Perceptual Categorization and Neural Representations of Vocal Stimuli

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Abstract

The ability to identify and categorize sensory stimuli is critical for the survival of organisms. While this behavior has been well characterized across multiple sensory modalities and multiple species, from invertebrates to humans, the neural mechanisms that drive this behavior remain a point of interest. In particular, we wanted to focus our efforts on how natural auditory stimuli are categorized, how those categories shift over time, and how those categories are represented in the auditory cortex. To study this, we took advantage of pup retrieval, a maternal mouse behavior where maternally experienced female mice will locate and retrieve pups separated from their home nest by utilizing ultrasonic vocalizations emitted from the pups.

I describe how we studied this in this thesis, divided into five chapters. In the first chapter, I provide an overview of the field in regards to the auditory cortex and how sensory stimuli are categorized, as well as why pup retrieval can be a valuable model to study auditory categorization. In Chapters 2 and 3, I describe two behaviors, one freely moving and one a head-fixed Go No-Go task, which demonstrate how mice categorize auditory cues with pup calls. More specifically, I demonstrate how frequency seems to be one of the most important features in driving mice to categorize sounds as pup calls. However, if other spectrotemporal features of the sound are similar to that of the pup call, mice are more willing to tolerate differences in the frequency. Furthermore, I show that the presence of a low frequency band can inhibit the mouse's ability to categorize a sound as a pup call. In Chapter 4, I describe single unit electrophysiology data that demonstrates how auditory cortex neurons respond towards full pup call trains as well as other broadband stimuli. In particular, I show that neurons in the auditory cortex of surrogate females have

earlier responses towards pup calls compared to naïve females, and that these early responses also exist in response to narrow, high frequency bands. Furthermore, I show how low frequency sounds seem to be more correlated in their responses compared to high frequency ones both in naïve and surrogate females. I also show that following behavioral training, low frequency bandlimited noise becomes even more correlated, while high frequency bandlimited noise does not change. Finally, in Chapter 5, I describe my results in the context of the field as well as propose several follow up experiments which would support and expand our findings.

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Contents

List of Figures

List of Abbreviations

2AFC	2 Alternative Force Choice Task
A1	Primary Auditory Cortex
A2	Secondary Auditory Cortex
AAF	Anterior Auditory Field
AC	Auditory Cortex
BF	Best Frequency
DF	Dorsoposterior Field
GNG	Go No-Go
ISI	Intersyllable Interval
OC	Olfactory Cortex
PC	Pup call
PV	Parvalbumin
PSTH	Peristimulus Time Histogram
ROC	Receiver Operating Curve
USV	Ultrasonic Vocalization
V1	Primary Visual Cortex
VOT	Vocal Onset Time
UF	Ultrasonic Field

Chapter 1

Introduction

1.1 Overview

In an ever-changing environment, animals must be able to respond to a variety of different stimuli in order to survive. These stimuli can range across all different modalities, and often are not uniform in their properties. Therefore, organisms need to be able to identify the key features of these stimuli and group them into behaviorally relevant categories. These categories must be malleable, as these features can also change and evolve over time, thereby requiring the individual to adapt its understanding of what constitutes a category.

Experiments studying how these categories are shaped and represented in the brain have been done in the past across multiple sensory modalities, including visual, olfactory, and auditory. Often times, these experiments utilize artificial stimuli in order to carefully probe specific features of the stimuli, allowing the researchers to minimize the number of variables being modulated at once. However, while using these artificial stimuli has led to a number of valuable insights, these artificial stimuli are often simplistic in nature. Rather, most organisms will be barraged with more complex stimuli, which can cause more variable responses in the neurons. To further complicate matters these complex stimuli can show responses highly variable from what one may suspect based on the results from previously

tested artificial stimuli. Therefore, understanding how these more complicated sensory stimuli are represented and categorized in the sensory cortices remains an important question to be answered.

One way in which we can address this question is by taking advantage of the maternal behavior pup retrieval, where a female mouse with pup experience will utilize ultrasonic vocalizations (USV) emitted by the pup to locate and return it back to a home nest. Importantly, naïve females, or female mice without pup exposure, will unreliably perform this behavior, thereby suggesting that these pup USVs gain behavioral relevance following pup exposure. We can therefore take advantage of this to study behaviorally which sounds maternally experienced females will recognize as categorically similar to pup calls, as well as how those sounds are represented in the auditory cortex.

The work described in this thesis will aim to better understand the role the auditory cortex plays in categorizing natural stimuli and how those representations change over time. This question will be addressed using a pup retrieval model as a framework, and will look at this question using a combination of behavioral and neural data. Chapter 1 will review the complicated nature of how sensory neurons respond to sensory stimuli, the neural underpinnings that drive categorization of these sensory stimuli, and how maternal experience can be used to probe auditory categorization. In Chapter 2, I examine the boundaries of maternal recognition of pup calls using a freely moving behavioral paradigm. In Chapter 3, I utilize a high throughput head-fixed behavioral paradigm to further probe which sounds are grouped as pup calls. In Chapter 4, I follow up my behavioral observations by studying how neural activity in the auditory cortex changes in naïve and surrogate females both before and after behavioral training. In Chapter 5, I discuss the

implications of this work on the field as well as how this work might be expanded on in the future.

1.2 Sensory Neurons are Complex and Subject to Modulation

One of the fundamental goals of the sensory systems is to transform sensory stimuli into perceptual realities, often in the form of some discrete motor action. In order to accomplish this, these sensory systems utilize neurons in the brain that respond to key features of the given stimulus. For example, in the visual cortex, neurons will respond strongly to things like the orientation or direction of motion (Britten et al. 1993; Niell and Stryker 2008) while those neurons in the auditory cortex will respond to the frequency or direction of a sound (Stiebler et al. 1997; Trujillo et al. 2011) and neurons within the olfactory cortex are tuned towards single or a specific odor combination (Yoshida and Mori 2007). Classically, responses towards these sensory stimuli are reported based on the firing rates of the neurons being measured, with an increase in firing representing excitation and a decrease representing inhibition (Adrian and Zotterman 1926). However, information can still be encoded even if the neuron fails to fire towards the stimuli. This was demonstrated in a recent study that showed that even neurons that do not classically respond to an auditory stimulus can still provide information about the stimulus being presented by using the interval between spikes, or the interspike interval (ISI) (Insanally et al. 2019).

While it may seem that sensory neurons are simply reporting the presence of a feature, this explanation is far too basic. Rather these sensory neurons can be heavily modulated by a number of different things. For instance, it hass been demonstrated that the presence of an anticipatory sound can increase the firing of neurons within the auditory

cortex (AC) towards a target sound in both mice and rats, demonstrating how temporal expectation can alter the firing of AC neurons (Jaramillo and Zador 2011; Peng et al. 2012). Furthermore, engagement in a behavioral task can also alter the firing of AC neurons, as shown in studies where neurons presented the same auditory cue had different firing rates depending on whether the cue was presented prior to a behavioral task or during a behavioral task (Miller et al. 1972; Carcea et al. 2017; Xin et al. 2019). Even the presence of other sensory modalities can impact AC neurons, as the presentation of olfactory or visual stimuli can sharpen the perception of auditory cues presented in tandem (Cohen et al. 2011; Atilgan et al. 2018).

This modulation is not restricted to the auditory cortex. For instance attention can increase neurons within V4's response towards a preferred stimulus, while decreasing its response to nonpreferred stimulus (Moran and Desimone 1985). Moreover, the presence of an auditory stimuli can sharpen V1 neurons response to visual stimuli in their preferred orientation while simultaneous decreasing the spike number to stimuli in the orthogonal direction (Ibrahim et al. 2016). The olfactory cortex (OC) shows similar findings, whereby neurons within the olfactory tubercle, a region within the OC, are directly modulated by whether a rat was in an odor-related behavioral task or not (Carlson et al. 2018).

Even controlling for the context of how a sensory stimulus is presented though, the actual content of the sensory stimuli can alter how sensory neurons fire beyond just the basic features that make it up. In particular, there has been a growing amount of research in regards to how sensory neurons respond towards simple, artificial stimuli compared to more complex, natural stimuli. Despite the fact that artificial stimuli provide a level of control and precision, very rarely are such simple stimuli found in nature. Rather, most natural stimuli consist of a combination of simple features that create a complex stimulus,

making them potentially more difficult to process and classify. Nonetheless, rats are able to correctly categorize movies containing rats from movies that do not contain rats, suggesting that they are able to utilize the higher level features found in natural stimuli (Vinken et al. 2014). Furthermore, humans were better able to classify a natural visual stimulus compared to an unnatural visual stimulus, suggesting that despite the added complexity of natural stimuli, the brain may have ways in which it is better able to encode and represent those stimuli (Li et al. 2002).

How the brain is encoding these natural stimuli, however, is not a straightforward question. For instance, the response of a neuron towards a simple stimulus does not necessarily predict how it will respond to a more complex stimulus containing that simple stimulus. This was well demonstrated by a report that showed that when a neuron in the auditory cortex would respond towards a chord, but not to the tones that made up that chord (Wang et al. 2020). These results also hold true in the reverse, as a neurons response towards a pure tone does not imply it will respond to a more complex, natural stimulus that contains power at the pure tones frequency (Machens et al. 2004; Bitterman et al. 2008). Once again, this feature is not unique to the auditory system, as a V1 neurons response towards an artificial stimulus was unable to predict its response to a more natural one. (David et al. 2004).

1.3 Categorization of Sensory Stimuli

While sensory neurons can provide information regarding the features of a stimulus, an important question that the brain must answer is, behaviorally, what the stimulus actually is. One way in which this can be answered is by assigning the stimulus to a

category. When novel stimuli appear, those stimuli that are similar to ones already belonging to a category can be assumed to have similar behavioral relevance. This process, called categorization, allows one to quickly and efficiently determine where novel stimuli stand, as well as enabling one to withstand variation among a prototypical stimulus. Put in another way, this enables the individual to exaggerate differences between objects in different categories, while diminishing differences in objects within similar categories. If these differences are exaggerated or diminished enough, categorical perception can occur. In this case, changes along a continuum result in no changes in perception until a certain point along the continuum is reached, whereby a sharp categorical change occurs.

Behaviorally, one of the most well understood examples of categorical perception in humans behaviorally is human speech perception. The Vocal Onset Time (VOT), or the time between a stop constant and the onset of a sound being released, is a continuous spectrum, yet humans often create a clear boundary between two different sounds (one example being between the sound of 'ba' and 'pa') (Elmas et al. 1971). This VOT boundary is innate, as infants are able to distinguish between these sounds (Lasky et al. 1975). However, as infants grow up, the sounds and languages they use and hear will shift their categorical perceptions of various sounds, highlighting the brains plasticity in defining relevant categories (Werker et al. 1981). These shifts highlight how categorization exists from a young age, and that this categorization can be altered and refined over time.

This ability to learn categories is not unique to humans, and has been observed in a wide range of organisms, from non-human primates to invertebrates like crickets, suggesting that categorical perception is a conserved feature among many species (Wyttenbach et al. 1996; Tsunada et al. 2012). Moreover, categorization has been observed across multiple sensory modalities, from audition to olfaction (Christison-Lagay and Cohen

2018; Reinert et al. 2021). However, while the behavioral mechanisms have been well understood, the neural mechanisms that drive categorization remains unclear. Therefore, these model organisms provide researchers the opportunity to study how categorization is shaped and represented in the sensory systems.

One particularly prominent area that has garnered attention in answering this question is the visual cortex. Following behavioral training where mice were trained to group visual stimuli based on their orientation, neurons within V1 will alter their firing pattern, demonstrating improved neural discriminability and sharpened orientation tuning in response to these stimuli, allowing the subject to better perform this task (Jurjut et al. 2017). But while neurons will sharpen their responses to better separate stimuli into categories, they do not necessarily reflect the categories they are responding towards. For instance, neurons within the middle temporal and V4 region, two regions within the visual cortex, seem to be more selective towards the features of the stimuli rather than the category of the stimuli (Brincat et al. 2018). With that said, recent discoveries have identified a small region in the visual cortex, the postrhinal area, which appears to more tuned towards the category of the stimuli, suggesting that neurons within these sensory cortices may actually be playing a larger role in categorization than previously thought (Goltstein et al. 2021).

While only a small portion of neurons in the visual cortex seem to represent categories, a large number of downstream regions have been identified as important in visual categorization. For instance, when monkeys were asked to separate visual stimuli based on their direction, neurons in the lateral intraparietal cortex were found to have strong category selectivity (Swaminathan and Freedman 2012). Furthermore, neurons within the inferior temporal cortex in macaques demonstrated enhanced neuronal representation for features important for the categorization of multiple line drawings (Sigala

and Logothetis 2002). Finally, neurons in the prefrontal cortex have respond specifically to categories, even when the sensory stimulus previously associated with one category changes its representative category, emphasizing its role in visual categorization in both mice and primates (Freedman et al. 2001; Reinert et al. 2021). Thus, while the visual cortex may play a small role, downstream targets may be playing a much larger role in categorization.

Generally speaking, olfactory categorization follows a similar trend to visual categorization, but the categorization has seems to happen upstream of the olfactory cortex instead. Much like the visual cortex, the olfactory cortex itself seems to mostly encode for sensory change rather than categories, as evidenced by a lack of reward representation in the posterior piriform cortex (Millman and Murthy 2020). These results were supported by work done within the ventral tenia tecta, a region within the olfactory cortex, that demonstrated that while neurons can change their firing during goal-directed behavior, these changes were are mostly changes in the timing of firing, rather than being category specific (Shiotani et al. 2020). Similar to the visual cortex, most olfactory categorization occurs downstream, as demonstrated by human fMRI studies which have implicated that odor categorization is encoded in the perirhinal, orbitofrontal, piriform, and insular cortices (Howard et al. 2009; Qu et al. 2016).

However, rather than only appearing in the deeper layer projections, categorization seems to also take place earlier along the olfactory pathway, in the olfactory bulb. When passively presented combinations of odors along a continuum (100%/0, 90%/10, etc.), mitral/tufted cells demonstrate a smooth, continuous change in firing between presented odors (Khan et al. 2008). However, when mice were given the same basic task but presented instead as a behavioral paradigm where the mice needed to lick for a water reward

depending on the odor, mitral cells began to respond more in line with a category rather than the odors features, suggesting that these cells may be the first stage in odor categorization (Kudryavitskaya et al. 2021). This early categorization in the olfactory bulb may allow for more rapid categorization than found in other sensory stimuli.

1.4 The Auditory Cortex

Auditory stimuli can also be categorized, and the auditory cortex plays a major role in this function. The auditory cortex is composed of both excitatory and inhibitory neurons, with 3 groups of interneurons, PV, SST, and 5HT3aR ,making up almost all the interneurons (Rudy et al. 2011). Neurons within the auditory cortex are often defined and grouped by the frequencies which they respond to best (BF) (Stiebler et al. 1997). Classically, in rodents, the auditory cortex has been separated into 5 distinct regions: the anterior auditory field (AAF), primary auditory cortex (A1), secondary auditory cortex (A2), ultrasonic field (UF), and dorsoposterior field (DF) Neurons located in A1 and AAF have ordered tonotopic maps, with neurons smoothly transitioning from those with BF of 4 kHz to those with BF of ~41 kHz. On the other hand, A2 and DF do not have smooth tonotopic maps, but instead fired in a disordered manner to frequencies between 4 kHz to 65 kHz. These regions were instead characterized by their relative location. Finally, those neurons located in the UF region responded largely to those frequencies with characteristic frequencies greater than 50 kHz, although, much like A2 and DF, not in any particular tonotopic order (Stiebler et al. 1997). Recently however, the presence of a UF region has been challenged, with some suggesting that this region is in fact a high frequency subpart of the A1 and AAF region (Guo et al. 2012). Alternatively, using fluorescence imaging, recent work has suggested that UF can in

fact be further separated, into a dorsomedial field (DM) and the dorsoanterior field (DA) (Tsukano et al. 2015).

These regions are generally stable if left unperturbed, as the frequency selectivity of these regions did not change after 30 days (Romero et al. 2020). However, if perturbed, the auditory cortex demonstrates remarkable plasticity and will shift its firing responses based on the presence of an external stimulus. For example, following maternal experience, areas less tuned to ultrasonic vocalizations became suppressed in responses to the ultrasonic vocalizations that pups will emit (Shepard et al. 2016). Furthermore, when exposed to a 22-psi blast exposure, mice had distorted A1 cortical maps, with some mice demonstrating increased representation of low frequencies, and others demonstrating increased representation of high frequencies (Masri et al. 2018). Thus, while these regions are generally stable, the representative frequency cortical maps can be shifted depending on the circumstance.

In addition to these regions, the auditory cortex can also be separated laterally, into the left and right AC, with each side tuned to more specific sounds. Using c-fos staining, an increase in c-fos positive neurons was found in the right AC when mice were presented with sweeps. On the other hand, when mice were presented with vocalizations, significantly more c-fos cells were found in the left AC, suggesting the role of the left AC in social communication (Geissler and Ehret 2004; Levy et al. 2019). These differences in lateralization are recognized not just by the neurons being activated, but also by the underlying circuitry amongst the inhibitory neurons within the cortices (Oviedo 2017; Neophytou et al. 2021). Taken together, these results seem to suggest that the left AC plays a role in vocalizations, while the right AC may be working to integrate more broad features. Behaviorally, these results also seem to hold true, as mice who had hearing from their right ear blocked off were

unable to distinguish between pup calls, which are a high frequency sound, and a low frequency tone (Ehret 1987). Furthermore, when muscimol was injected into the left AC, but not the right, pup retrieval behavior was inhibited (Marlin et al. 2015). Because pup retrieval relies on the high frequency USVs emitted by the pups, these results lend further credence to the idea that the left AC is vital for social auditory cues (Hernandez-Miranda et al. 2017).

While the name suggests that the auditory cortex plays a role in hearing, and indeed a large amount of work supports this idea, there have been studies which call into question the actual role that the auditory cortex plays. For example, work in rats has showed that even when the auditory cortex is inactivated, rats are still capable of engaging in a sound-frequency categorization task (Gimenez et al. 2015). These results are supported in mice, where inactivation of the auditory cortex via optogenetics demonstrated that mice were still capable of discriminating between two pure tones (Ceballo et al. 2019). To better understand what the auditory cortexes role actually is, the same group looked at whether auditory cortex was required for more complicated stimuli. While auditory cortex inactivation was insufficient to disrupt simple frequency discrimination, it was able to disrupt discrimination of frequency modulated sounds (Ceballo et al. 2019). Thus, the auditory cortex may not be required for simple auditory discrimination, but instead important for discriminating more complex auditory sounds.

1.5 Categorization of Auditory Stimuli

The concerns about the auditory cortex's role in hearing notwithstanding, the AC has been shown to play a crucial role in the categorization of auditory stimuli. One way in which it does this is by modulation its firing depending on whether an auditory stimulus

belongs to a behaviorally relevant category or not. For instance, humans trained to categorize monkey calls demonstrated sharpened tuning in response to these calls in the left AC, suggesting that those sounds had gained salience following behavioral training (Jiang et al. 2018). This sharpened tuning has been seen in non-human primates as well, as when monkeys were tasked with separating two auditory stimuli apart from one another, neurons in A1 would modulate their firing in a manner directly correlated with the monkeys choice (Christison-Lagay and Cohen 2018). This sharpening of a response occurs not just in response to auditory stimuli that the subject is trained on, but also towards other auditory stimuli close to the boundary between the trained stimuli. For example, in rodents, it has been demonstrated that auditory stimuli near a category boundary will elicit stronger responses once categories have been formed (Xin et al. 2019). These results together suggest that when presented with a categorical choice, the AC will modulate its firing to improve its ability to place auditory stimuli into correct, meaningful categories.

