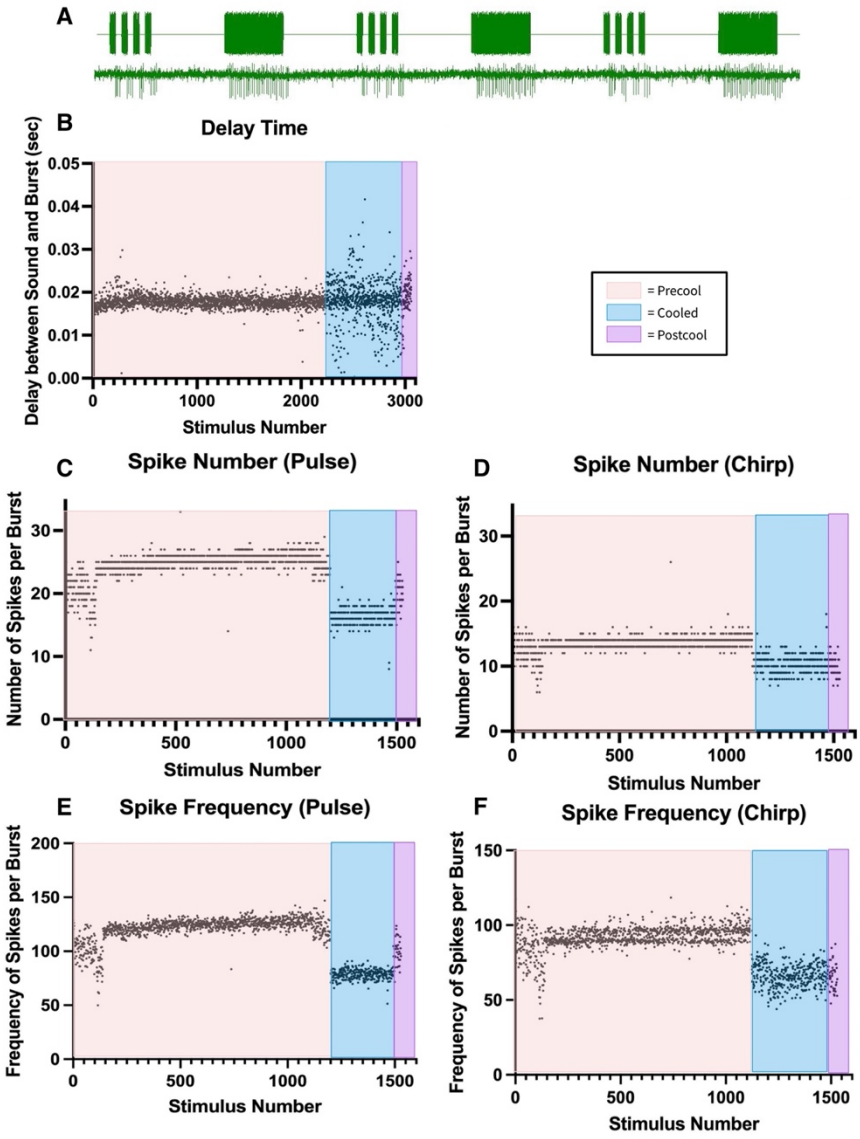

Figure 12: Field potential recordings (A) and analysis of the changes in delay time (B), spike number (C/D), and spike frequency (E/F) in cricket 210614, a female, brown morph cricket, throughout the experimental process, without analysis of the periods during cooling and during warming. (A) A raw trace from the cooled portion of the experiment. (B) Cold-deactivation resulted in an increase in the variability of the delay time, and warming the ear partially returned the delay time to baseline levels. (C/D) Cooling decreased the number of spikes in response to both stimuli but more so in response to pulses (C) than chirps (D), with a partial restoration of baseline levels after warming only in response to pulses. (E/F) Similarly, cold-deactivation decreased the spiking frequency in response to both sound stimuli, but more so in response to pulses (E) than chirps (F), with a partial restoration of baseline levels after warming only in response to pulses.

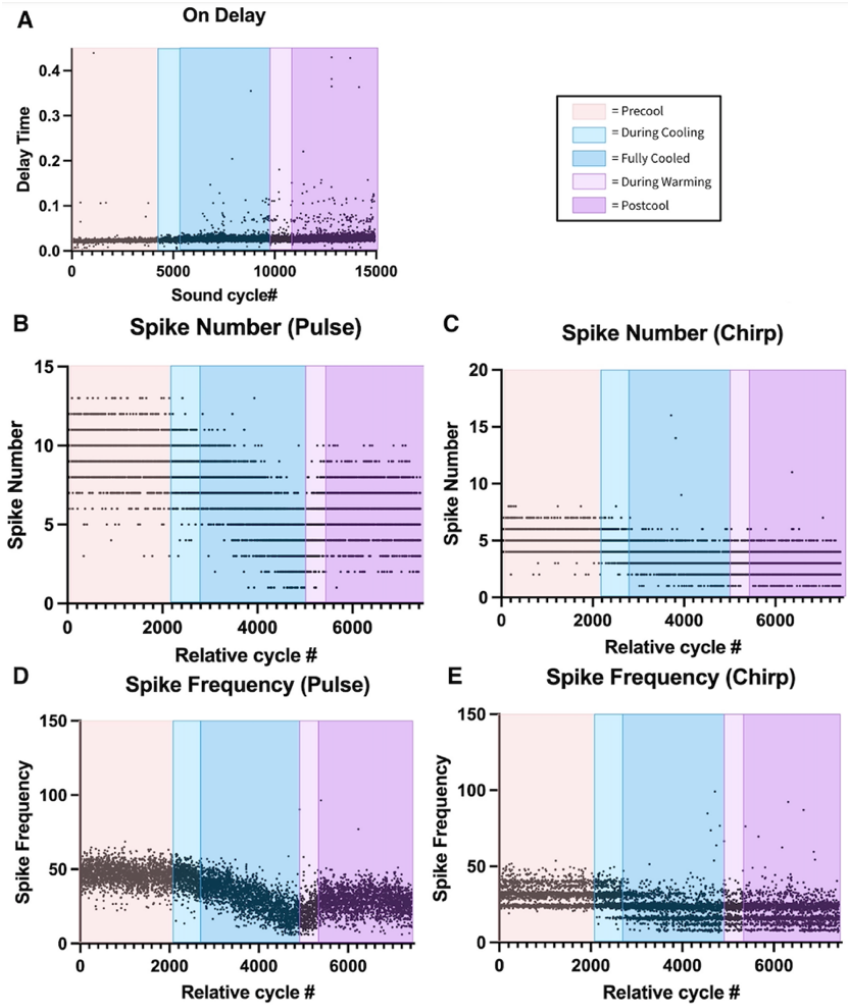

Figure 13: Analysis of the changes in delay time (A), number of spikes (B/C), and spike frequency (D/E) in cricket 210714, a female, black morph, cricket throughout the entire experimental process. (A) Cold-deactivation increased the delay time, and warming did not return it to baseline levels. (B/C) Cold-deactivation decreased the number of spikes per burst in response to both stimuli, but especially in response to pulses (B). Warming partially returned spike number to baseline in response to the pulse stimulus (B), but it had no effect on the chirp stimulus (E). (D/E) Cold-deactivation also decreased the spike frequency in response to both stimuli, but warming somewhat restored this response more so in response to pulses (E) than chirps (E).