While neurons have sharpened responses towards categorically relevant stimuli, the auditory cortex also contains neurons specific for the category rather than the actual stimulus features themselves. In studies done in primates, these category selective neurons were more likely to be interneurons than excitatory ones (Tsunada et al. 2012). These category specific neurons exist in gerbils, represented by peaks in the neurons response only after behaviorally relevant categories are formed (Ohl et al. 2001). More recently, similar results have been observed in mice, as neurons in the auditory cortex were detected to only become active when an auditory cue became associated with a category in a decision-making task in mice, suggesting that those neurons showed stronger selectivity towards category membership than passive stimulation (Xin et al. 2019). Furthermore, in mice, LFP from the auditory cortex showed responses towards changes in an auditory pattern,

strongly supporting the idea that those neurons were responding towards the temporal category, rather than the specific features of the stimuli (Astikainen et al. 2014). Finally, experiments done in ferrets have demonstrated that some neurons in the AC are driven by categorical information rather than features of the stimulus, and also showed that these neurons maintain their categorical responses even in a non-task condition, highlighting how these neurons persist outside of the context (Yin et al. 2020). Together, these results highlight the importance of the AC in categorization, not just in terms of modulation of firing in response to category specific sounds, but also to category specific firing.

While the presence of category specific neurons in the auditory cortex has been discovered, it is important to at least briefly highlight that categorization also exists in brain regions outside of the AC as well. When an auditory stimulus is presented from two different learned categories, neurons selective for those two categories are observed in the lateral prefrontal cortex (Jiang et al. 2018). Moreover, neurons in the ventrolateral prefrontal cortex also demonstrated categorical preference towards auditory sounds (Russ et al. 2008). Finally, the orbitoprefrontal cortex was implicated in auditory categorization, as optogenetically disrupting projections to the auditory cortex was sufficient in disrupting the mouse's ability to perform a categorization task (Liu et al. 2021). Thus, while the AC plays a crucial role, auditory categorization requires a number of different brain regions.

1.6 Maternal Behavior and the Auditory Cortex

Newborn mammalian animals are largely helpless, requiring the help of their parents to survive until they are able to fend for themselves. While the manner in which animals may engage in parental behavior is different, this kind of behavior is largely

conserved, making it an attractive behavior to study. In rodents, this parental behavior can take the shape of a number of behaviors, including grooming, licking, nestbuilding, or retrieving the pups when they are out of the nest (Capone et al. 2005).

One particular act of maternal behavior, pup retrieval, has been heavily studied in regards to understanding plasticity in the brain. When pups are separated from their home nest, they emit an ultrasonic vocalization, which mothers will use to locate the pups and bring them back to the home cage (Hernandez-Miranda et al. 2017; Krishnan et al. 2017; Schiavo et al. 2020). Despite the fact that adult mice are able to separate out cues from their own pups, they still are capable and willing to retrieve both other pups, suggesting that there are innate changes that occur in the female brain following motherhood that drives them to these calls (Ostermeyer and Elwood 1982; Mogi et al. 2017). Interestingly, this pup retrieval behavior is not exclusively done by mothers. While naïve virgin females can learn to retrieve pups by themselves, they learn quicker when in the presence of a mother, becoming surrogate females (Krishnan et al. 2017; Carcea et al. 2021). This is accomplished when mothers encourage the virgin female to spend time in the nest with the pups, thereby activating oxytocin neurons within the hypothalamic paraventricular nucleus and in turn, accelerating the onset of pup retrieval (Carcea et al. 2021). While male mice typically do not engage in pup retrieval, they can be taught to do so by upon hearing specific female vocalizations (Liu et al. 2013).

Pup retrieval induces activity in a number of different regions in the brain, include some deeper layer structures such as the medial preoptic area, the ventral tegmental area, the basal amygdala, the temporal association cortex, and the locus coeruleus (Fang et al. 2018; Kohl et al. 2018; Tasaka et al. 2020; Dvorkin and Shea 2021; Nowlan et al. 2022). However, pup retrieval is a multisensory task that also requires input from multiple sensory

cortices. While there has been some work on the visual system and how that system may play a role in learning this behavior, most work has focused on the auditory and olfactory cues that pups will emit and how those cues drive a behavioral and neural response (Carcea et al. 2021). Thus, it makes it an attractive option to study what changes occur between naïve and surrogate females in the brain that drive this behavioral difference.

The auditory cortex is particularly interesting as pups will emit a USV to alert the mother of its presence. Following maternal experience, neurons in the auditory cortex exhibited stronger excitatory responses to those pup calls compared to AC neurons in naïve females, (Lau et al. 2020). These changes are not exclusive to the excitatory neurons, as PV-positive neurons were suppressed following maternal behavior, as well as having their best frequency shifted upwards an entire octave (Cohen and Mizrahi 2015; Lau et al. 2020). These responses are not due to an increase in size of the USV region, but rather due to neurons responsive to lower frequencies becoming more suppressed when pup USVs are being presented (Shepard et al. 2015; Shepard et al. 2016). Taken together, these changes allow mothers to more efficiently discriminate pup calls from other auditory cues. The presence of olfactory cues have also plays a critical role, as maternal experience has can improve odor processing by enhancing synaptic integration of new neurons into the olfactory bulb circuitry(Belnoue et al. 2016). Furthermore, olfactory cues in combination with auditory cues can act to enhance neural responses to pup USVs in the auditory cortex (Cohen et al. 2011; Cohen and Mizrahi 2015).

While maternal experience is certainly important for pup retrieval, the pup must also play its role in this behavior. If a pup is separated from the nest and does not vocalize for any reason, mothers will typically not retrieve the pups, strongly consistent with the hypothesis that these pup calls are an important piece in the pup retrieval process

(Hernandez-Miranda et al. 2017; Schiavo et al. 2020). These pup calls are diverse in a number of different features, including the frequency, call rate, and call duration, strongly pointing to the idea that maternally experienced mice are able to identify important features of these pup calls and categorize them together (Rieger and Dougherty 2016). Therefore, the question becomes which of these features is important for pup call recognition. Upon building a library of pup calls and using signal detection theory, one lab generated a receiver operating curves (ROC) and hypothesized that in order to distinguish pup calls from other high frequency sounds, the frequency of the pup call was the most important feature, followed by the intersyllable length and finally the duration of the call (Liu et al. 2003). These results were at least partially confirmed behaviorally, where an increase in intersyllable interval was shown to be important for pup call recognition, as when the interval increases or decreases past a certain point, maternally experienced females will begin to respond less frequently to those calls (Schiavo et al. 2020).

In addition to the frequency, intersyllable interval, and duration of pup calls, an additional area that some people have looked at are the spectrotemporal features of the individual syllables. It has been suggested that these pup call USVs are composed of approximately ten distinct syllables (Grimsley et al. 2012). Furthermore, studies centered around these pup call syllables have demonstrated that an autism mouse model has unique pup call USVs which directly impact mother ability to retrieve their pups (Takahashi et al. 2016). These results suggest that the spectrotemporal features of these syllables may play a unique role in communication between pups and their mothers. However, while there is credence to the idea that some of these features are important, there is also some work that suggests the opposite. When presented with pup calls and broadband noises, researchers found that certain broadband noises were equally likely to attract the mother as a natural

pup call, suggesting that the previously mentioned spectrotemporal features may not be as important currently believed (Ehret and Haack 1981). Thus, which features are truly critical for pup call recognition remains unclear.

1.7 Summary and Thesis Goals

Taken together, I have highlighted the role of the auditory cortex in the categorization of auditory stimuli. Pup retrieval is a prime way to investigate this further, as we can take advantage of the pup calls that pups emit when separated from the nest. While some studies have suggested features of these calls that could be important, very few have behaviorally tested these in a high throughput manner.

Thus, in my thesis I will showcase a freely-moving behavior task I have developed to investigate which stimuli mothers will associate with a pup call (Chapter 2). I will then support these results using a high throughput head fixed Go No-Go behavioral task (Chapter 3). Finally, I will use single unit electrophysiology to demonstrate how surrogate and naïve females respond differently to pup call trains, as well as how training on the Go No-Go behavioral task impacts how neural responses are correlated with one another (Chapter 4).

Chapter 2:

Auditory Categorization in a Freely Moving Behavior

2.1 Introduction

A critical component for the survival of a species is the ability to group behaviorally relevant sounds into distinct categories. One way in which this is observed is in mice via pup retrieval behavior. As described in Chapter 1, pup retrieval is when maternally experienced females locate pups that are separated and return them back to the home nest. One of the critical features that allow this behavior to occur are the pup calls that the pups emit. Therefore, we wanted to take advantage of this behavior and use it to probe what sounds mothers will recognize as pup calls.

Pup calls consist of groups of syllables within the ultrasonic range. The ultrasonic vocalizations (USVs) naturally exhibit variability in regards to their frequency, intersyllable interval (ISI), and syllable category, strongly suggesting that mothers are able to generalize their response across multiple calls (Liu et al. 2003; Takahashi et al. 2016). However, it remains unclear which of these features are important as well as how much variance within these features maternally experienced females can withstand while still recognizing the sound as a pup call. While some studies have hypothesized which features may be important, very few have behaviorally tested it (Liu et al. 2003; Castellucci et al. 2018). An early study which did behaviorally test this utilized a two-alternative choice task, where

they showed that mice have a preference towards USVs compared to low frequency band limited noise, but show no preference between USVs and high frequency band limited noise (Ehret and Haack 1981). While these results strongly suggest that surrogate females can group these sounds into the same category, one major downside is that the mouse is forced to make a choice between two options. Therefore, only a few stimuli can be compared together at once, as increasing the number of stimuli rapidly increases the number of comparisons that would need to be done. Perhaps more importantly, this experiment is naturally low throughput, as mice can quickly become habituated towards auditory stimuli if no reward is present (Scourse and Hinde 1973).

A recent study has looked to solve this dilemma by utilizing pups which have been cooled and, consequently, do not emit USVs (Schiavo et al. 2020). By doing so, they were able to conduct a large number of trials without having to worry about habituation, as the maternally experienced female was receiving a reward, the pup, in response to an auditory stimulus played from a speaker. The study highlighted the role of the ISI in pup call recognition, demonstrating that even when a pup call with an ISI almost 200ms shorter or longer than a prototypical pup call was played from the speaker, surrogate females would still go and retrieve a cooled pup nearby. We wanted to expand this work and identify other features, such as the frequency of the pup call or the presence of other low bandlimited noise that may impact the way in which maternally experienced females classify sounds as pup calls.

To do this, we developed a high throughput freely moving behavioral assay where we could probe which stimuli mice would categorize as a pup call. Using this behavioral task, we were able to train mice to selectively approach a speaker in response to a pup call

for a pup reward, but not to an 8 kHz tone. In doing so, we found that mice tended to approach the speaker when the sounds were spectrally more similar to the pup call.

2.2 Methods

2.21 Animals

Experiments were performed on female CBA/J (approximately 10-12 weeks old) mice. Mice were maintained on a 12/12 h light dark cycle (lights on 19:00) and provided with food and water ad libitum. Females were introduced to male mice when they were approximately 8 weeks old. After about 18 days, males were removed from the cage. Females typically dropped pups 21 days after males were introduced.

2.22 Behavioral Rig

The behavioral rig (18″ x 18″ x 18″) was made out of plexiglass. The rig was placed inside a sound proof chamber to prevent noise from the outside disrupting the experiment. Small holes were drilled in the plexiglass where the speaker was placed so that sound could pass through, but the mouse could not disrupt the speaker. Holes were also drilled near the wall that divided the arena so that sound could pass throughout the entire arena freely. During experiments, bedding and nesting material was placed within the rig.

2.23 Auditory Stimuli

Four different pup calls trains were used. These pup calls were recorded from postnatal day 1 pups using an ultrasound microphone (Avisoft). The four tones used were either 8 or 18 kHz and matched the temporal structure of the pup calls. Each catch trial was one of two stimuli, temporally modulated towards two of the pup calls. The 65 kHz tone, 45 kHz tone, 60-70 kHz broadband sound, and 20-70 kHz broadband sound were generated using Matlab. The 55 kHz pup call and 40 kHz pup call were generated using a phase vocoder within Matlab. All stimuli were normalized and presented at ~ 70 dB SPL from the home chamber.

2.24 Auditory Stimuli Presentation

Stimuli were presented using an ED1 Electrostatic Speaker Driver and an ES1 Electrostatic Speaker (TDT).

2.25 Data Analysis

All plots, data analysis, and statistics were done in MATLAB. Cumulative distribution plots were compared using a Kolmogorov-Smirnov test. Average response latency was compared using a 2-tailed t-test. Multiple comparisons tests were performed when appropriate.

2.3 Results

2.31 Designing the Behavioral Setup

When designing our behavioral rig, our primary goal was to design a set up where mice would approach a speaker if it heard a sound that was deemed appropriately similar to that of a pup call, and avoid approaching the speaker in all other instances. Furthermore, we also wanted to ensure that the behavior was high throughput by providing the mouse with a reward for approaching a pup call, as mice will quickly learn to stop approaching the speaker if no reward is given to the mouse.

Therefore, we designed a behavioral box that was partially separated into two parts using a wall (Figure 2.1). The behavioral box was placed within a sound proof chamber to prevent outside noise from interfering with the experiment. On one side, which we henceforth refer to as the home area, the base of the box was covered in bedding and the mouse was given nesting material. A small opening in the wall connected the home area to the second area, which contained a speaker. Following pup birth, mice were habituated to the behavioral box with their pups on postnatal days 1 and 2. Starting on postnatal day 3, we began the experiment. After a one-hour habituation period, 4 pups were scattered around the box and 1 was placed nearby the speaker to confirm that the mouse was able to retrieve pups back to the home nest. If the mouse successfully retrieved all 5 pups, the pups were removed from the box and was allowed to habituate for 5 minutes. Afterwards, one of four auditory stimuli, a pup call, tone, or one of two test stimuli, was presented and the mouse was given 45 seconds to approach the platform. If the mouse approached the speaker in response to a pup call, a pup was given to the mouse as a reward and the mouse was

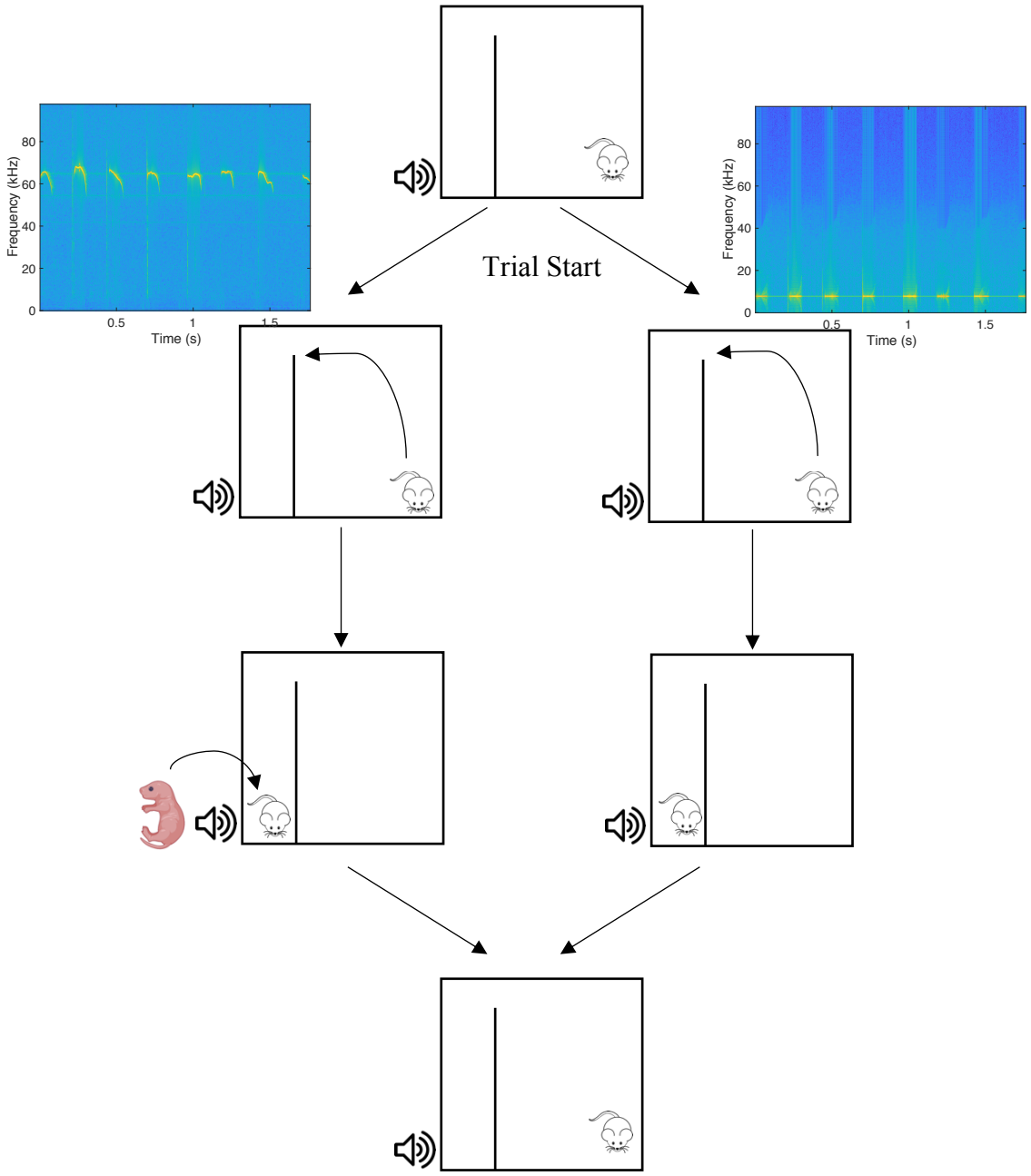

Figure 2.1: **Freely Moving Behavioral Paradigm**. Trials begin when the mouse is in the home area. An auditory stimulus is presented from a speaker located on the outside the rig. If the subject responds towards a pup call stimulus playing from the speaker, they are rewarded with a pup. Otherwise, responses towards any other stimulus are unrewarded. When the mouse returns back to the home area for at least 1 minute, the next trial begins.

allowed to return the pup back to the nest. If the mouse approached the speaker to any other stimuli, no reward was given. After the stimuli was presented and the mouse returned back to the home area for at least 1 minute, the next trial was manually started. After 40 trials or if the mouse failed to approach the speaker following five consecutive pup call trials, the session was concluded. This experiment was repeated on postnatal days 4, and 5.

Our initial attempts at designing the behavioral rig allowed the mouse to walk freely between the home area and the speaker. However, we quickly discovered that we were unable to observe any differences between the average time it took for mice to approach the speaker in response to pup calls compared to tones (Figure 2.2A). Based on our observations of the behavior, we suspected that this was due to mice walking between the home area and speaker too casually and that making this behavior more difficult may increase discrimination between the two stimuli. Therefore, we attempted to make the path to the speaker more difficult to dissuade the mouse from walking to the speaker, but not so difficult that the mouse would refuse to walk towards the speaker at all. We attempted several different iterations of this behavioral chamber, including shining a light in the hallway leading up to the speaker, as mice have an innate aversion towards bright areas, or adding Velcro to the floor, which would make walking slightly more uncomfortable. However, we found that both attempts were unable to deter mice from approaching the platform, as both instances showed no difference between the time to approach the platform in response to pup calls compared to a tone (Figure 2.2 B, C; paired t-test $p>0.05$).