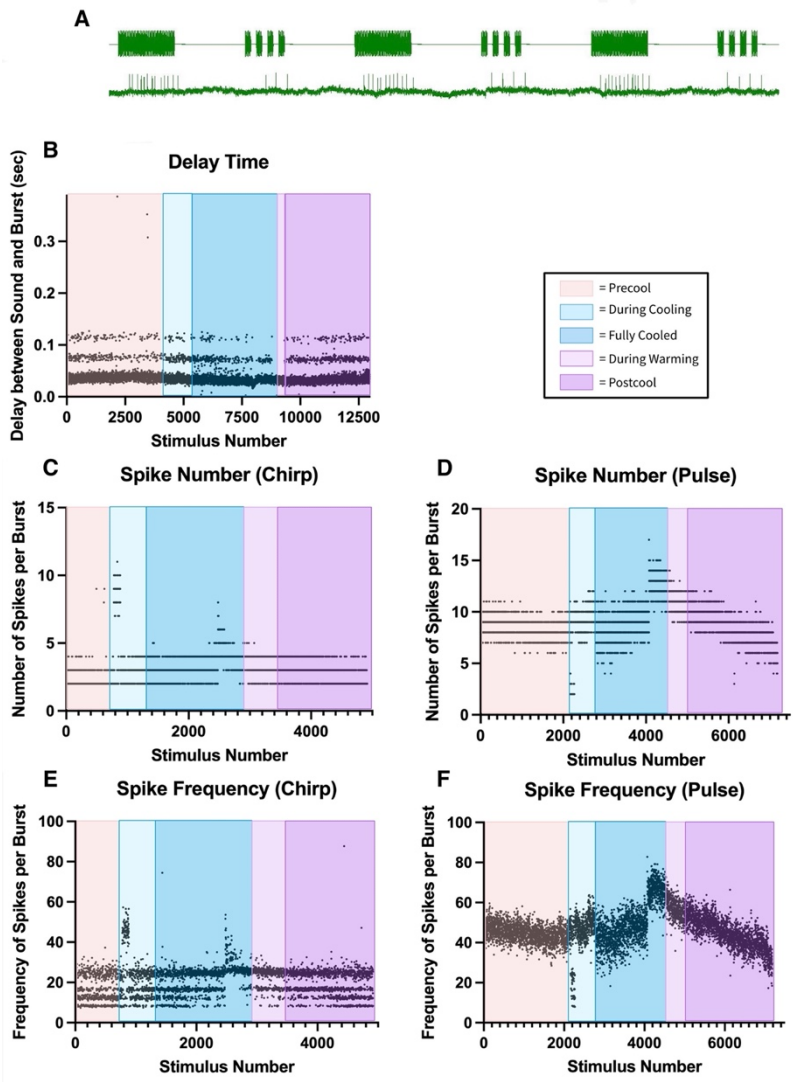

Figure 14: Intracellular recordings (A) and analysis of the changes in delay time (B), spike number (C/D), and spike frequency (E/F) in cricket 211019, a female, black morph cricket, throughout the experimental process. (A) A raw trace from the cooled portion of the experiment. (B) Cold-deactivation had no noticeable effect on the delay time. (C/D) Cold-deactivation seems to have marginally decreased the number of spikes per burst in response to pulses (D), but not chirps (C). (E/F) Cold-deactivation also seems to have slightly decreased the spike frequency in response to pulses (F), but not chirps (E).

Behavioral Results

Similar to the physiological results, behavioral responses were also quite varied and inconsistent. Across crickets, the ability to respond effectively to the sound was variable, which is surprising given the relative simplicity of negative phonotaxis and its crucial role in survival. Nonetheless, only 6 out of the 9 total crickets responded in the correct manner at least half of the time (a score of 0.5 or higher) (Fig. 15). Even within a single cricket, the latency to turn varied dramatically both across and within stimulus presentations (Fig. 16). Although, in some instances, such as cricket 220214 G, the latency increased during the stimulus presentation

(which would be consistent with habituation to the stimulus), this is by no means universal. (Fig. 16). For some crickets, like cricket 220218 G, the amount of time between the onset of the sound stimulus and the initiation of the turn actually decreased over the course of the stimulus presentation, and for others, there appeared to be no discernable pattern at all (Fig. 16).

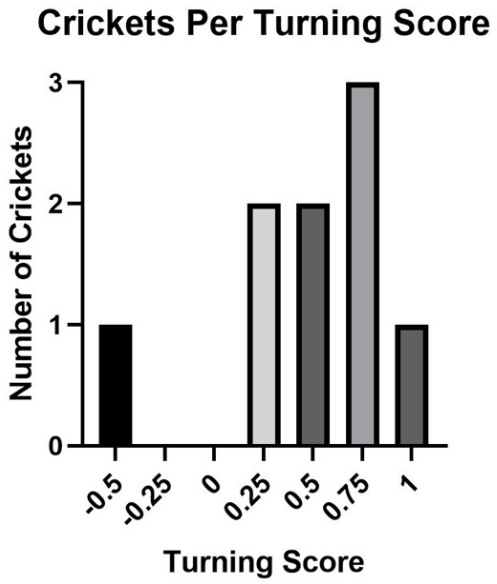

Figure 15: Histogram depicting the number of crickets with each turning score, from -0.5 (at least two incorrect turns and no correct turns) to 1 (all four correct turns). Of the 9 crickets, most showed a generally correct response, with most scoring between 0.25 and 0.75, but there was still clear variability in response accuracy.

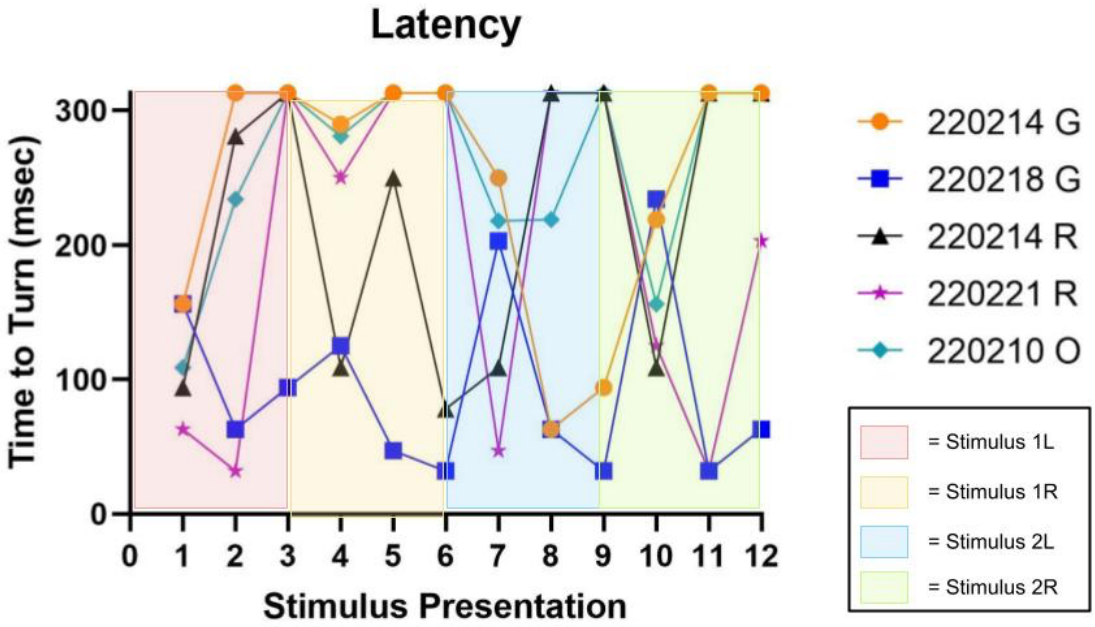

Figure 16: The turning latency for five crickets in behavioral experiments. The latency was recorded at three timepoints (the beginning, middle, and end) for each stimulus presentation. Since there were two stimuli and each was generated from each speaker, this led to a total of four stimulus presentations. Each timepoint is represented on the x axis. The latency to turn was capped at 313 msec, and times above this range were recorded as 313 msec. For each cricket, there is a large amount of variability in response, and, though some crickets appeared to habituate to the stimulus, this is not the case for every individual.