We eventually decided to try and take advantage of the natural aversion mice have towards open and elevated areas, as often seen in elevated plus mazes (Komada et al. 2008). To do this, we placed a narrow walkway between the platform and the speaker (Figure 2.3A, Left). We found that when using this walkway, we were able to see modest differences

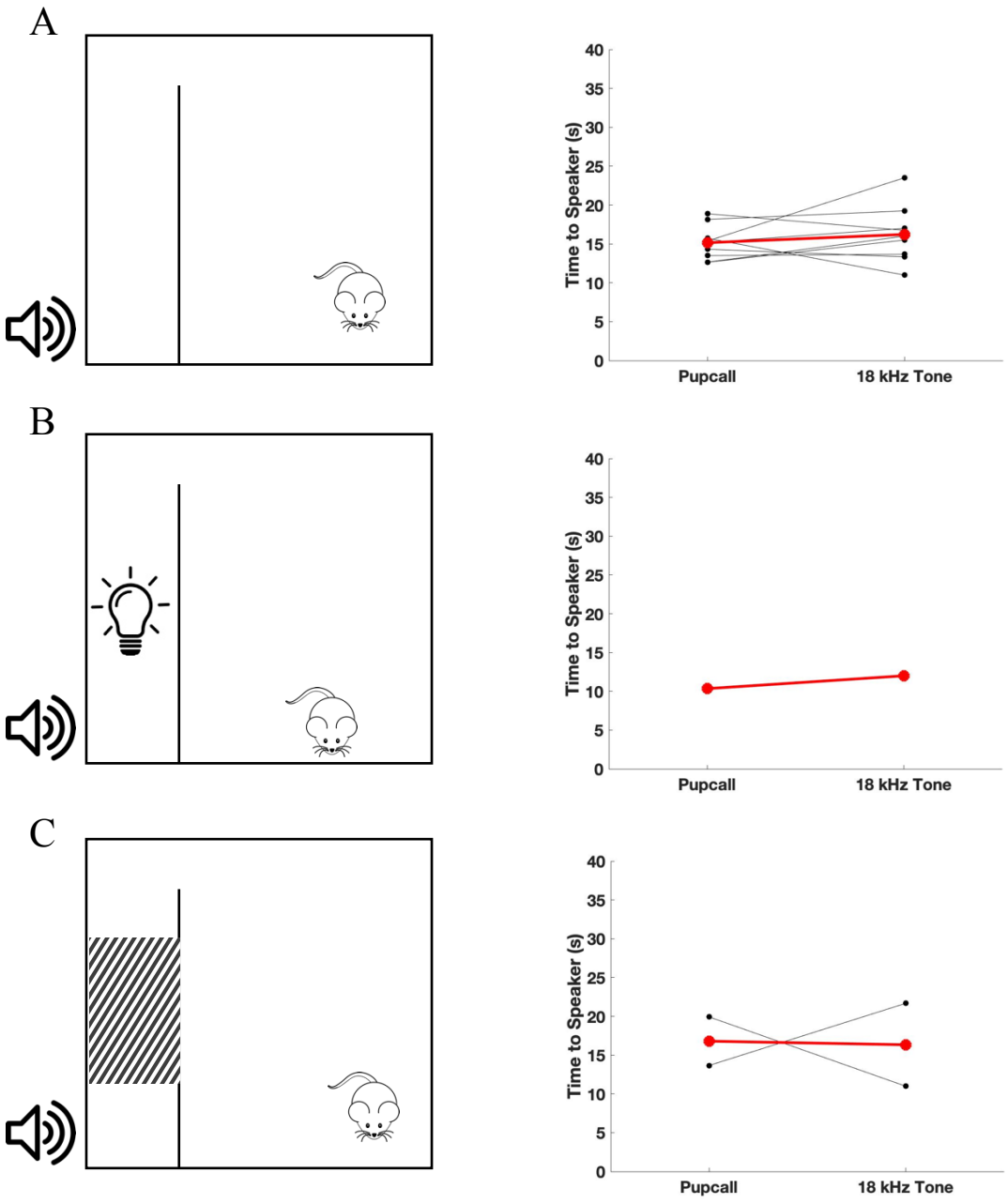

Figure 2.2: **Initial Attempts at Designing a Behavioral Rig.** (A) Left: Base behavioral box schematic. Right: Average time to approach speaker when auditory stimulus is presented. Black lines represent individual mice, red line indicate average of all mice (n=9). Pairwise t-test p>0.05. (B) Left: Behavioral box with light shining on narrow walkway schematic. Right: Same as A (n=2). (C) Left: Behavioral box with velcro on platform schematic. Right: Same as A (n=1). Pairwise t-test p>0.05.

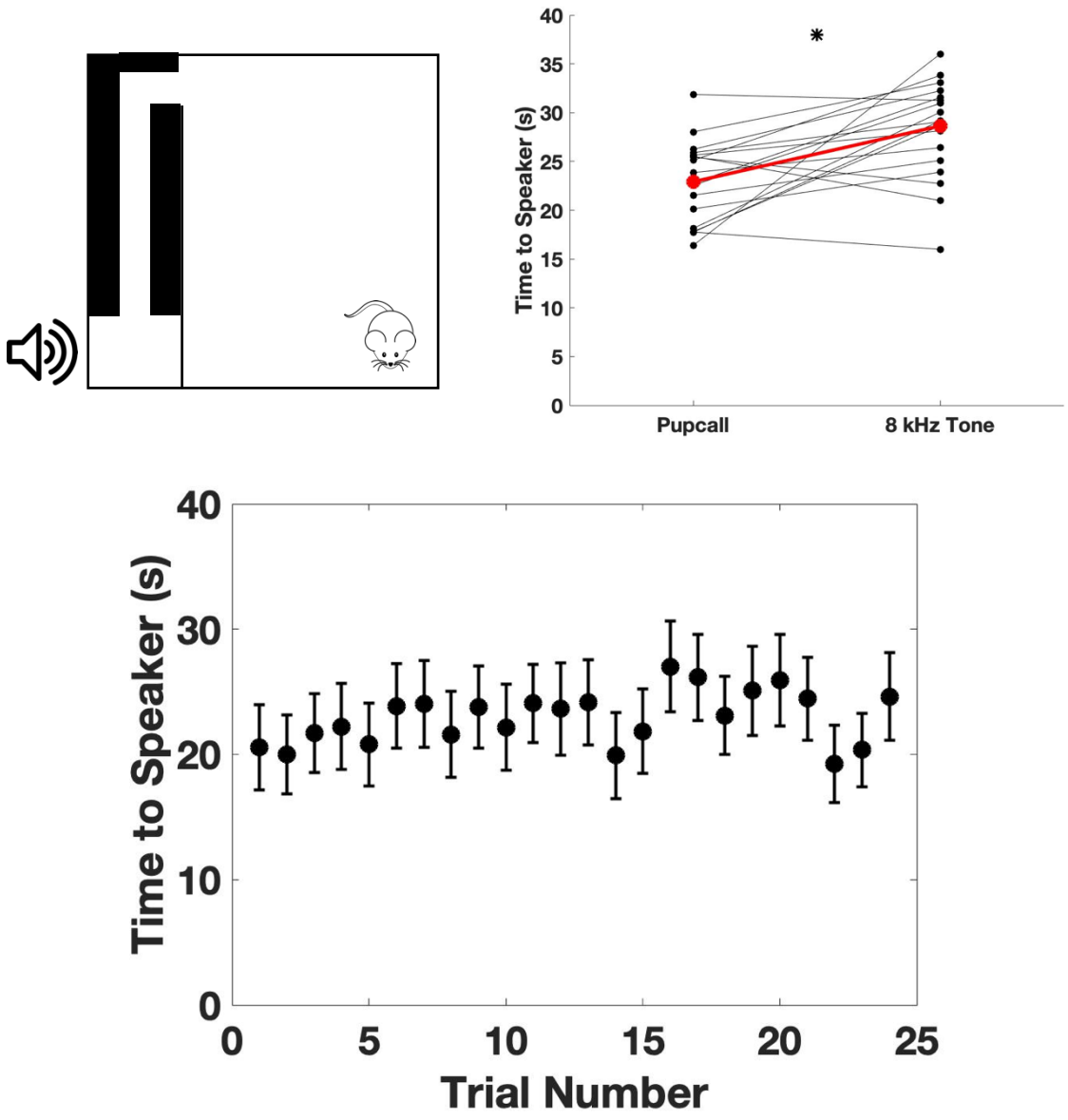

Figure 2.3: **A narrow walkway is sufficient to demonstrate differences between pupcalls and tones.** (A) Left: Design of behavioral box. Right: Average response time of mice to approach speaker. Black lines represent individual mice, red line indicates average of all mice (N=18). Pairwise t-test, $p<0.005$. (B) Average time to to approach speaker during pupcall trials, sorted by trial number during a session.

between responses towards pup calls and tones, suggesting that this iteration would allow us to see differences when we introduced catch trials in the future (Figure 2.3A, Right; paired t-test $p<0.05$). Furthermore, we found that mice took approximately 22 seconds to respond to pup calls across all trials within a session, demonstrating that our usage of 40 total trials was appropriate, and that mice were not getting slower or more tired within a session (Figure 2.3 B).

While adding an elevated platform helped us differentiate responses towards pup calls and tones, we found that surrogate females were unwilling to walk across the platform. This effectively made it so that surrogates would never walk towards the speaker, regardless of the stimuli being presented. Mothers generally tend to show less anxiety, which we were also able to observe based on their willingness to walk across the platform (Maestripieri and D'Amato 1991). Therefore, for future experiments in this chapter, we opted to use mothers.

2.32 Mice can Learn to Categorize Sounds with Natural Pup Calls

Three groups of six mice were presented with one of three different stimuli sets: those that included broadband noise, those that included pitch shifted pup calls, and those that included tones. These stimulus sets included one high frequency auditory stimuli and one low frequency auditory stimuli. Broadband sounds included a 60-70 kHz and 20-70 kHz band limited sound, pitch shifted pup calls included a 55 kHz and 30 kHz pitch shifted pup call, and tones included a 65 kHz and 45 kHz tone. Across the 40 trials presented within each session, each mouse heard 24 pup calls, eight 8 kHz tones, four of one high frequency catch trial, and four of one low frequency catch trial, with both catch trials being from the

same set. Stimuli were presented pseudo randomly such that within a block of 10 trials, every stimulus was heard at least once. Furthermore, all stimuli were temporally matched to one of the pup calls, such that mice would be unable to use the temporal structure of the stimulus to inform their decision.

We plotted cumulative probability plots of the time that mice took to respond to each stimulus for each trial. We also plotted the average response time mice took to respond to each stimulus. When we presented mice with pitch shifted pup calls, we found that on the first two days of experiments, mice responded to all stimuli similarly (Figure 2.4 A, B). However, by the third day of the experiments, we started to see differences emerge, as mice responded to the pup calls quicker than tones (Figure 2.4 C). While not statistically significant, we observed that mice seemed to be grouping the pitch shifted pup calls with the tone, suggesting that mice were separating them away from the natural pup call.

Next, we presented mice with tones of varying frequencies to see whether the spectrotemporal features of the call would be relevant for their responses. Similar to the pitch shifted pup calls, we found that mice tended to respond to all stimuli equally on the first two days of experiments (Figure 2.5 A, B). On the third day of the experiment though, we started to see signs of categorization as, based on the cumulative probability plots, mice were grouping the pup calls with the 65 kHz tone and the 45 kHz tone with the 8 kHz tone (Figure 2.5 C Left. Two-tailed Kolmogorov-Smirnov test $p>0.05$). Furthermore, when we looked at the average response across mice, we find that they responded towards both the pup call and the 65 kHz tone significantly faster than the 8 kHz tone, further supporting the notion that the high frequency tone was being grouped together with the pup call (Figure 2.5 C Right. One-way ANOVA $p<0.05$).

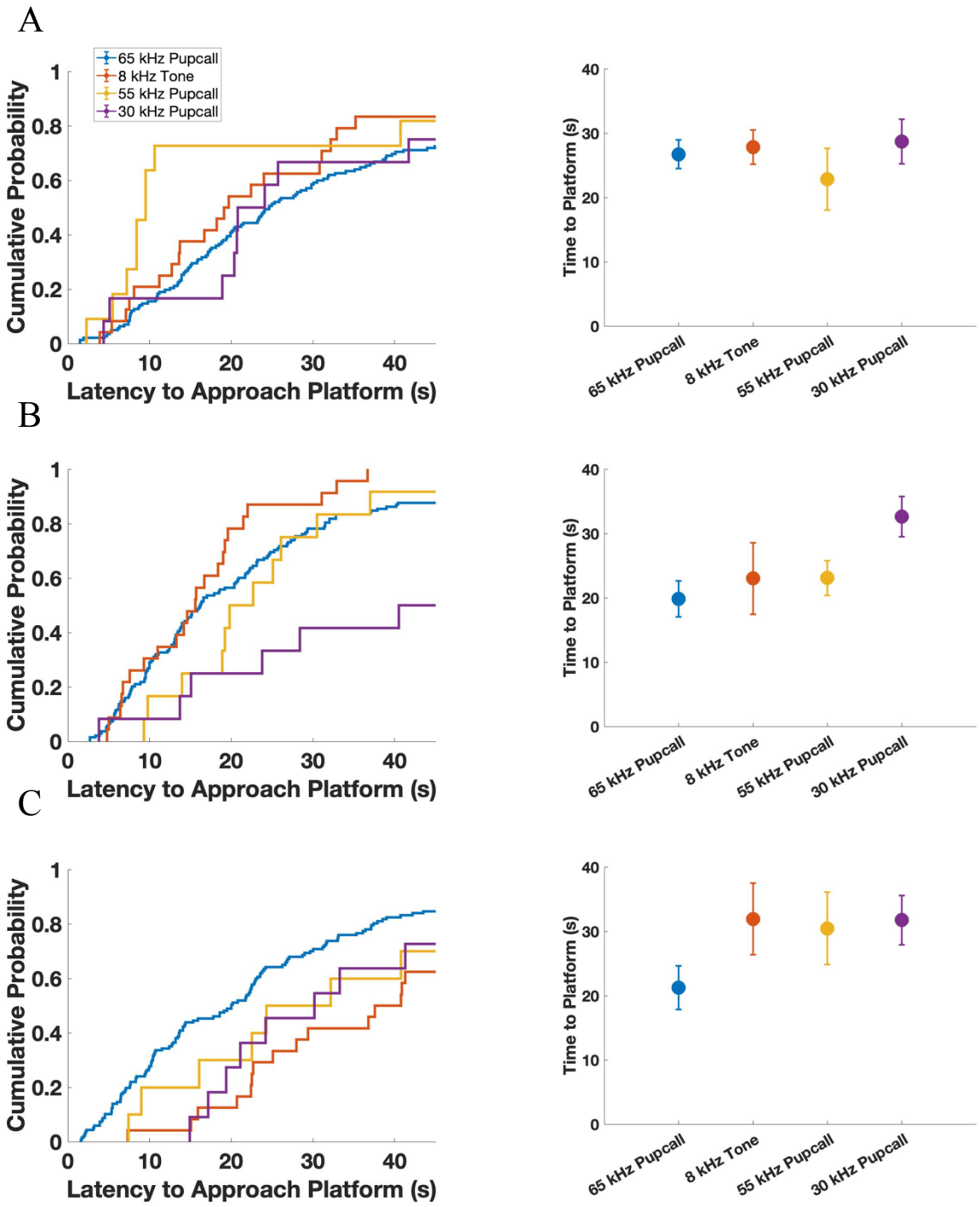

Figure 2.4: **Mice learn to differentiate pup calls from pitch shifted pup calls**: (A) Left: Cumulative probability plot for mice on the first day of the experiment for the broadband stimulus set. Each line represent a different stimulus and each point on the line represents a single trial. Right: Average time for mice to approach the platform where the speaker is located for each stimulus. Points represent average across 6 mice and errorbars represent standard error of the mean. (B) and (C): Same as A, but for experimental days 2 and 3.

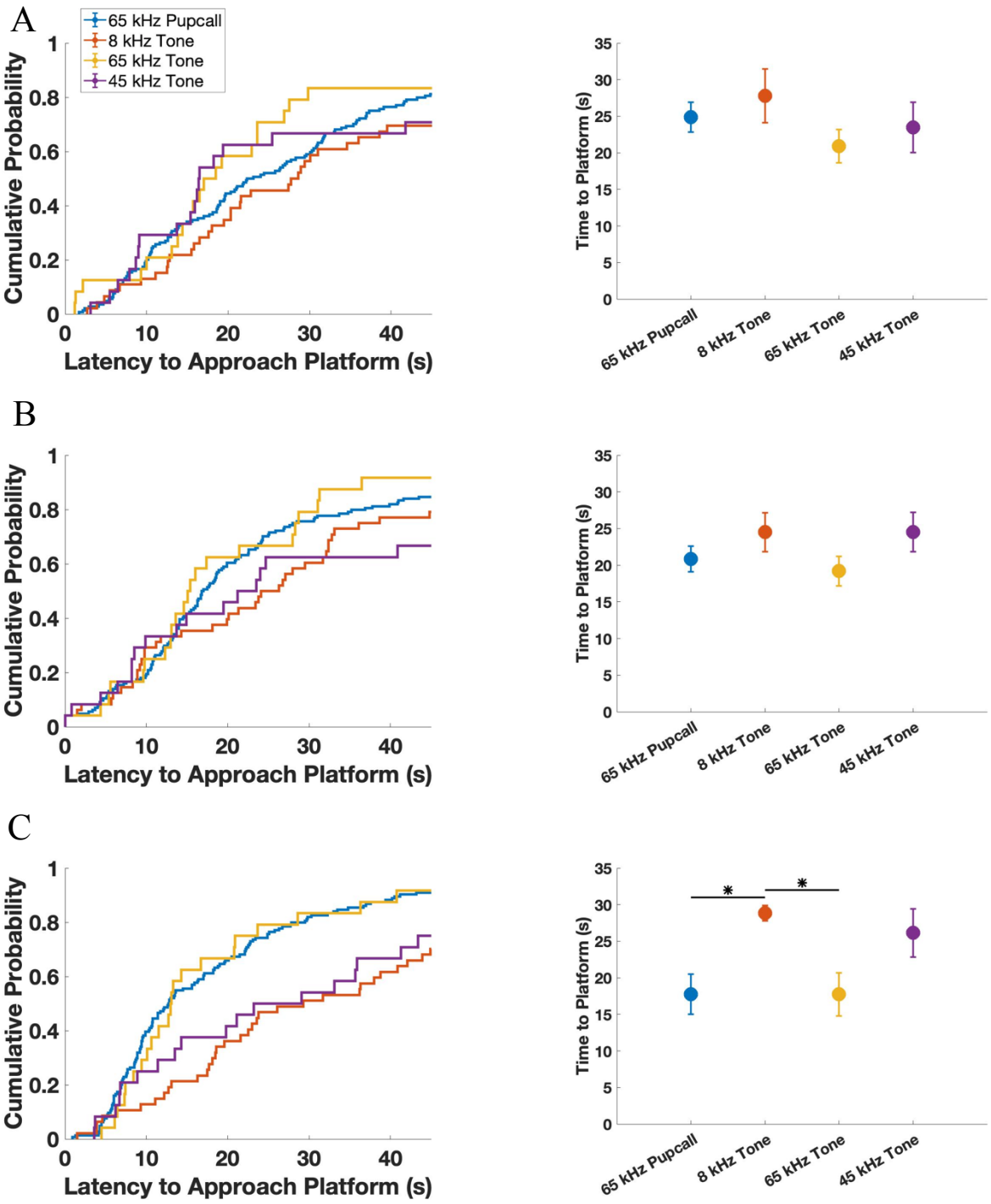

Figure 2.5: **Mice learn to group a 65 kHz tone with a natural pup call**: (A) Left: Cumulative probability plot for mice on the first day of the experiment for the broadband stimulus set. Each line represent a different stimulus and each point on the line represents a single trial. Right: Average time for mice to approach the platform where the speaker is located for each stimulus. Points represent average across 6 mice and errorbars represent standard error of the mean. (B) and (C): Same as A, but for experimental days 2 and 3, respectively. *P<0.05, one way ANOVA.