Correlating Behavioral and Physiological Results

In relating behavioral and physiological responses, I had expected to see a correlation between the delay time of the physiological response and the turning accuracy and latency of the behavioral response. Given that the physiological response does not need to be sent to the brain and then the muscles, I expected there to be a substantial difference in magnitude between the two responses, but I expected that, if an AN-2 cell responded quickly to the sound stimulus, so too would the cricket. I did not find this to be the case. Although both behavior and physiological data were collected for only one cricket, that cricket did not show any sort of correlation between the two datasets (Fig. 17). The cricket exhibited some variability in physiological delay time, but much more variability in turning latency, and there was no relationship between the two in terms

of orders of magnitude (Fig. 17). Though this is a single cricket, it seems to be consistent with trends seen in other crickets. Across the board, there appears to be very little variability in delay time within a single cricket's physiological responses, and a great deal of variability in a single cricket's behavioral responses. The example of the individual shown in Figure 17 only serves to strengthen this observation.

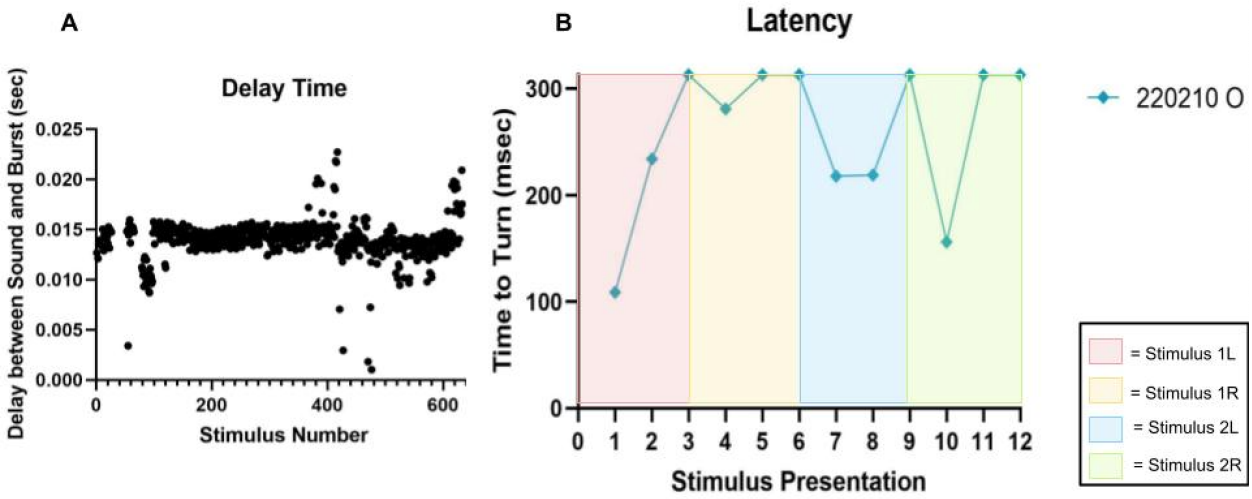

Figure 17: (A) The delay time for a single cricket, without cooling. Though there is some variability in response, there is generally very little, with most responses occurring between 120 and 150 msec after the onset of the stimulus. (B) The latency response for the same cricket, which shows a great deal of variability in response time, with most turns occurring between 200 and 300 msec after the onset of the stimulus, regardless of stimulus presentation. Though there appears to be some degree of habituation for each stimulus, it is not true in every instance.

DISCUSSION

Interpretation of Physiological Results

This study explored the firing of AN-2 in response to different sound stimuli as influenced by cold-deactivation. Analysis across ten crickets prior to cooling and four during and after cooling revealed great variability in individual responses to sound, with minimal trends developing as a result of cold-deactivation (Fig. 8, 9, 10). Although this may make it appear as though cold-deactivation is ineffectual, it is consistent with previous data found by the Horch

Lab that, across behavioral, morphological, and physiological measures, crickets respond to sound with varying degrees of intensity and specificity (Edwards, 2021). In fact, investigating the molecular reasons for this individual variability presents an exciting avenue for further research, with implications for developing better individualized treatments in humans.