Finally, we presented mice with the broadband stimulus set. Again, much like the previous two stimulus sets, mice showed no difference in their response towards the stimulus on the first two days of the experiment (Figure 2.6 A, B). By the third day however, mice were responding towards the pup call significantly quicker than the tone (Figure 2.6 C, Right. One-way ANOVA $p<0.05$). Furthermore, when we looked at the cumulative distribution curves, we observed that the 60-70 kHz was significantly different than the curve of the tone and that the cumulative distribution curve of the 20-70 kHz band limited noise was also significantly different than the curve of the pup call (Figure 2.6 C, Left. Two-tailed Kolmogorov-Smirnov test $p>0.05$). Together, these results suggest to us that over time, mice learn to separate out the 8 kHz tone and pup calls from one another and that they are generalizing their responses towards other stimuli that they believe matches a pup call.

2.4 Discussion

In the work described above, mice were trained on a freely moving behavioral task where mice learned to respond to a pup call to receive a pup as a reward. Through multiple iterations of this behavior paradigm, we discovered that we were able to generate the best results when we used a narrow walkway to separate the home area from the speaker. While mice initially responded to all stimuli equally, we found that over time, mothers became more selective in their responses and learned to approach the speaker more often towards pup calls and less often towards the 8 kHz tone. Using this behavioral paradigm, we were able to identify that mice generally responded to those stimuli with high frequencies, while responding less to all other stimuli. Overall, these results highlight the ability for pups to be

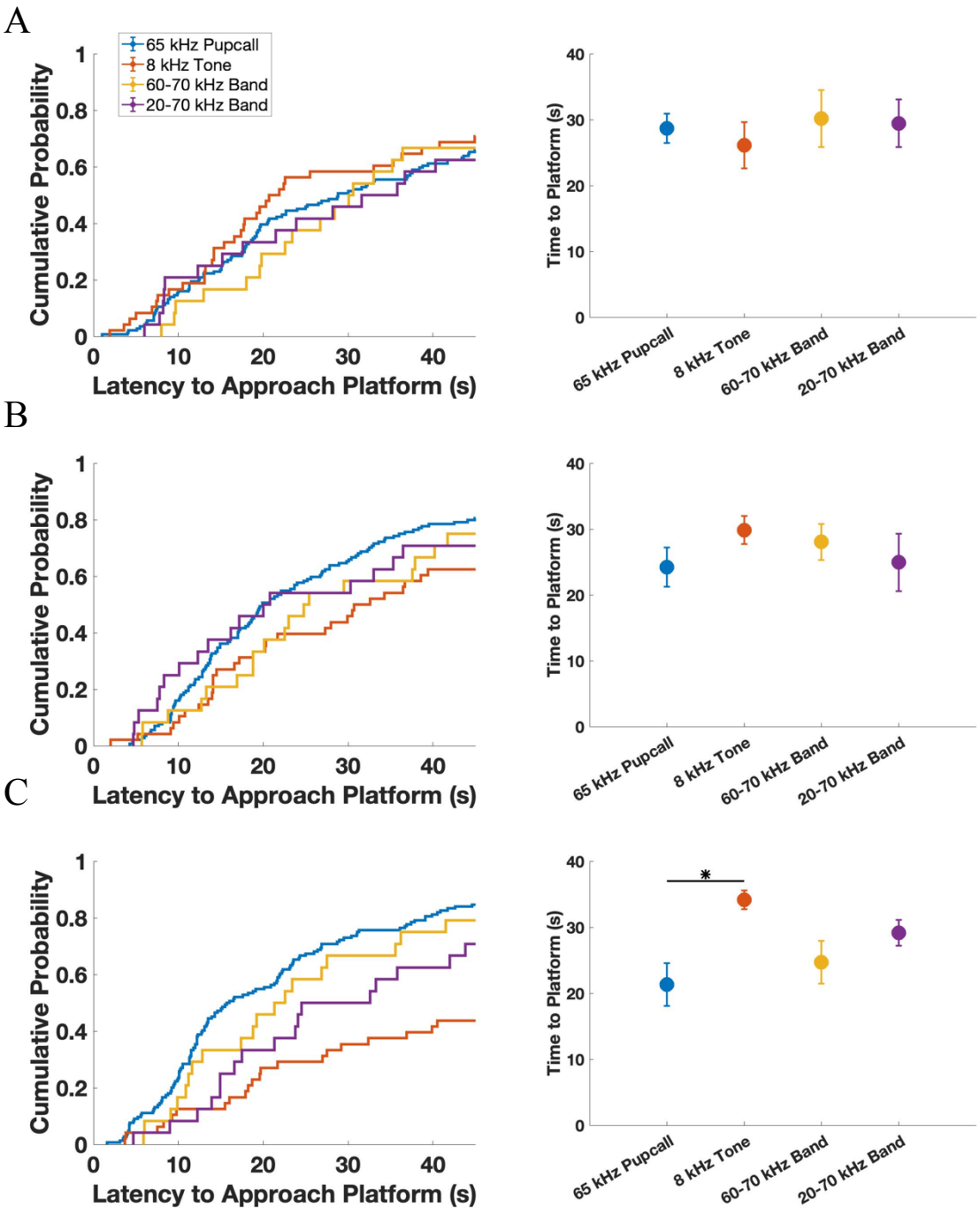

Figure 2.6: **Mice learn to group certain broadband stimuli with pup calls**: (A) Left: Cumulative probability plot for mice on the first day of the experiment for the broadband stimulus set. Each line represent a different stimulus and each point on the line represents a single trial. Right: Average time for mice to approach the platform where the speaker is located for each stimulus. Errorbars represent Standard Error of the Mean. (N=6). (B) and (C): Same as A, but for experimental days 2 and 3. *P<0.05, one way ANOVA.

used as a positive reinforcement tool, as well as which stimulus mothers may classify pup calls with.

Previous studies had suggested that frequency may be one of the more critical features of an auditory stimulus that mice use to categorize sounds (Chen et al. 2021). Our results here support those studies, as evidenced by the strong preference towards the 65 kHz tone. while also demonstrating that the other spectrotemporal features of the call may not be critical. Furthermore, studies have also demonstrated how pup contact can elicit a dopamine response, demonstrating that pup retrieval may be positively rewarding. (Curry et al. 2013; Xie et al. 2022). By demonstrating that mice improve over time to a pup call reward, we show behaviorally that pups can act as positive reward for mice, a result that supports previous work (Besosa et al. 2020).

Most previous studies have used surrogates in their experiments. This is because pregnancy itself has been associated with a number of changes in the brain, in regards to both hormones, such as prolactin, and gene expression (Ray et al. 2015; Ladyman et al. 2021). Nonetheless, we still feel that our use of mothers is still appropriate as both mothers and surrogate females have demonstrated the ability to retrieve pups quickly and with few errors (Krishnan et al. 2017). Furthermore, both mothers and surrogates will utilize pup calls to retrieve pups, suggesting that both are able to group other stimuli into the same category as pup calls. Despite this, we recognize that differences may still exist in regards to the sensitivity in which mothers and surrogate females categorize these sounds. To investigate this further, experiments could be done which provide an increased habituation time for surrogates within the behavioral chamber, or using a boundary between the home area and the speaker that is less anxiety-inducing.

Furthermore, we saw gradual improvement of the behavior over time, which might suggest that longer experimental trials would lead to further improvements in our results. We opted to do three days of experiments as by the fourth day of the experiment, which correlates with postnatal day 6, mice typically move around a lot and would often fall off the platform. To combat this issue, we could try and rotate the pups we use to always use younger pups, as mice were able to retrieve pups that were not their own (Wu et al. 2014)

These results provide us with valuable insight in regards to which sounds maternally experienced mice will associate with pup calls. We expand on these results by presenting more stimuli in a head-fixed manner, allowing us to improve the contrast between different categories as well as present an increased number of stimuli (Chapter 3). Furthermore, we explore changes in the auditory cortex that lead to these differences (Chapter 4).

Chapter 3

Categorization of Pup USVs using a Go No-Go Task

3.1 Introduction

Early work comparing pup calls and broadband stimuli showcased an extremely sharp, categorical boundary between 37.5-60 kHz bandlimited noise and 36-60 kHz bandlimited noise (Ehret and Haack 1981). More recent work looking at changes in the ISI between syllables also seems to show sharp boundaries behaviorally, as mice often responded towards pup calls with an ISI between 75 and 375 ms, but responded infrequently pup calls with an ISI of 575 ms (Schiavo et al. 2020). While I demonstrated in the previous chapter that, following training, mice were able to separate pup calls and tones from one another, we were unable to present a large variety of stimuli, largely because we only presented eight catch trials per session. This severely limited the number of catch trials we were able to present to each individual subject. Consequently, this made it difficult to see how a spectrum of stimuli that varied along a single axis were perceived by mice and whether those perceptions were categorical or not. In addition to this, despite our best efforts our data still demonstrated high variability, as mice still approached the speaker in response to a tone both often and relatively quickly.

Therefore, in order to push these results further, we wanted to use a higher throughput method that would also allow us to present both a larger variety of stimuli as well as a greater number of stimuli. To this end, we trained both naïve and surrogate

females to perform a Go No-Go (GNG) task where mice were asked to lick in response to a pup call for a water reward and withhold licking in response to a tone to avoid a time out. We then presented a number of unrewarded, untrained stimuli to probe how the mouse would respond to them and, in turn, what sounds they would group with the pup call.

Overall, the results described in this chapter demonstrate that mice are able to utilize a Go No-Go task to separate pup calls from a tone. When faced with catch trials, we find that generally, naïve and surrogate females respond systematically with how different the catch trials were from the pup calls, using a number of spectrotemporal features of the auditory stimuli to drive their decision. Furthermore, we find that the presence of 60-70 kHz band limited noise was sufficient for mice to regularly group sounds as pup calls, while the addition of lower frequency band limited noise inhibited the mouse's response. Finally, although we found no differences in how naïve and surrogate females responded to the catch trials, we did see differences in how mice responded to early catch trials compared to later ones.

3.2 Methods

3.21 Mice

Experiments were performed on female CBA/J mice (approximately 10-12 weeks old at start of training). Mice were maintained on a 12/12 h light dark cycle (lights on 7 PM) and provided with food ad libitum. Mice were provided with a minimum of 1 ml of water each day.

3.22 Surgery

Mice were headfixed with headbars using dental acrylic. Mice were anesthetized with an 80:20 mixture (1.25 ml/kg) of ketamine (100 mg/ml) and xylazine (20 mg/ml). A 1:10 mixture of meloxicam was used as an analgesic and was given to the mice immediately prior to surgery and on the following two days. Following surgery, mice were water deprived for one week before beginning behavioral training.

3.23 Auditory Stimuli

Auditory stimuli were collected and generated in the same manner as described in Chapter 2.

3.24 Auditory Stimuli Presentation

Stimuli were presented in the same manner as described in Chapter 2. Stimuli were presented at 70 dB SPL.

3.25 Behavioral Training

Naïve female mice were trained on a Go No-Go task where they were asked to lick in response to a Go cue, one of four pup calls, and withhold licking in response to a No-Go cue, one of four temporally modulated 8 kHz tones. If the mouse licked correctly to the Go cue, she was rewarded with a small amount of water (~3 ul). If the mouse incorrectly licked

to the No-Go cue, the mouse was punished with a timeout and unable to continue the session for 7 seconds. Stimuli were presented every 7.5 seconds and mice were required to withhold licking for at least 1.5 seconds before the next trial would begin. Prior to behavioral training, naïve females were water deprived for at least 1 week and allowed to habituate on a rotating wheel for ~ 30 min each day.

After 1 week of water deprivation, training began (Figure 3.1 A, B). First, we introduced the lick spout to the mouse and presented a series of Go and No-Go cues to her. Water was presented to the mouse following the presentation of the Go Cue regardless of her response, and no punishment was given. After two days, the difficulty of the task was increased, where half of all Go cue and No-Go stimuli required licking or withholding of licking to receive a reward/avoid a punishment, respectively. After two more days, mice were required to lick to the Go Cue to receive a reward and withhold licking to the No-Go cue to avoid a punishment.

Once mice had reached criteria on this behavior, which we marked as achieving an 80% hit rate and 80% correct rejection, we made the task probabilistic, whereby licking to the Go Cue did not guarantee a reward. This was done so that when catch trials were introduced, which were always unrewarded and unpunished, mice would be unable to determine whether those particular stimuli were from an unrewarded trial, or whether it was a catch trial. To train mice on this, 10% of trials were made probabilistic. After reaching stable criteria for two consecutive days, the task was made 20% probabilistic. Mice were again checked to ensure they reached stable criterion for 2 consecutive days.

Finally, mice were given water ad libitum for 3 days and separated into two groups, naïve and surrogates. Mice grouped as naïve females were water restricted again. The surrogate group was introduced to pups for 30 minutes for 3 days. At the end of the third

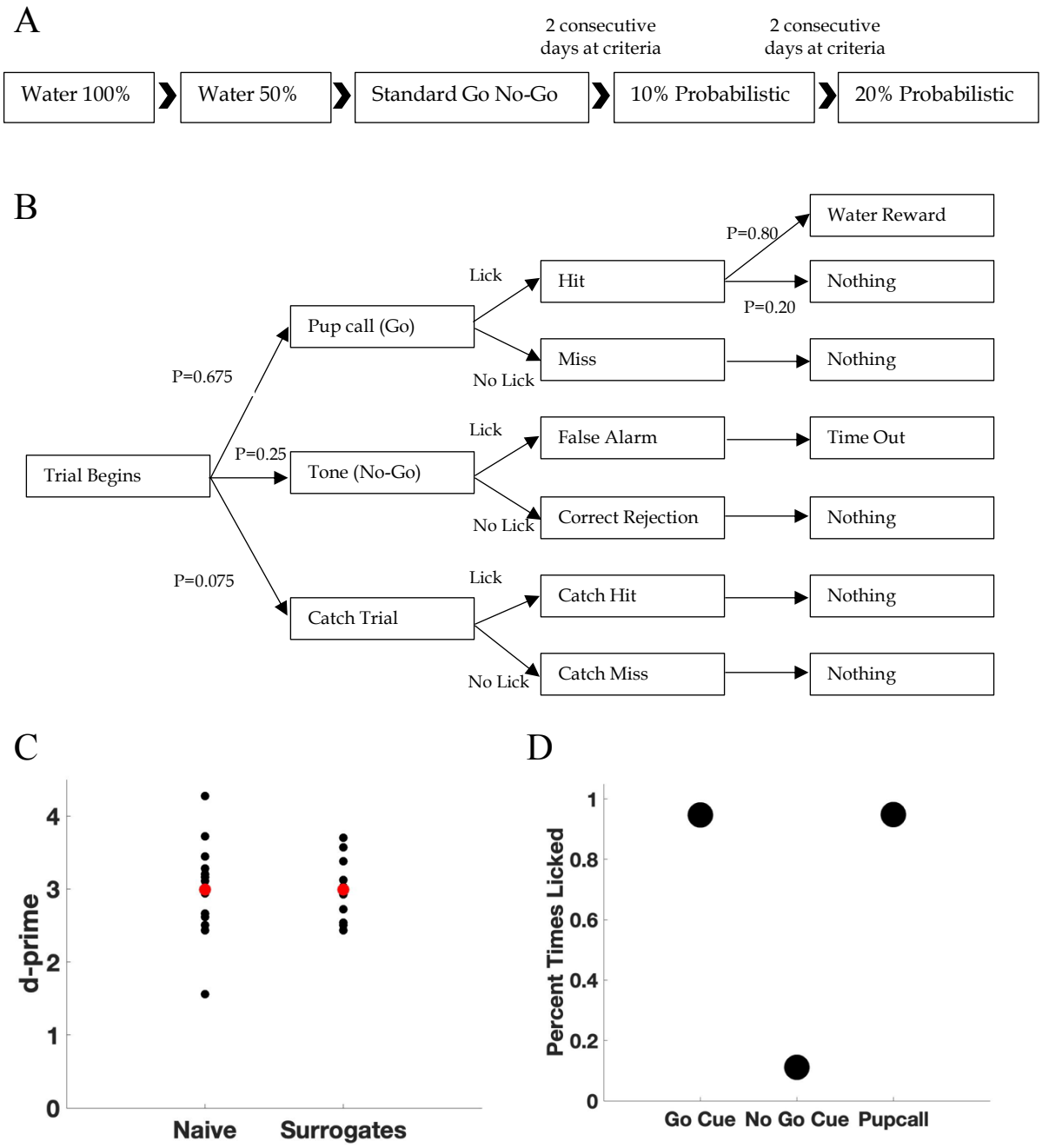

Figure 3.1: **Mice learn to do a Go No-Go task and generalize their responses**: (A) Behavioral training regimen. Mice were trained across several training blocks (described in section 3.25) before the experiment began. (B) Flow chart showing the possible outcomes for each trial, with percentages. (C) Mice learn to distinguish between pup calls and tones. D-prime (d') is plotted for each mouse across the experiment. Black dots represent individual mice, red dots represent average of all mice. (Mean d' for naïve: 2.99 ± 0.18; n=12. Mean d' for surrogate: 2.99 ± 0.14; n=14). (D) Mice can generalize the task to other stimuli. Black dots represent average represent average. n=26. (Mean percent licked across stimuli from left to right: 94.7 ± 0.6%, 11.1 ± 1.5%, 94.9 ± 1.4%)

day, mice were checked for their ability to retrieve pups, which was used a proxy for surrogacy. If they were able to retrieve the pups, the mice were water restricted again and, once they reached stable criterion on the probabilistic Go No-Go task again, the experiment was started.

3.26 Data Analysis

Data was collected using Spike2 (Cambridge Electronic Design). Plots, data analysis, and statistics were done in MATLAB. Comparisons were computed using either a paired t-test or a two-tailed t-test. Multiple comparisons tests were performed when appropriate.

3.3 Results

3.31 Mice are Able to Perform a GNG Behavioral Task and Generalize Responses Towards Other Stimuli

Naïve female mice were water restricted before being trained to lick in response to a Go cue for a water reward and withhold licking in response to a No-Go cue to avoid a time out punishment. In our experiments, the Go cue was one of four 65 kHz pup calls and the No-Go cue was an 8 kHz tone temporally modulated to one of the pup calls. This was done so that mice would be unable to use the temporal features of the stimulus to inform them of their decision. The task was made to be probabilistic, so that even if the mouse licked correctly towards the Go cue, there would only be an 80% chance a water reward was given. This was done to both make the task more challenging and to make it more difficult for mice

to learn how to respond towards future catch trials (explained in more detail in section 3.32). Mice were trained on this task until they had an 80% hit rate and an 80% correct rejection rate for 2 consecutive days. Afterwards, we separated mice into a naïve and surrogate group, where the surrogate group was allowed to interact with pups for 1 hour each day for 3 days. We found that following behavioral training, mice were able to perform this behavioral task well, which we measured by looking at the discriminability index (d'), a measure which effectively compares the hit rate to the false alarm rate for each mouse. We looked at all trials during the experiment and found that both naïve and surrogate were able to perform the task well, with naïve females having an average d' score of 2.99 ± 0.18 and surrogate females having an average d' score of 2.99±0.14 (Figure 3.1C).

We also wanted to ensure that our mice were capable of generalizing their responses towards other stimuli. To check this, we presented mice with pup calls that they were not previously trained on. These stimuli, and all other future catch trials, were never rewarded or punished, so mice received no feedback based on their response towards these stimuli. Nevertheless, we found that when presented with these unrewarded pup calls, mice responded to them 94.9%± 1.4% of the time which was a similar rate when compared to their response towards the Go Cue (Figure 3.1D, paired t-test, $p>0.05$). Taken together, we concluded that mice were able to perform this Go No-Go task and that they are able to generalize their responses towards other stimuli to match the categories of previously learned stimuli.

3.32 Hit rate and Lick rate were Equally Informative in a GNG Task

We next set out to see how mice responded to various other auditory stimuli. We presented four groups of auditory stimuli: high broadband noise, low broadband noise, pitch shifted pup calls, and pitch shifted tones (Figure 3.2). Much like the untrained pup calls from the previous section, mice were never rewarded nor punished for responding to these stimuli. To prevent mice from learning that licking to these stimuli would not result in a reward, in addition to the behavior being probabilistic, we presented these catch trials extremely infrequently (7.5% of all trials). A minimum of thirty trials per stimulus was presented to each mouse across the entire experiment.

The most natural way for us to analyze this data was to look at the percentage of times mice responded towards each auditory stimulus. However, we postulated that lick rate may be a more informative measure, as licks per second is a more continuous measure than hit rate. This would provide insight into the confidence of mice in how they group stimuli. For instance, mice may impulsively lick towards the start of an auditory stimulus which it does not classify with the Go-Stimuli, but stop licking shortly afterwards. While these results would show up as a hit because the mouse licked to the stimulus, we would be able to see a decreased lick rate compared to a stimulus that the mouse continued to lick towards. However, we generally found that across all mice, hit rate and lick rate were equally informative in how mouse responded towards the catch trials (Figure 3.3). Therefore, we opted to use hit rate as our measure of responsiveness for the remainder of this chapter.

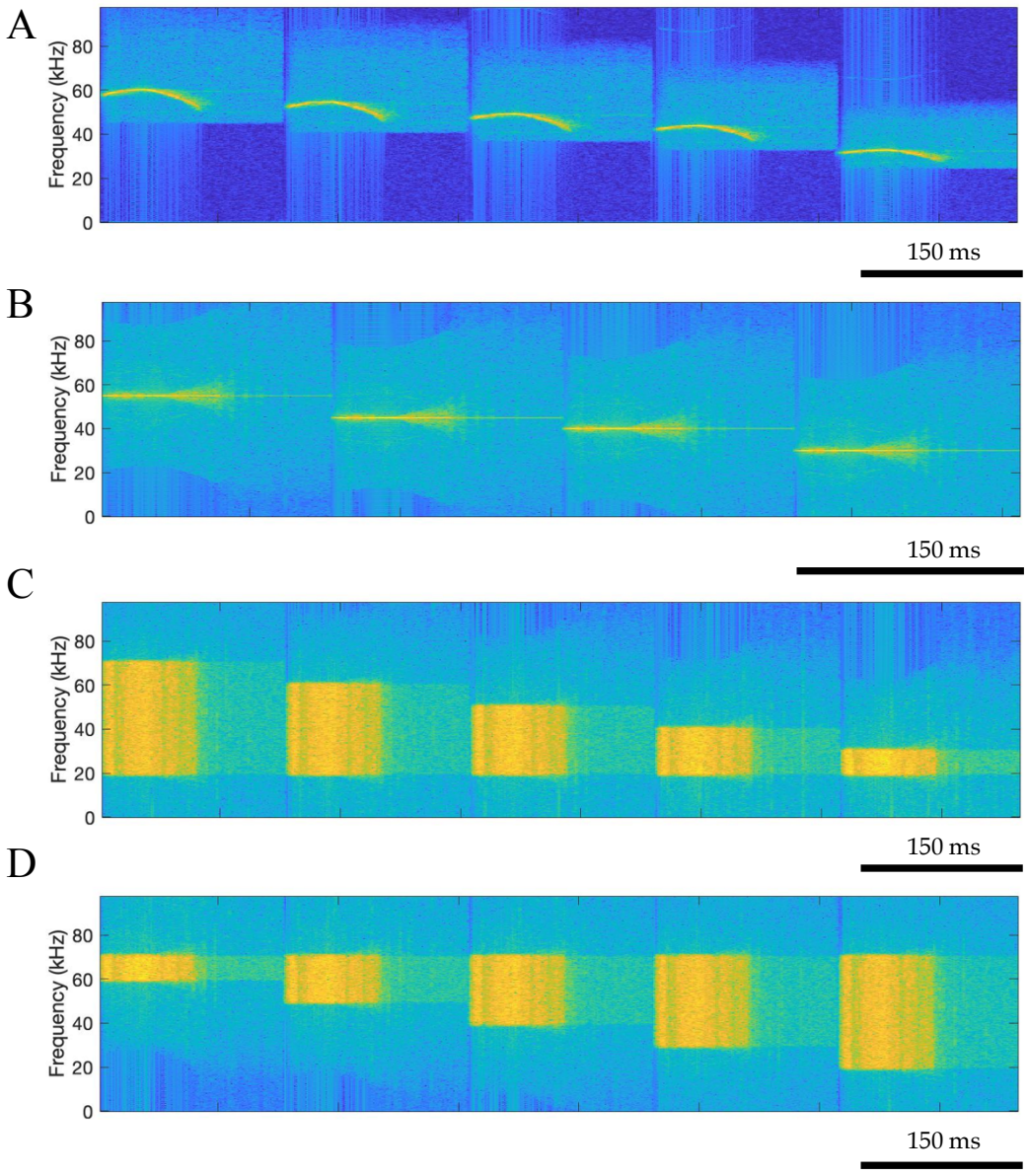

Figure 3.2. **Auditory stimulus sets used during experiment**: (A) Pitch shifted pup calls. From left to right: 55 kHz, 50 kHz, 45 kHz, 40 kHz, 35 kHz. (B) Pitch shifted tones. From left to right: 65 kHz, 55 kHz, 50 kHz, 45 kHz. (C) High broadband stimuli set. From left to right: 60-70 kHz, 50-70 kHz, 40-70 kHz, 30-70 kHz, 20-70 kHz. (D) Low broadband stimuli set. From left to right: 20-70 kHz, 20-60 kHz, 20-50 kHz, 20-40 kHz, 20-40 kHz. Black bars represent 150 ms.

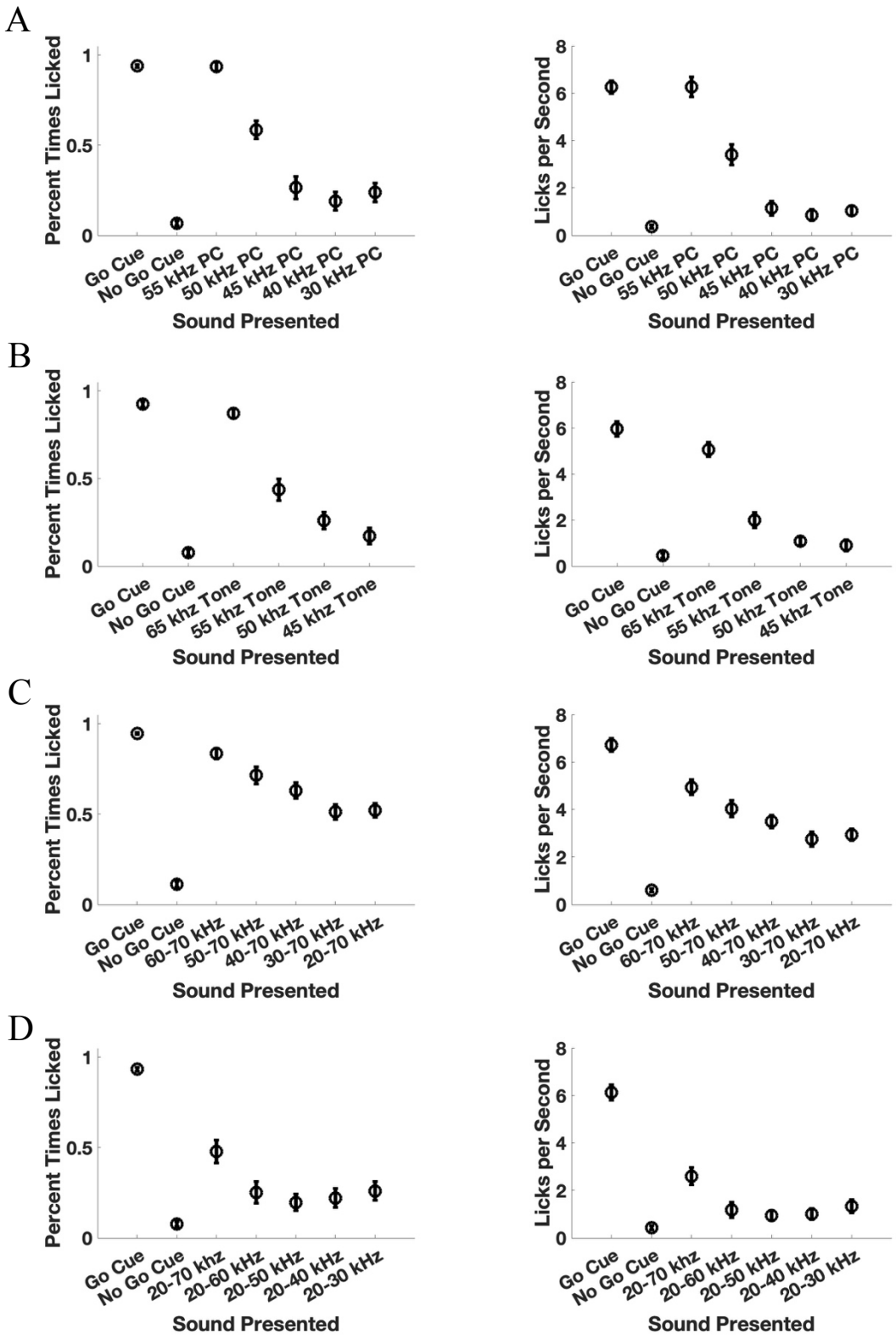

Figure 3.3: **Hit rate and licks per second are equally informative.** (A) Hit Rate in response to pitch shifted pup calls. Right: Licks per second in response to pitch shifted pup calls. (B, C, D) Same as A, but for tones, high frequency broadband noise, and low frequency broadband noise, respectively.

3.33 Mice Respond Systematically towards Pitch Shifted Pup calls and Tones

First, we presented mice with pup calls that had been pitch shifted downwards. We plotted the percentage of times naïve and surrogate mice responded towards each auditory stimulus (Figure 3.4A). We found that mice responded towards the 55 kHz pup call 93.6± 2.4% of the time, which was statistically similar towards their response to the Go Cue (Figure 3.3A; paired t-test, $p>0.05$). This response quickly drops off with mice only responding to the 30 kHz pup call 23.8±5.2% of the time.

Interestingly, when we presented pitch shifted tones, we saw similar trends compared to the pitch shifted pup calls but not at the same frequency levels as those of the pup calls (Figure 3.4B). More specifically, we found that mice responded to the 65 kHz tone 87.1 ± 2.6% of the time. As the frequency of the tone decreased, mouse responses started to decrease as well, responding only 17.2 ± 4.7% of the time to the 45 kHz tone. While the curve looks similar between the pitch shifted pup calls and tones, we observed that there was a significantly higher response towards the 55 kHz pup call compared to the similarly pitched tone (Figure 3.4C; paired t-test, $p<0.05$). This demonstrates that beyond just the frequency being important, the spectrotemporal features of the call are also important for the mouse in how it categorizes these stimuli.

While we presented these sounds to both naïve and surrogate females, we actually found no significant differences between how the two groups of mice. These results held true not just for the pitch shifted pup calls and tones, but for all other stimuli that we presented (Figure 3.4A; 3.4B; 3.6A; 3.6B). However, while we did not see any differences between surrogate and naïve females, we did see differences in how mice responded to

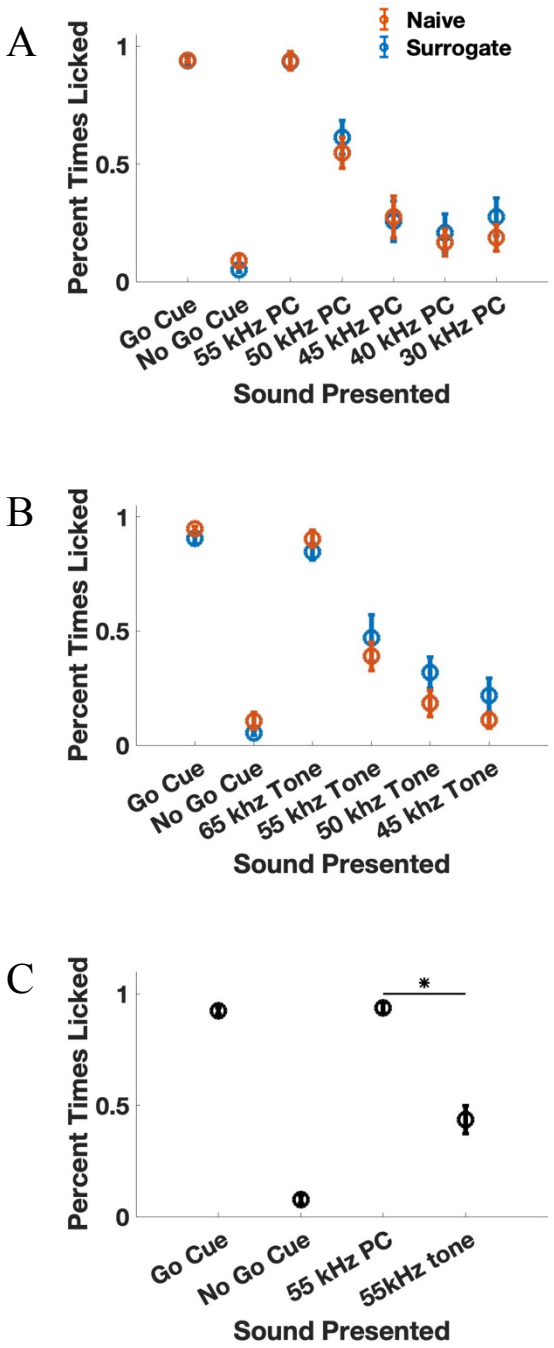

Figure 3.4: **Mice categorize based on the frequency and the spectrotemporal features of the call**. (A) Percentage of times mice licked to each pitch shifted pup calls between Naïve (Red; n=6) vs Surrogate (Blue; n=8). (B) Same as A, but for pitch shifted tones. (C) Mice respond differently towards similar frequency pup calls and tones. Circles represent mean, and error bars represent standard error of the mean. *p<0.05, 2-tailed t-test.

early trials compared to later trials, as mice responded less frequently towards later trials compared to early one. Once again, these results held true not just for the pitch shifted pup calls and tones, but also for the other presented auditory stimuli as well (Figure 3.5A) We quantified these results by averaging the response of all mice to the first 15 trials of each catch trial and the last 15 trials of each catch trial (Figure 3.5B). Doing this, we found a significant difference between the hit rate of the early trials compared to the late trials (paired t-test, $p<0.05$)

3.34 Low Frequency Band Limited Noise Inhibit Behavioral Responses

Next, we wanted to see how mice responded towards broadband stimuli (Figure 3.6A). Previous work had demonstrated that mothers would respond equally to pup calls and certain broadband stimuli, and we wanted to see whether we could replicate those results in our experiments (Ehret and Haack 1981). When mice were presented with a 60-70 kHz band, which was the same frequency range that our pup call Go-cues exist in, mice responded $83.5 \pm 3.0\%$ of the time. As the bandlimited noise became larger, mice tended to respond less and less, as we would have expected given that the stimulus was becoming less similar to the Go-Cue. However, interestingly enough, even at the largest band size (20-70 kHz), mice still responded almost $52.1 \pm 3.9\%$ of the time, significantly more than the No-Go stimulus (paired t-test, $p>0.05$). These results suggest that the mere presence of a high frequency band is sufficient in driving a response.

To test this idea further, we presented mice with progressively smaller bands, each missing the upper 60-70 kHz band (Figure 3.6 B). We found that, as soon as we removed the upper frequency band, mice began to respond much less, licking towards the 20-60 kHz

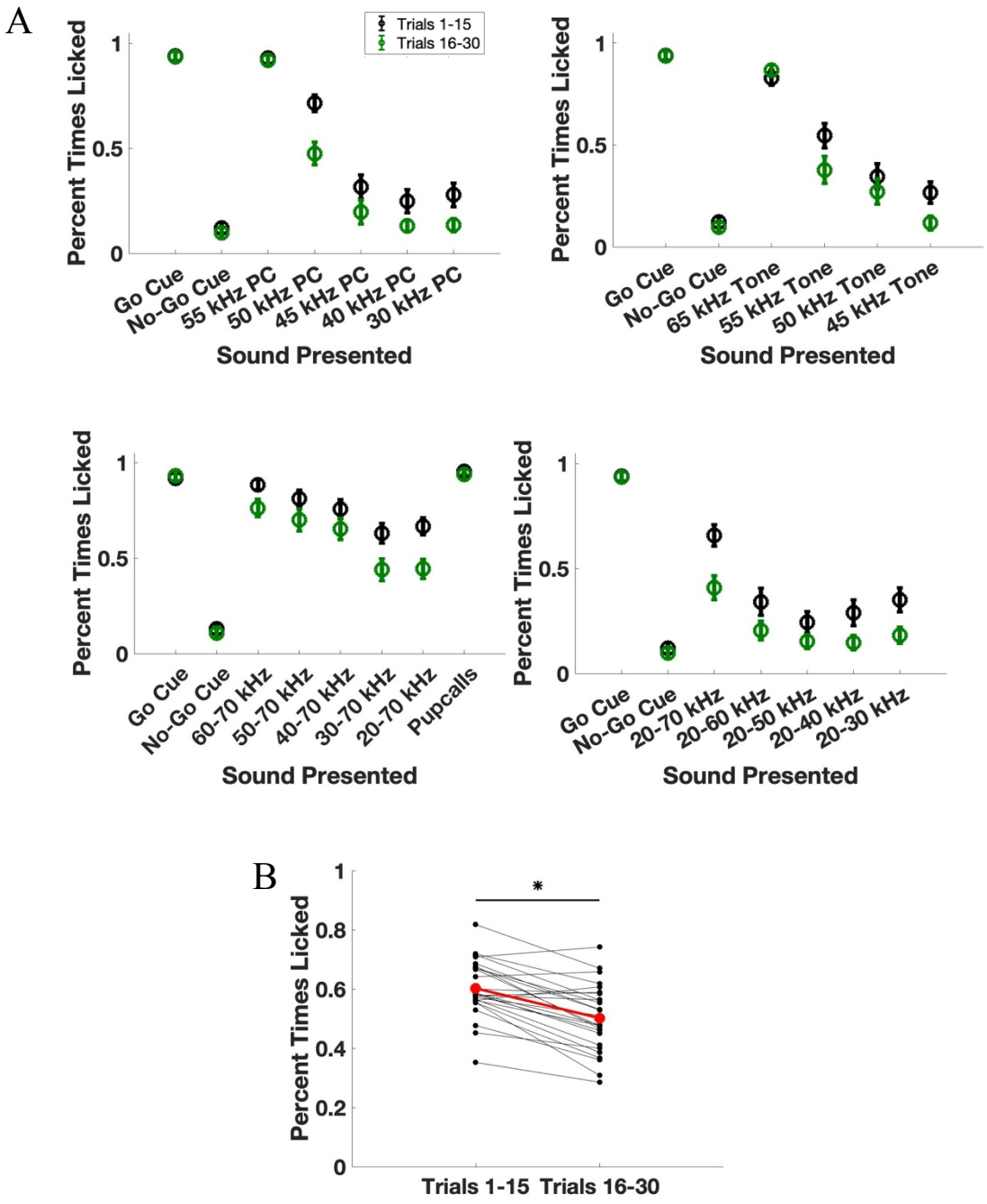

Figure 3.5: **Mice lick more to early catch trials than late catch trials.** (A) Percentage of times mice licked auditory stimuli between first 15 trials (Green) vs last 15 trials (Black). n=26 for bottom left, n=14 for rest. Circles represent mean, and error bars represent standard error of the mean. (B). Average response towards first 15 catch trials and last 15 catch trials (n=26, * $p<0.05$, paired t-test)