The within-cricket analysis, on the other hand, was more elucidating as to the specific effects of cold-deactivation. Although delay time was the main analysis parameter, it was a less consistent measure than spike number and spike frequency. In two crickets, delay time did not inncrease, but rather stayed the same on average, while increasing in variability (Fig. 12, 13). In another, the delay time barely changed (Fig. 14), but in the last cricket, the delay time did increase dramatically, consistent with the presence of DOPE (Fig. 11). However, none of the crickets exhibited the prolonged-excitation also indicative of the DOPE response. In every case but one, warming failed to return the delay time to pre-cooled levels (Fig. 12, 13, 14). Generally, cooling expectedly decreased both spike frequency and spike number in response to both sound stimuli, although this effect was greater in response to the pulse stimulus than the chirp stimulus for both parameters (Fig. 11, 12, 13). Similarly, responses to the pulse stimulus generally indicated some recovery after warming, though this recovery was incomplete in every case but one (Fig. 11, 12, 13). Although this differential recovery was unexpected, it makes sense that the pulse stimulus would show such changes earlier and to a greater effect than the chirp stimulus. This is due to the fact that the pulse stimulus is more intense than the chirp, which due to the silence it contains, does not stimulate the neuron as much as the longer, uninterrupted pulse. Given this difference, it is possible that AN-2 would be more sensitive to the pulse than the chirp, so such changes would be more noticeable in response to it.

It is, however, still unexpected that there would be such little recovery after warming in most individuals. One of the primary advantages of cold-deactivation over traditional deafferentation (through the removal of the leg) is that its reversibility allows for the visualization of which changes within the system are permanent and which are not, so the fact that it was not entirely reversible in this case is concerning. Previous research has not maintained cooling for such an extended period of time (i.e. 90 seconds as opposed to the 30 minutes used here) (Zhang and Hedwig, 2019), but here we felt that a long period was necessary in order to fully examine the effects of cooling and determine whether or not DOPE was present. However, we found that such cooling causes irreversible damage in many crickets, even before the fully cooled temperature is established, though this was not the case for every cricket (Fig. 6, 7). This is inconsistent with previous results, which poses a methodological problem moving forward, unless measures can be taken to ensure that the electrode does not move through the cell membrane as the cell moves due to contraction of the PTG. That being said, the fact that some cells survived the entire cooling and warming process, and, in one recording, warming even returned all parameters to baseline levels indicates that cooling may not be an insurmountable obstacle as long as enough experiments are performed to account for the fact that many of them will not be successful.

Interpretation of Correlational Results

Using this baseline characterization of the DOPE response as background, I then aimed to contextualize the DOPE response by comparing behavioral and physiological experiments. I hoped that such a comparison would specifically probe the variability found in physiological recordings and other experiments performed by the Horch Lab. The fact that AN-2 is tuned to the high frequencies emitted by predatory bats means that its activation mediates a conserved

survival response (negative phonotaxis), which, given its integral role in survival, would likely not be highly variable (Moiseff et al., 1978). Despite this, I expected that the great variability in physiological response would translate to some behavioral variability as well. I found that this was indeed the case, as there was a great deal of variability with regard to both turning accuracy and latency in behavioral experiments (Fig. 15, 16). However, unexpectedly, there was no relationship between the observed variability in physiological and behavioral responses in the one cricket for which this data was collected (Fig. 17). The fact that these results are both from an uncooled, control cricket further suggests that comparing physiological and behavioral results does not seem to yield additional insight.

If, however, a similar dissonance were seen with correlated physiological and behavioral data, it would call into question the hypothesis elucidated previously that DOPE is caused by PIR. Given the hypothesis that crickets that exhibit a stronger DOPE response will also show stronger negative phonotaxis, because of the tightly regulated inhibition by ON-1, such results would suggest that DOPE may not be a result of ON-1 mediated inhibition, suggesting that it is not due to PIR at all. Alternatively, DOPE could still be a PIR-induced phenomenon, but some degree of higher order processing modulates this response in such a way that does not correlate with behavioral outcomes.