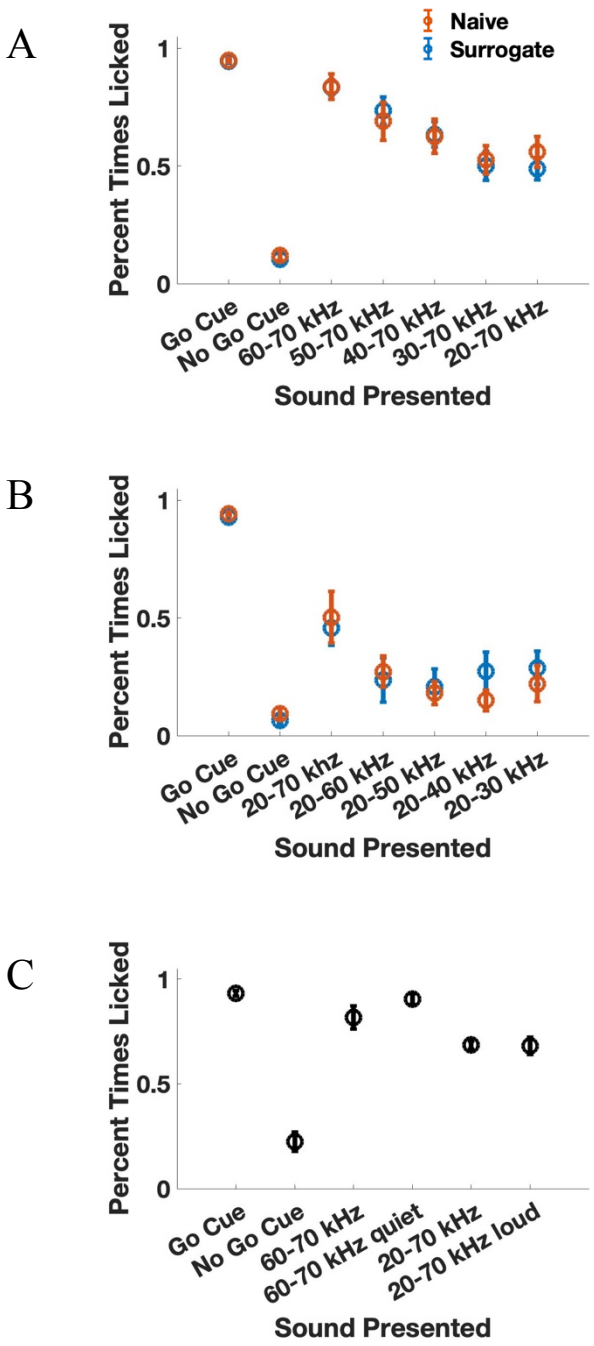

Figure 3.6: **Low frequency bands inhibit responses towards auditory stimuli**: Response towards high frequency broad band stimuli in Naïve (Red; n=12) vs Surrogate (Blue; n=14). Circles represent mean, and error bars represent standard error of the mean. (B) Same as A, but for low frequency broadband stimuli (Naïve n=6, Surrogate n=8). (C) Response towards 60-70 kHz bandlimited noise scaled 5x quieter and 20-70 kHz broadband stimuli scaled 5x louder. Circles represent mean, and error bars represent standard error of the mean. n=4.

band only 25.1 ± 6.0%of the time. These responses continued to stay low as the bandlimited noise got smaller, strongly supporting our hypothesis that the presence of a high frequency band was sufficient to drive a response.

As we changed the size of the bandlimited noise, we made sure that the overall power of the band was the same throughout. This meant that large bandlimited sound, like the 20-70 kHz stimulus, had less power in the upper frequency range than the smaller bandlimited noises, like the 60-70 kHz stimulus. Therefore, we wanted to identify whether the decreased response to larger bands was due to the presence of lower frequency bands or whether it was due to decreased power in the upper frequency bands. To test this, we presented a group of mice with a 60-70 kHz stimulus that overall had five times less power and a 20-70 kHz stimulus that overall had five times more power compared to the 60-70 kHz and 20-70 kHz stimulus we presented earlier (Figure 3.6 C). We found that there was no difference between how mice responded to the original and quieter 60-70 kHz band, nor was there any difference in regards to how they responded to the original and louder 20-70 kHz band (paired t-test, $p>0.05$). Thus, we concluded that mice had a decreased response to the 20-70 kHz band due to the presence of a low frequency bands rather than due to lower power in the high frequency band.

3.4 Discussion

Overall, our results suggest that mice are capable of using a head-fixed Go No-Go behavioral task to distinguish pup calls and tones from one another and are able to generalize their responses towards other stimuli. While GNG has issues, such as an inherent dissymmetry between responses and rewards (lick/no-lick, reward/punishment), we found

that mice were still able to learn and perform the behavior well with few false alarms. When presented with a number of different catch trials, including pitch shifted pup calls, tones, high broadband stimuli, and low broadband stimuli, we found that using both hit rate and lick rate demonstrated that mice generally respond to these stimuli based on the presence of a high frequency band, although the other spectrotemporal features of the call can also influence the mouse's decision. Moreover, the presence of low frequency bands was able to suppress the response of mice on this behavior. Furthermore, we found no differences between naïve and surrogate females in this behavior. Finally, we found that despite presenting these catch trials extremely infrequently, mice were still able to learn that these trials were unrewarded and subsequently responded less.

One of our primary goals with this experiment was to expand on our findings in freely moving mice in Chapter 2. Overall, we found that these results largely align together well, with mice grouping pup calls together with high frequency broadband stimuli and high frequency tones. One discrepancy we found was in regards to the pitch shifted pup calls, where mice grouped the 55 kHz pup call together with the unchanged pup call in the head-fixed behavior, but not in the freely moving behavior. We propose two possibilities for this difference: the mice used and the context. We used surrogate females in this experiment, but mothers in the previous one. Thus, it could be that mothers are more sensitive towards these calls than surrogates are. While we recognize that previous work has demonstrated that mothers and surrogates retrieve pups at equal efficiency, it is not clear whether these mothers and surrogates perceive and categorize these sounds the same (Krishnan et al. 2017).

In addition to the mice we used, the context of the experiments could drive different responses. While we and others have demonstrated pups can act as a positive

reinforcement, it is potentially not as a strong of a reinforcement tool as water when water deprived (Besosa et al. 2020). Given that in this Go No-Go task mice were motivated by survival, the desire to respond towards the catch stimulus may have been stronger than when motivated by a pup, thereby leading to an increased propensity to respond. Alternatively, our use of a GNG task may have caused this, as GNG tasks often suffer from an increase in false positives due to mice needing to suppress an action it wants to do, in this case lick. To combat this, a similar experiment could be done using a 2 Alternative Forced Choice Task (2AFC), which requires the mouse to make an action in response to both control stimuli.

While we expected to see differences between naïve and surrogate females, our inability to find any differences could be due to the behavioral training we do prior to experimentation. By repeatedly presenting pup calls to naïve females, we may be training mice to learn and respond to these calls in a similar manner to how surrogate females learn to retrieve pups. Therefore, one control experiment we hope to do in the future is to present naïve females with pup calls and see how they perform on a pup retrieval task. Alternatively, it could be that both naïve and surrogate females are able to perceive pup calls and categorize them in a similar manner, but only surrogate females assign behavioral significance towards the stimulus. To combat this, we could try and do our behavioral task in a more natural setting, an idea I explore more in Chapter 5.

Taken together, these results provide insight into how mice categorize natural sounds. These differences in how the stimuli can be categorized may be represented in the brain, and we speculated that we may see these differences manifest themselves within the auditory cortex (Chapter 4).

Chapter 4

Single Unit Recordings in Auditory Cortex Towards Pup calls and Broadband Stimuli

4.1 Introduction

Categorization of auditory stimuli has been well observed behaviorally, from organisms as simple as crickets to more complicated ones like humans (Wyttenbach et al. 1996; Jiang et al. 2018). However, the neural mechanisms that drive this behavior remains a point of interest. While a number of downstream structures have been identified to play a role in categorization, such as the lateral prefrontal cortex and ventrolateral prefrontal cortex, recent research has demonstrated that neurons within the sensory cortices themselves can be altered following behavioral training, with and some demonstrating a preference towards the categories themselves(Russ et al. 2008; Jiang et al. 2018; Xin et al. 2019; Yin et al. 2020). For instance, when mice are trained on a behavioral task to categorize specific tones, neurons tend to demonstrate stronger responses towards those tones that represent either category (Jiang et al. 2018). Furthermore, the auditory cortex also had neurons that demonstrated stronger responses towards those sounds at the categorical boundary, suggesting more energy is being expended to separate sounds into distinct categories(Xin et al. 2019).

However, many of these experiments have been conducted using simple stimuli. While these simple stimuli provide valuable information, often times neural responses

towards natural stimuli cannot be predicted based on their responses towards these artificial, simple stimuli (Laudanski et al. 2012; Talebi and Baker 2012; Chen et al. 2021). Therefore, seeing how natural stimuli are categorized in the brain could provide us with novel, unpredicted results.

Finally, most studies which explore auditory categorization have done so by training subjects to separate stimuli into distinct categories (Jiang et al. 2018; Wang et al. 2020). This training step has the benefit of setting clear rules for mice to learn as well providing well defined boundaries for the stimuli. However, a lot of learning is done in a more natural setting, such as that seen in early language learning or pup retrieval in mice (Lasky et al. 1975; Carcea et al. 2021). While the behavioral principles between structured and natural learning are similar, such as learning to identify the important features of the stimulus, the neural underpinnings that drive these changes may be different. Furthermore, how these two different kinds of learning will interact at the neural level remains unclear.

To study these questions, we utilized loose patch clamp single unit electrophysiology. As described in Chapters 2 and 3, I showed that other sounds can be categorized with pup call USVs. Therefore, we wanted to see how neurons within the auditory cortex represent auditory cues that fall into those categories. Furthermore, we wanted to see whether after behavioral training (GNG task), neurons within the AC change their firing patterns relative to those categories. Therefore, we utilized four separate states: naïve and surrogate before behavioral training and naïve and surrogate after behavioral training and presented a number of different auditory stimuli to awake head-fixed mice. We then compared firing rates of neurons both within and across states and looked at correlations in how those neurons responded between stimuli. We found that before behavioral training, surrogate female neurons in the AC respond earlier to pup call trains

compared to naïve females. Furthermore, when looking at how neurons correlate with one another, we found responses towards low frequency bandwidth stimuli were strongly correlated with one another, and that, following behavioral training, more sounds became correlated with the lower frequency bandwidth stimuli.

4.2 Methods

4.21 Mice

All experiments were performed on female CBA/J mice. Mice were maintained on a 12/12 h light dark cycle (lights on 7 PM) and provided with food and water ad libitum.

For those mice that were in the group before behavioral training, mice were approximately 10-12 weeks old. Surrogacy was induced by placing mice with mothers 2.5 weeks pregnant. After pups were born, we checked to see if surrogates could retrieve pups. For those mice in the group after behavioral training, electrophysiology was conducted one week after behavioral training.

4.22 Surgery

Mice were anesthetized and headbars were affixed onto the mouse's head as described in Chapter 3. Craniotomies were done above the left auditory cortex. Craniotomies were done at the same time as headbars were affixed for those mice in the prior to behavioral training

group. For those mice who were looked at after behavioral training, craniotomies were done following behavioral training.

4.23 Electrophysiology

Neural recordings were done via single unit loose patch clamp electrophysiology. In short, borosilicate micropipette tips were filled with an intracellular solution (125 mM potassium gluconate, 10 mM potassium chloride, 2 mM magnesium chloride, 10 mM HEPES, pH 7.2). Neuron were patched on to and spiking was recorded using a BA-03X bridge amplifier (npi Electronic Instruments). Data was acquired using Spike2 software.

Mice were habituated to the electrophysiology setup for at least 2 days for at least 30 minutes each by head barring them to a wheel which allowed the mouse to freely move in one direction. Electrophysiology was done several hours after craniotomies were done and conducted for ~4 hours each day.

4.24 Auditory Stimuli

Auditory stimuli were generated in the same manner as described in Chapters 2 and 3.

4.24 Auditory Stimuli Presentation

Auditory stimuli were generated in the same manner as described in Chapters 2 and 3. Stimuli were presented at 70 dB SPL.

4.25 Data Analysis

Spike rates for individual neurons were z-scored and individual trials where the same stimuli were presented were averaged together. Neurons were identified as responsive by averaging their response towards the target stimulus. This value was compared with a matrix that contained 1000 values generated by taking the average of an equal length section of the spike train that had been randomly shuffled. If the response towards the target stimulus was greater or less than the 97.5^{th} of 2.5^{th} percentile, the neuron was tagged as responsive.

Correlation matrices were created by first getting PSTH's of the z-scored spike rate for each individual neuron response towards each stimulus. PSTH's from a single neurons response towards two different stimuli were plotted against each other and the correlation coefficient was obtained. This was repeated until the correlation coefficients for each stimulus pair for each neuron was calculated. Finally, the correlation coefficients were averaged together and placed into the correlation matrix.

4.26 Statistics and Data Analysis

Data was collected using Spike2 (Cambridge Electronic Design). Further analysis and statistics were conducted using MATLAB.

4.3 Results

4.31 Recording from and Selecting Responsive AC neurons

Four groups of mice were used for this analysis: naïve females without behavioral training, surrogate females without behavioral training, naïve females after behavioral training, and surrogate females after behavioral training. Behavioral training in this case refers to the Go No-Go task described in Chapter 3. This enabled us the unique opportunity to see how natural training, i.e. surrogacy, compared to artificial training, i.e. our GNG task when both types of training tune the subject to the same stimulus (in this case, a pup call). In short, mice were head-fixed and loose patch clamp electrophysiology was used to locate neurons in the auditory cortex. Throughout the entire experiment, the mouse was awake and allowed to freely walk on a wheel in one-dimension. While loose patch clamp electrophysiology is low throughput, it allows us to record spiking activity from single neurons with very little post processing necessary. After neurons were located, we presented a pup call, high frequency bandlimited noise, and low frequency bandlimited noise. All stimuli were temporally modulated to be identical to the pup call.

After collecting our neurons, we set out to separate them into parvalbumin-positive and parvalbumin-negative neurons (PV and non-PV neurons, respectively) by looking at the spike shape of individual neurons. PV neurons have a distinctive spike shape, generally having a shorter distance between the peak and the trough in addition to having a shorter peak to trough interval when compared to non-PV neurons (Figure 4.1). Based on previous work from our lab (Lau et al. 2020), we classified neurons with a trough to peak amplitude

ratio greater than 0.5 and peak to trough interval less than 0.6 as PV neurons and all others as non-PV neurons (Figure 4.1B). Due to the small number

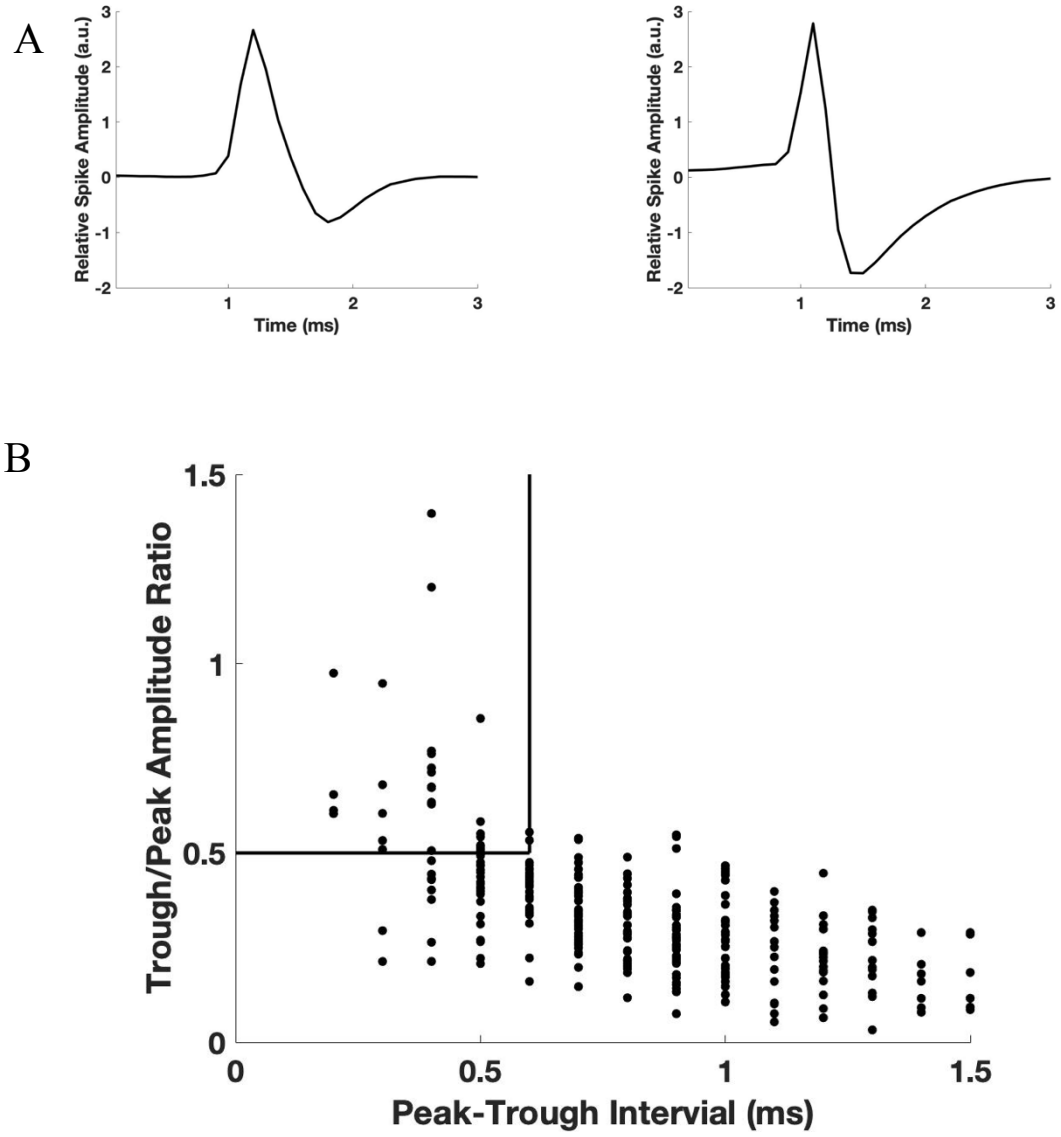

Figure 4.1: **Neurons can be separated into non-PV and PV neurons**: (A) Spike shape of sample neurons. Left: Sample non-PV neuron. Right: Sample PV neuron. (B) Neurons plotted with trough/peak amplitude ratio against the peak-trough interval (ms). Neurons greater than a 0.5 trough/peak amplitude ratio and less than a 0.6 peak-trough interval were defined as PV and others were defined as non-PV.

of PV neurons we were able to obtain, we will focus the rest of the chapter on the non-PV neurons.

We selected for those neurons that demonstrated a change in firing rate in response to an auditory cue. This in itself was an interesting dilemma, as we presented full pup call trains, with multiple instances of sound starting and stopping. By looking at the overall pup call firing rate across the entire train, we were concerned that we may miss neurons that only fired towards certain syllables. These concerns were not unfounded, as previous work had demonstrated that neurons may fire exclusively towards certain syllables and not others (Lau et al. 2020). We also saw neurons in our own data that followed this trend as well, as some neurons showing firing to certain syllables within the train, but not others (Figure 4.2A). Therefore, we opted to include any neuron that had a significant change in firing rate to any of the syllables within the train.

4.32 Neurons in Surrogates Demonstrate Early Excitatory Responses Towards Pup calls

After obtaining our neuronal dataset, we first wanted to see whether differences existed between naïve and surrogate females in their responses to full pup call trains. While a large amount of research has been conducted on naïve and surrogate females in regards to their response to individual pup call syllables, very few studies have looked at full pup call trains and whether responses to these full trains differ between naïve and surrogate females (Marlin et al. 2015).

To investigate this, we generated heatmaps of the z-scored firing rate of neurons in response towards a pup call. The firing rate was averaged during the entire auditory period

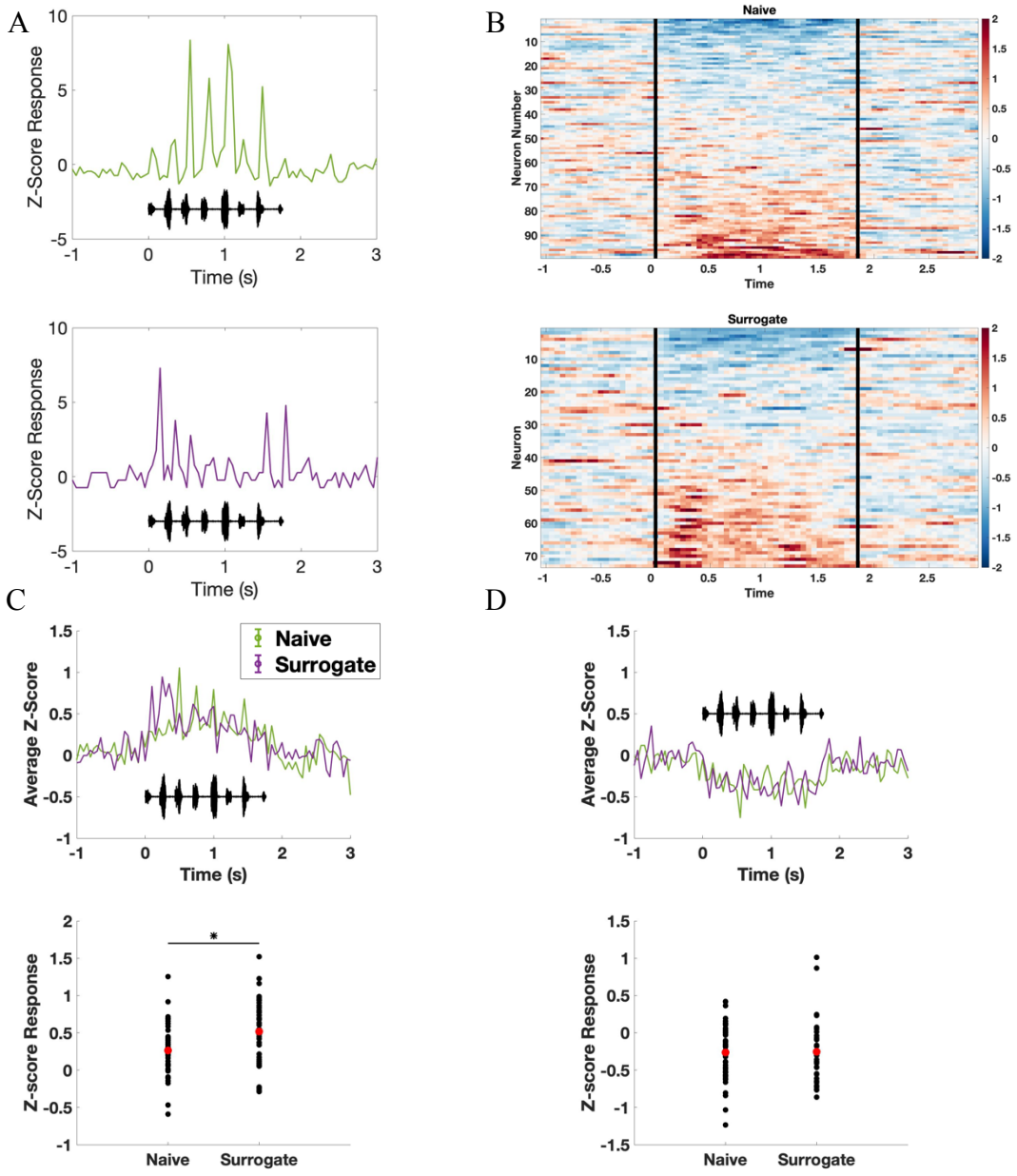

Figure 4.2 **Surrogate female excitatory neurons respond earlier to pup calls compared to naïve females:** (A) Single neurons response towards pup calls. Top: Sample neuron from naïve female. Bottom: Sample neuron from surrogate female. Black trace represents pup call. (B) Heatmaps of neurons in naïve females in response to pup calls. Neurons are sorted in increasing order by response to pup call Top: Naïve; n=99 neurons from 7 mice. Bottom: Surrogates; n= 73 neurons from 8 mice. Black bars represent start and end of stimulus. (C) Surrogate females have earlier responses to pup calls than naïve females. Top: Average trace of all neurons with an excitatory response towards pup calls. Bottom. Scatter plot of individual neurons response towards first two syllables of the pup call. Black points represent individual neurons. Red points represent average. Naïve: n=48 from 7 mice; Surrogate: n =39 neurons from 8 mice. *p<0.05 two-sample t-test (D) Neurons with an inhibitory response show no difference in response to first two syllables (Naïve n=51 neurons from 7 mice; Surrogate n= 34 neurons from 6 mice).

and the heatmaps were sorted from lowest to highest (Figure 4.2B). From these heatmaps, we identified groups of neurons in both naïve and surrogate females that had excitatory responses towards pup calls, as well groups that had inhibitory responses towards them However, something that immediately drew our attention was the increased response at the start of the pup call in surrogate females compared to naïve females, particularly in those neurons that had excitatory responses towards the stimulus. To quantify these results, we first separated out those neurons with excitatory responses from those with inhibitory responses to the entire pup call. We next averaged the total z-score responses from these neurons during the first two syllables. Overall, we found that there were stronger responses towards the first two syllables, but only in those neurons that had excitatory responses (Figure 4.2C, D).

We next wanted to see whether this early response existed for other stimuli. Therefore, we looked at whether neurons also demonstrated an increased response towards the first two syllables when presented with bandlimited noise. Interestingly, we found that this selective early response was present in the neurons that had an excitatory response towards the 60-70 kHz band, but not in response to any of the other band limited noise (Figure 4.3A). This strong response was not due to an overall increase in firing, as we found no difference in response between surrogate and naïve females in the response to the next two syllables across all stimuli (Figure 4.3B). Together, these results suggest to us that surrogate females have quicker responses towards narrow high frequency sounds.

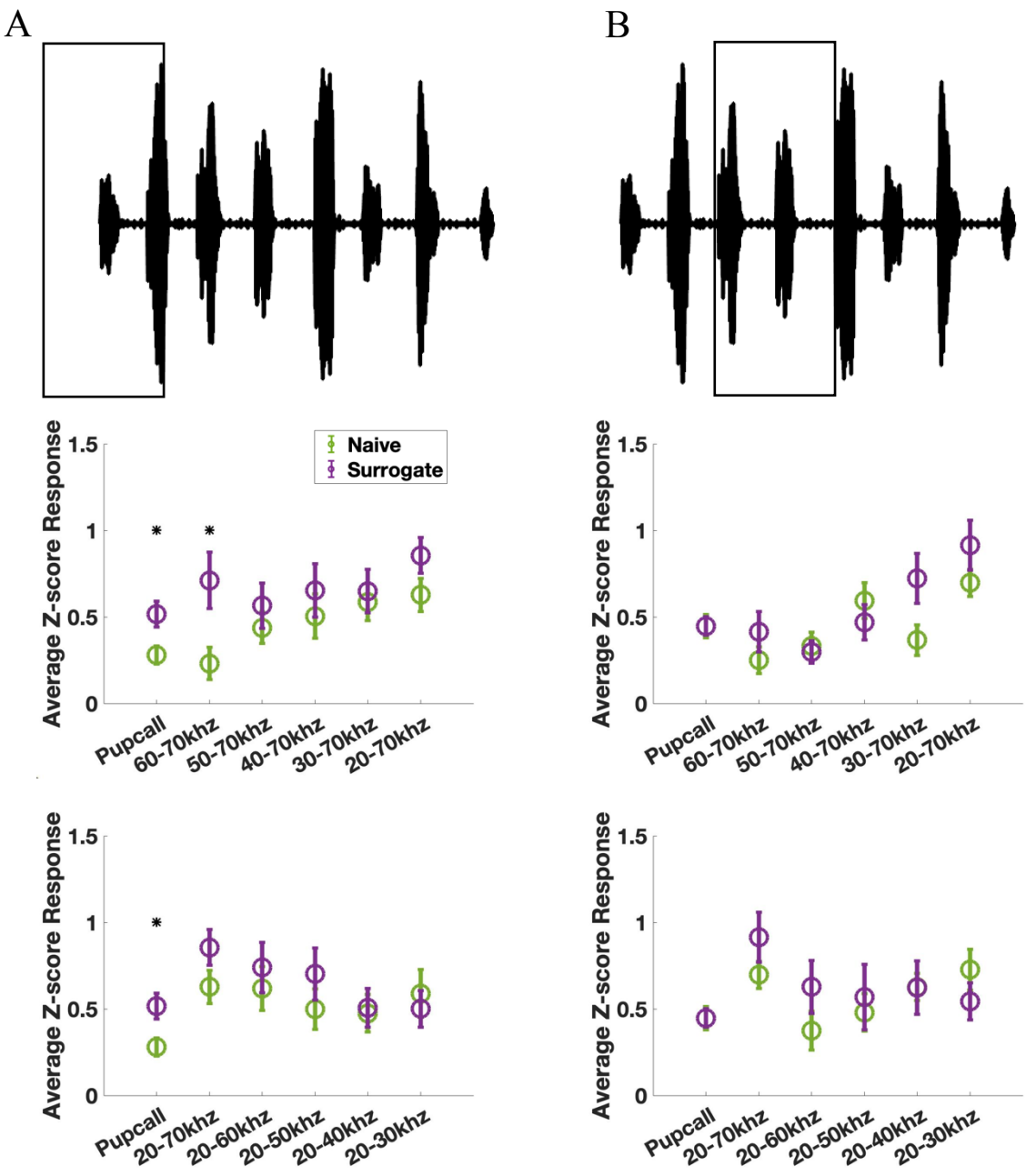

Figure 4.3**: Surrogate, but not naïve, females respond early towards narrow high frequency bandlimited noise.** (A) Top: Image of pup call. Box indicates area of pup call that is being averaged. Middle) Average response of neurons to first two syllables of high broadband stimuli. Bottom) Average response of neurons to first syllables of low broadband stimuli. * $p<0.05$; two-tailed t-test, corrected for multiple comparisons with Benjamin-Hochberg Procedure. (B) Same as A, but for second two syllables. Circles represent averages, error bars represent standard error of the mean.

4.33 Correlations Amongst Firing Rates in Response to Auditory Stimuli

Next, we sought to see whether correlations existed between how neurons responded to various auditory stimuli, and whether those correlations were altered by changes in behavioral state, such as surrogacy or behavioral training. If a neuron fires similarly towards two auditory stimuli, its ability to discriminate those stimuli apart would not be as good as if it fired differently to those stimuli. Thus, we hypothesized that those stimuli which are behaviorally categorized together would generate similar responses in some auditory neurons, while those stimuli which are not grouped together would have low correlations between each other.

To test this, we took individual neurons and generated the PSTH's of their responses towards each auditory stimulus that we presented to it. We took two PSTH's from the same neuron and plotted them against each other (Figure 4.4A). This enabled us to compare the responses of a single neuron to two different stimuli along the entire time interval when the stimuli were being played. Each bin of the PSTH for stimulus 1 was plotted against the corresponding time bin for the PSTH of stimulus 2 and the correlation coefficient was generated. This was repeated for every neuron and every pair of stimuli that was presented to each neuron. Finally, we averaged the correlation coefficients between each stimulus pair across neurons to give us our average correlation response between two stimuli. These values were then plotted into a correlation matrix (Figure 4.4B, 4.5).

We first looked at the correlation matrices for mice before training in response to high and low broadband stimuli (Figure 4.4 B). Interestingly, we found that pup calls were generally uncorrelated with any other stimulus presented, even the other narrow high frequency broadband stimuli. Moreover, we saw much more correlation between the low

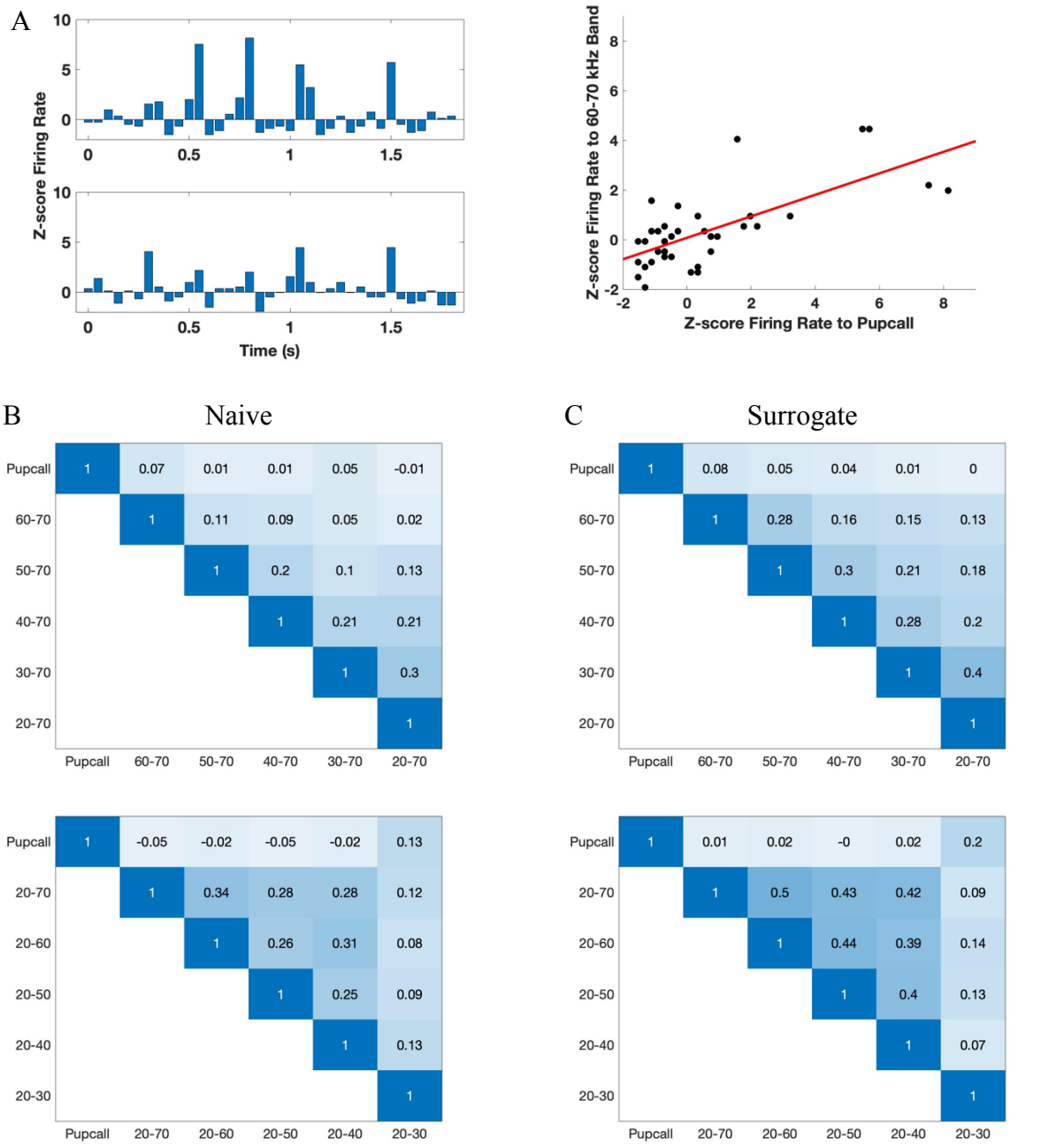

Figure 4.4 **Low frequency sounds are correlated together, while high frequency ones are not before behavioral training**. (A) Correlation coefficients are calculated. Left: PSTH's of individual neuron responses towards two stimuli are compared. Right: PSTH bins are potted against each other and the correlation coefficient is determined. Correlation coefficients for every pair of stimuli presented to every neuron is averaged. This process is repeated every neuron-stimulus pair. (B) Correlation matrix for naïve females before GNG. Top: Correlations matrix for high frequency bands from naïve females (n=51 neurons from 7 mice). Bottom: Correlation matrix for low frequency bands (n=44 neurons from 7 mice). (C) Same as B, but for surrogate females. Top: n= 38 neurons from 8 mice Bottom: n=33 neurons from 8 mice

frequency broadband stimuli than the high frequency ones, suggesting that mice were better able to discriminate these high frequency stimuli compared to the low frequency stimuli. Finally, surrogate females seemed to have stronger correlations than naive females, suggesting that naive females were better able to discriminate these sounds apart from one another.

We also looked at the correlation matrices between naïve and surrogate females following behavioral training (Figure 4.5). Generally speaking, we saw similar trends to those seen prior to behavioral training, with very low correlations in the high frequency bandlimited noise and stronger correlations to low frequency bandlimited noise. Interestingly, following behavioral training, our correlation matrices seemed to suggest naïve females had stronger correlations between stimuli than surrogate females did, a reversal from what was observed prior to behavioral training. One other difference that we did see emerge was how the 20-30 kHz band limited noise became more correlated with the other low frequency stimuli.