Regardless, these results problematize the potential power of correlational measures in crickets. This has huge ramifications for future research in the area of DOPE and other intracellular properties, but it also extends to other areas of research. If there is indeed no relationship between turning latency and delay time in response to these stimuli, it seems less likely that we can correlate behavioral and intracellular responses more generally. Unfortunately, this suggests that experiments that have been occurring in the Horch Lab, which aim to correlate

behavior, morphology, and physiology, may not show the expected relationships between these measures of analysis. This is not to say that such experiments are without value, but we should be cautious when considering the results that they produce.

Future Directions: Post-inhibitory Rebound

Moving forward, I hope that this research can be used to definitively determine the origins of the DOPE response. I still hypothesize that DOPE is most likely a manifestation of PIR. However, the lack of correlation between physiological and behavioral data means that this relationship may not be best investigated through such correlational experiments. Ideally, successful experiments would instead induce DOPE in both cooled and uncooled crickets by injecting currents of various amplitudes that match the timing and duration of the sound stimuli. If the DOPE response can be successfully evoked, it would support the hypothesis that DOPE is a facet of PIR. Presumably, there would be some variability of response in this experiment (as with the sound-based stimulus), but responses consistent with those seen in this study would point to such an explanation for DOPE. Additional experiments could then be performed to determine the mechanism of action behind PIR (if present) in this system through the use of ion replacement and blockers.

Future Directions: Priming

The characteristics of PIR might be altered through cold-deactivation and other manipulations of the system, especially if it is driven by contralateral input. Of particular note is the instance of priming, a phenomenon that occurs in sensory systems throughout the animal kingdom in which a certain stimulus is able to bias the system towards a distinct response (Schacter and Church, 1992). In humans, for example, priming an auditory stimulus that is supposed to later be remembered with one spoken in the same pitch leads to greater recall than

when the priming stimulus is in a different pitch (Schacter and Church, 1992). In the cricket auditory system, priming with various stimulus intensities as well as directionalities has been shown to influence the animal's behavioral response to stimuli mimicking bat ultrasound (Engel and Hoy, 1999). When low-intensity stimulation is used to prime the neuron's response, this results in greater attenuation to the actual stimulus than when higher-intensity priming stimuli are used, and prior stimulation on one side of the animal encourages a response on the ipsilateral rather than contralateral side (Engel and Hoy, 1999). Additionally, the temporal pattern of the priming stimulation can affect the firing of auditory neurons (and particularly AN-2) (Samson and Pollack, 2002). For example, long trains of stimulation with little silence between the individual tones as a priming stimulus causes increased firing latency and a decreased number of spikes in response to each later recorded stimulus (Samson and Pollack, 2002). This means that the primed response is less acute than the initial response, indicating that temporal priming of this sort results in habituation on a scale as small and precise as the electrophysiological response of a single neuron.

As such, it would be interesting to explore the impacts of priming with sound stimuli of different frequencies on this response. Given the frequency-specific responses of AN-1 and AN-2, priming the stimulus with an equal stimulus of a different frequency both when cooled and in control conditions should help elucidate the role of contralateral inhibition on AN-2's response. As such, one could perform an experiment in which the contralateral ear is cooled (which is the direct opposite of what has been shown here, where the ipsilateral ear undergoes cooling), thereby functionally removing the inhibition that AN-2 receives from ON-1. When such inhibition is removed through cooling, the effects of priming should be decreased, as the PIR mechanism would no longer be relevant.

Elucidating the origins of the DOPE response and the impacts of priming on AN-2 will help clarify models of the circuitry of the *G. bimaculatus* auditory system, and this expanded understanding of the circuitry and specific properties of the neurons within it will provide insights into how this auditory system is capable of such profound plasticity. Not only will this research help expand what is known about AN-2, but it will also provide a basis for further exploring the intrinsic properties of other neurons in the system, especially through reversible cooling and current injections. Ultimately, better understanding the mechanisms behind compensatory plasticity in *G. bimaculatus* from an electrophysiological perspective will expand opportunities to further explore such mechanisms in other species, potentially with notable ramifications for treatments for human neuronal injury.

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