4.4 Discussion

In this chapter, we utilized single unit electrophysiology to examine how neurons in the auditory cortex responded to complex auditory stimuli and how those neural responses change over time. We found that generally, surrogate females responded to the very start of the pup call train, while naïve females only started to show response later. These early responses persisted with other high frequency band-limited noises, but were suppressed by the presence of lower frequencies. Furthermore, we generated correlation matrices and discovered that neurons in naïve and surrogate females seemed to correlate low frequency

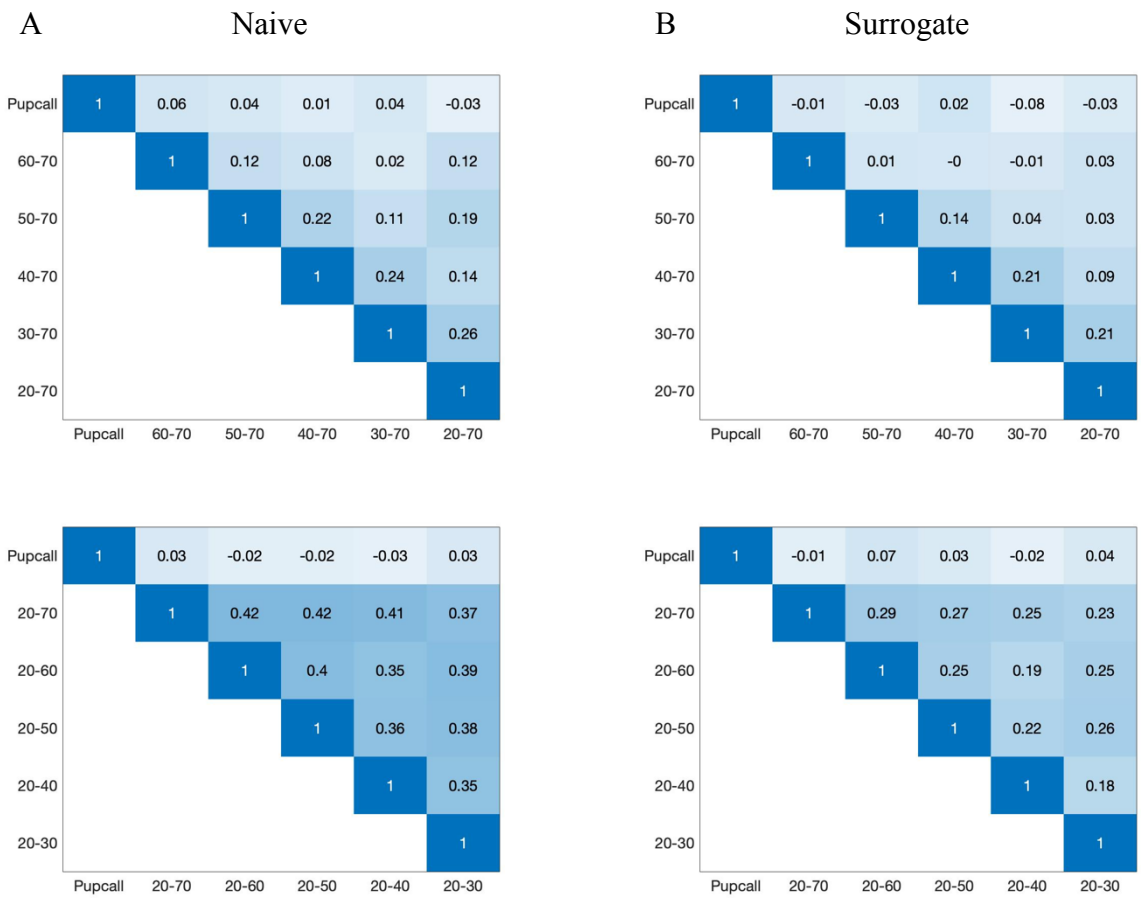

Figure 4.5: **After behavioral training, low frequency sounds are more correlated**. (A) Correlation Matrices for naïve females. Top: Correlation matrix for high frequency bandlimited noise (n= 18 neurons from 4 mice). Bottom: Correlation matrix for low frequency bandlimited noise (n=24 neurons from 5 mice). B) Same as A, but for surrogate females. Top: n= 27 neurons from 4 mice. Bottom: n=18 neurons from 2 mice

sounds together more strongly than high frequency sounds. These correlations generally seemed to be stronger in surrogate females compared to naïve females. Interestingly, this tendency reversed following behavioral training, with stronger correlations being seen in naïve females compared to surrogates. Finally, following behavioral training, the 20-30 kHz band limited noise became more correlated with the other low frequency stimuli.

A large number of studies have looked at how maternal experience effects neural response within the auditory cortex in response to pup calls. For instance, maternal experience can drive increased firing in non-PV neurons in the auditory cortex in response to pup call syllables (Lau et al. 2020). These changes can occur outside of just changes in firing rate, as one study showed how pup call syllables cause earlier peaks in surrogate females compared to naïve females (Liu and Schreiner 2007). However, our work is the first to show that this early response exists not just in response to pup call syllables, but extends to the entire train. These results are interesting, as it may partially explain why an increase in firing is seen in the non-PV neurons in response to pup call syllables, as repeatedly playing pup call syllables could be thought of as continually playing the start of the pup call train (Lau et al. 2020). This may also hint at differences in the spectrotemporal modulation of early pup calls compared to late ones. Moreover, it suggests that the temporal firing of these neurons could play a role in how these pup calls gain behavioral significance.

We also looked at correlations between firing rates of AC neurons in response to various auditory stimuli. Previous studies have demonstrated how a neurons response towards a chord does not necessarily indicate how it will respond towards the tones that make it up (Wang et al. 2020). Our results generally support these ideas, as we saw low correlation between the 20-70 kHz bandlimited noise and the other high frequency band limited sounds that encompass it. However, we were surprised to see that while there was

very little correlation found in the high frequency band-limited noise, strong correlation was exhibited in the lower frequency band-limited noise. These results suggest that mice may naturally have a stronger need to distinguish and separate out high frequency sounds, especially given that many USVs carry some behavioral significance for the mouse (Portfors 2007; Mun et al. 2015). Furthermore, the high correlation amongst the low frequency sounds could suggest that those noises are less important to distinguish apart from one another, although more work would need to be done to confirm this. One outlier in the low frequency broadband noise existed, which was the 20-30 kHz band limited noise. This stimulus was not correlated with any of the other low broadband stimuli prior to behavioral training in both the naïve and surrogate female mice. This suggests that this particular sound may have some behavioral significance that requires mice to distinguish to from other sounds, although what that significance is remains unclear.

Previous work has demonstrated how, following behavioral training, auditory cortex neurons responses towards frequency modulated wriggling calls became more decorrelated in mice trained to differentiate between natural calls and altered ones (Maor et al. 2019). While we failed to see decorrelation between pup calls and other stimulus, partially because pup calls were uncorrelated with all presented stimuli before behavioral training, we did see an increase in correlation between the low frequency sounds and the 20-30 kHz band limited noise. These results suggest to us that following behavioral training, mice are grouping all low frequency band limited noise together. However, the high frequency sounds remained uncorrelated, which may point to how mice are able to perceive these sounds as different, but are grouping them together behaviorally. In this case, categorization may be occurring not in the AC, but further downstream instead.

Overall, these results highlight the role the AC plays in categorization and that the temporal response towards pup call trains could drive behavioral differences between naïve and surrogate females. Furthermore, we show how responses towards low frequency band limited noise are better correlated compared to responses towards high frequency band limited noise. These results, as well as some future experiments to better elucidate these results, are elaborated on in Chapter 5.

5 Conclusions and Perspectives

5.1 Summary

I have presented behavioral and neural data which describes how natural auditory sounds are categorized and how those sounds are represented in the auditory cortex. In Chapter 2, I described a freely moving behavioral task that asked mother mice to discriminate natural pup USVs from other sounds to receive a reward of a pup. We found that while mothers initially responded equally towards all different stimuli, they eventually became more adept at the behavior and began categorizing similar auditory cues together. More specifically, mothers tended to associate those sounds that were more similar to pup calls, such as narrow high bandpass noise or high frequency tones, together with the pup calls.

In Chapter 3, we sought to design a higher throughput technique to expand our previous results. To this end, we utilized a Go-No Go behavioral paradigm where naïve and surrogate female mice were asked to lick to a pup call for a water reward and withhold licking to a temporally modulated tone to avoid a timeout punishment. In addition, we presented a number of other untrained, unrewarded catch trials and monitored how mice responded to those trials. We found that this behavioral task was able to recapitulate our results in the freely moving behavior, and that mice respond systematically towards catch trials depending on how different they were to the pup call. These results held regardless of whether we used the hit rate or lick rate as our readout. While the frequency of the stimulus was important, if the spectrotemporal modulation of the call was the same as the Go Cue, mice were more tolerant of changes to the frequency of the catch stimulus. In addition, we

found that the presence of low frequency noise acted to inhibit responses towards catch trials. We also showed that there were no differences between surrogate and naïve females in their responses to these catch trials. Finally, we found that despite infrequent presentations of these catch trials, mice still learned that these catch trials were unrewarded and responded less frequently towards them over time.

In Chapter 4, we used single unit electrophysiology in naïve and surrogate females both before and after behavioral training and compared the neural responses between neurons in the auditory cortex to a number of different broadband stimuli. We found that non-PV neurons in surrogate females generally had earlier excitatory responses towards pup call trains compared to naïve females. These early responses were also seen in surrogate females in response towards the 60-70 kHz band limited noise, but not to any of the other band limited sounds we presented to the mice. Furthermore, we observed that while responses to low frequency broadband stimuli correlated with one another, responses to our high frequency broadband stimuli tended not to in both naïve and surrogate females. Following behavioral training, we saw the 20-30 kHz bandlimited sound correlate better with the other low frequency band limited stimuli, suggesting that these sounds were beginning to be categorized together.

5.2 Impact on the field

5.21 The Role of Various Spectrotemporal Features in Pup call Categorization

Previous work has looked at how changes in a pup calls ISI effect a surrogate female's response towards those calls (Schiavo et al. 2020). Our work complements this well, demonstrating how changes in other spectrotemporal features impact how pup call USVs are perceived. Previous results had suggested that frequency was one of the most important features for the categorization of auditory stimuli (Chen et al. 2021). The results here support these claims, as changes in the frequency of the stimulus dramatically affected how mice responded to the stimuli and that mice behaviorally responded similarly towards a 65 kHz tone and a natural pup USV. These behavioral results align well with previously discovered neural data which found that firing rates in response to pup calls were similar to firing rats in response to tones at the same frequency (Liu et al. 2006). However, our work is the first to our knowledge to present pup calls that had been pitch shifted downwards to mice. These results highlight how mice are more tolerable to changes in the frequency of the call if the structure of the signal is maintained, suggesting that mice utilize multiple features of the call to inform their decisions.

While the spectrotemporal features of the call can be important if the frequency of the sound deviates too far from a prototypical pup call, our work also shows that so long as a high frequency band that encompasses the pup calls frequency is present, mice will often group this sound as a pup call, as demonstrated by responding over 50% of the time towards stimuli containing high frequency bands in our GNG task. These results partially

support early work which demonstrated that mice would approach a pup call the same amount as a high frequency band (Ehret and Haack 1981). One difference between our results and their work is that they observed very sharp, categorical changes between what mothers would recognize as a pup call or not. We failed to see these differences, instead observing a smoother curve from our narrow high frequency band-limited noise to the large band-limited sounds. One explanation for this difference is that our results lay on a continuous scale (percentage of hits), while Ehret and Haack's results were more binary (pup call or not pup call), which may hint that changes along this axis are more continuous than previous thought.

Despite our work suggesting that the spectrotemporal modulation of the call may not be critical, a large amount of literature has suggested the opposite. For instance, mothers will show a preference towards pup USVs from their own pups compared to pups from a different litter, suggesting that they are able to discriminate these calls apart from one another (Mogi et al. 2017). However, we postulate that while mothers may be able to discriminate these different calls apart from one another, they may still categorize those sounds together. This idea is supported by neural data which demonstrated that neurons in the auditory cortex that are responsive towards a played pup call USV were also responsive towards others pup call USVs, regardless of whether the presented call was from the same strain or even if the call was flipped (Tasaka et al. 2018). Furthermore, our own neural correlations seem to show that high frequency sounds were decorrelated with one another, supporting the idea that these mice are able to distinguish high frequency stimuli apart from one another. Therefore, while maternally experienced females may be able to differentiate these different syllables apart from one another, they may still be functionally recognized as pup calls.

All together, these results suggest that the presence of sound at a similar frequency to the pup call USV is sufficient for mice to recognize it as a pup call, regardless of other spectrotemporal features of the call. However, if those features of the call are maintained, mice are more willing to tolerate frequency changes and categorize those sounds as pup calls.

5.22 Neural Changes in Response towards Bandlimited Noise

We show surprising findings that surrogate females had earlier responses towards pup call trains compared to those responses in naïve females. This early response extended beyond just pup calls, but also towards the 60-70 kHz band limited noise as well, suggesting that surrogate females may generally have stronger responses towards high frequency sounds. These temporal dynamics have been seen in pup call USVs before. While they only present pup call USV syllables, multiunit recordings in mice have demonstrated that neurons have stronger, earlier responses when these USVs gain behavioral significance (Liu and Schreiner 2007). Thus, the early responses to high frequency sounds could be a directly responsible for pup call recognition, although future experiments are certainly necessary to confirm this hypothesis.

In addition, we found that neurons in the AC were less correlated among high frequency band limited noise compared to the strong correlations we saw in low frequency band limited noise. These results imply that mice are better able to distinguish high frequency sounds from one another, while tending to group the low frequency sounds together, which was a confusing finding for us. This could be due to mice being exposed to more high frequency sounds in general, thereby having a stronger need to discriminate out

those sounds containing a 60-70 kHz band limited noise. Indeed, a large number of the social calls that females hear are in the 70 kHz range, from other males, females, and pups, suggesting a need to be able to separate out those calls from one another (Lahvis et al. 2011). Along the same line, it may be that other stimuli at lower frequencies do not require such precise discrimination, or that this discrimination of lower frequency sounds is handled further downstream. Regardless, future work will look to better understand these differences, perhaps by tasking mice with discriminating between low frequency band limited sounds and seeing whether the correlations we observed remain or become more decorrelated, as well as if mice have difficulty doing this task.

5.3 Future Directions

5.31 Studying Pup Call Categorization in More Naturalistic Settings

While these results provide insight into the role categorization may play in pup retrieval, there are a number of studies that can be done which would push this work further along. One such experiment we would like to do is try and make our behavioral task more natural. While we did use a freely moving behavioral task, that task relied on giving a pup in response to an auditory stimulus, rather than having the pup already be there, and also had fairly high variability with mice approaching the speaker even when no stimulus was present. Numerous studies have showed that maternally experienced females will not retrieve pups if they are not vocalizing (Hernandez-Miranda et al. 2017; Schiavo et al. 2020). Therefore, we could take advantage of this behavior by using non-vocalizing pups and presenting sound from a speaker nearby. This could be done through a permanent genetic

mutation to make the pup mute, or through a transient change in state, either by cooling the pups or by using isoflurane to anesthetize them, both of which would cause the pup to stop vocalizing (Drobac et al. 2004; Hernandez-Miranda et al. 2017; Schiavo et al. 2020). This would allow us to significantly decrease the number of false positives we saw while still allowing us to present a large number of auditory stimuli for each mouse.

In addition to making our behavioral setup more naturalistic, it would also be beneficial to record from neurons while they are performing our behavioral task. It has been well documented how neurons in the AC can change their firing depending on the context in which an auditory cue is presented (Miller et al. 1972). In our experiments, we utilized single unit loose patch clamp electrophysiology to record from neurons in the auditory cortex. While this enables us to get strong single unit data with little post processing necessary, it comes with several drawbacks. Namely, these recordings require the animal to be headfixed, which make it impossible to record neurons while the mouse is actively engaging in pup retrieval. Furthermore, it is basically impossible to record from the same neurons across days. Finally, single unit recordings are fairly low throughput, which can make it challenging to record from enough neurons during our behavioral task. To rectify this, we could use something like the Neuropixels 2.0, which would allow us to chronically record multiple neurons from freely moving mice (Steinmetz et al. 2021). This would have the benefit of seeing how neurons in the auditory cortex respond towards our catch trials while the mouse is actively engaging in one of our behavioral tasks. Furthermore, because these recordings could be chronic, we could also record how neurons change their responses as mice transition from naïve to surrogate, particularly in regards to the early responses we see surrogate neurons have in response to pup calls.

5.32 Identifying the Role PV Neurons Play in the Categorization of Pup Calls

In addition, we would also like to see how other neuronal subtypes effect these categorical changes. One particular group of neurons we can focus on are PV-positive neurons. PV-positive neurons make up almost 40% percent of all inhibitory neurons in the auditory cortex, suggesting that they play a critical role in sound processing in the AC (Rudy et al. 2011). These neurons have been implicated in pup retrieval, as maternal experience in mice causes a suppression of PV neuron auditory response. (Lau et al. 2020). Furthermore, dysregulation of these PV neurons has been linked to an inability to retrieve pups, demonstrating that they play a major role in the processing of pup calls in the AC (Krishnan et al. 2017). To test this, we would like to increase our neuronal sample size in order to record from more PV positive neurons. While doing more electrophysiology would be one way to accomplish it, it would also require a fairly significant number of neurons to be recorded from. One way in which we could get around this is by using fiber photometry to monitor the activity of PV positive neurons during a behavioral task, which would trade some temporal resolution for spatial resolution. We could also use optogenetics to inhibit PV neurons within the AC and see whether the performance of mice would be impacted in our behavioral tasks and, if so, how the performance changes.

5.33 Potential Role of Lateral Inhibition in Categorization

One of the most interesting parts of our data is that the presence of low frequency bandlimited noise seemed to inhibit, but not completely destroy, the perception of pup calls.

This suggests that there may be some lateral inhibition occurring in the auditory cortex that drives this behavior. Lateral inhibition is the process whereby pyramidal cells recruit local interneurons to suppress firing of other neurons with different tuning properties. Given the results of our GNG behavior, one possible explanation for our results is that when the 60-70 kHz band limited noise is played, other neural responses are laterally inhibited, which in turn sharpens the tuning properties of neurons excited by this stimulus. If the 60-70 kHz band limited noise is treated like a pup call, which our behavioral data suggests it is, than this is not unreasonable, as neurons that were tuned towards frequencies below pup USVs were inhibited in the presence of pup calls in mice that found these calls behaviorally relevant (Galindo-Leon et al. 2009). As we increase the size of our band limited noise, lateral inhibition becomes weaker as more and more neurons become excited, causing mice to treat the stimulus less and less like a pup call.

At the moment, all of the above is conjecture. However, one way in which we could test this hypothesis is by looking at a population of neurons within the AC, perhaps with a Neuropixel 2.0, and presenting our high frequency band limited stimulus set. In doing so, we could identify whether we see increases in inhibition when mice are presented with a 60-70 kHz band limited noise. As we increase the bandwidth of the stimulus, we would expect to see less inhibition as lateral inhibition becomes weaker. Additionally, we could optogenetically activate SOM neurons and see how the GNG behavior changes. SOM neurons have been heavily implicated in controlling lateral inhibition in the AC. (Kato et al. 2017). Therefore, activation of SOM neurons could increase lateral inhibition even in the presence of lower frequency band limited noise. Thus, we would anticipate that when presented with our high frequency band limited stimulus set, mice would respond to all stimuli at similar levels to the Go Cue.

5.4 Closing Remarks

In conclusion, the results described here help describe how natural, complex auditory sounds are categorized and how neurons within the auditory cortex may drive those categories. We hope that these results provide new possible behavioral paradigms that can be used to probe auditory categorization, as well as provide insight into how those sounds are processed in the auditory cortex. Taken together, these results provide insight into how natural experiences drive behavioral and neural changes, improving our understanding of speech processing and learning.

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