UNIVERSITY OF CALIFORNIA
RIVERSIDE

Intensity Selectivity in the Auditory Cortex of the Pallid Bat, *Antrozous pallidus*.

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Neuroscience

by

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December 2014

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Acknowledgements

I would like to acknowledge my committee for their guidance over the past five years. I would not have completed my PhD without their continued support. I consider Dr. Hickmott, Dr. Adams, and Dr. Razak as mentors and friends. Dr. Hickmott was always there for me with advice and unwavering confidence and I thank him for that. Dr. Adams was there for me in my toughest times as a student and believed in me when my belief in myself faltered. I would like to additionally acknowledge my advisor, Dr. Khaleel Razak, someone who is responsible for making me the scientist, educator, and person I am today. I will be forever grateful for his kindness, patience, willingness to challenge me.

I would also like to thank my family for their support through these years. To my mother, Heather Measor: I thank you for your support and love, I am the man I am today because of you. To my son Ignatius Nicanor Measor: I love you with all my heart and everything I do is for you. To my wife Nikki Measor: You have been my biggest supporter in this journey and I could not have done it without you. You are my best friend and have held my hand through the toughest times. You are my inspiration both as a parent and a professional and I write this dissertation for you.

Chapter 3, Parvalbumin and Calbindin Expression in the Parallel Thalamocortical Pathways in the Pallid Bat, has previously been published in the Journal of Comparative Neurology. It has been reproduced in this dissertation with permission from the principal investigator, Dr. Khaleel Razak, in its entirety and without alteration. The authors,

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Campo, Heather Martin, Kevin Measor, and Khaleel A. Razak. "Parvalbumin and calbindin expression in parallel thalamocortical pathways in a gleaning bat, Antrozous pallidus." *Journal of Comparative Neurology* 522.10 (2014): 2431-2445.

ABSTRACT OF THE DISSERTATION

Intensity Selectivity in the Auditory Cortex of the Pallid Bat, *Antrozous pallidus*.

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University of California, Riverside, December 2014
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Adaptations that allow for greater discrimination of low intensity sounds may be important in the echolocation behavior of bats. In this dissertation, we used the pallid bat, *Antrozous pallidus*, a gleaning bat of the western United States, as a model to study the adaptations for intensity selectivity present in the auditory cortex. We performed in-vivo extracellular recordings in the auditory cortex of the pallid bat, to determine the cortical organization and mechanisms of intensity selectivity. Downward frequency modulated (DFM) sweeps that approximated echolocation calls were used to study intensity selectivity using a behaviorally relevant sound. We also examined the distribution of Parvalbumin (PV) and Calbindin (CB) expressing cells in cortical regions and thalamic nuclei in the auditory pathway. Immunohistochemical staining was used to determine if the distribution of these calcium binding proteins in cortical and thalamic regions that have been implicated in processing of echolocation calls and how that distribution is different from non-echolocating regions. Lastly we examined the thalamocortical projections to intensity selective neurons in the echolocation region of

the auditory cortex. Retrograde tracing from intensity selective and non-selective neurons was used to identify the thalamic nuclei that provided input to those neurons. We show that the region of the auditory cortex that is selective for echolocation calls contains a majority of neurons that are highly selective for low intensity sounds. We discovered that in the pallid bat intensity selectivity is enhanced by using behaviorally relevant stimuli and that high-frequency inhibition in the bat's echolocation call is responsible for this increased selectivity. This suggested a spectrotemporal integration mechanism that can shape intensity selectivity. Cortical mapping, in this study, revealed a systematic organization of intensity selectivity measures. We also discovered a differential staining pattern of calcium binding protein in the cortex as higher percentage of PV+ cells compared to CB+ cells was found in both echolocation call- and non-call regions. CB+ neurons where found in all of the regions of the medial geniculate body of the auditory thalamus, while PV staining was limited to the suprageniculate (SG), a region known to project to echolocation selective regions of the cortex. This study also confirmed previous results showing that echolocation selective regions of the cortex receive projections from all nuclei of the MGB except the lemniscal ventral nucleus, however an organization related to intensity selectivity was not able to be determined in the projections. The results from this dissertation highlight the special adaptations that are present in the auditory cortex of the pallid bat that may be important for processing the low-intensity echolocation call. Deviations from a general mammalian plan or even from other bat species in the properties that lead to these adaptations may strengthen the

notion that general patterns of cortical processing and organization may be altered through evolution to support the unique behavioral needs of a species.

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Chapter 1: Intensity Selectivity in the Mammalian Auditory System

1.1 Introduction

For most mammals, hearing makes up a significant portion of their sensory experience. They use this sense to help facilitate communication with conspecifics, for mating, aggression and general social interactions. Hearing is also used to locate prey and avoid predation. All sensory information is coded in the brain using parameters that can be directly detected by the sensory epithelium. For the auditory system, the cochlea can extract the timing, intensity, and frequency of a sound. These three parameters are used by the central nervous system to make meaning out of the sound that comes into the outer ear. The intensity of a sound is a property present in nature that can distinguish one sound from another and provide context and information for what that sound represents. Humans and many mammals, including bats can perceive sounds that differ over a large range of intensities. They can also discriminate small differences in intensity over most of this range (see review in (Popper and Fay, 1992)). This ability allows them to experience a large number of unique sounds in their environment thus providing a much higher fidelity comparison of what they perceive to the actual sounds present in nature.

An important part of understanding how we process sounds in nature is by examining how different levels of the auditory pathway respond to different parameters of the sound or how they extract information by the use of intrinsic calculations about the stimuli. The mammalian primary auditory cortex is a higher level center in the auditory pathway and thus can build upon processing in earlier levels to extract information from more complex stimuli. Early electrophysiological studies in the auditory cortex described neurons that

responded less strongly to high intensity sound than to low intensity stimuli (Davies et al., 1956, Evans and Whitfield, 1964, Brugge et al., 1969). Neurons that have a non-monotonic rate-level response to increasing sound stimulus intensities are considered to be intensity selective (Greenwood and Maruyama, 1965) . A non-monotonic response is one characterized by cessation or reduction of response to an increase in the sound intensity, whereas a monotonic response would be one that increases or saturates as sound intensity increases. Spike-count intensity functions of auditory nerve fibers are all monotonic (Winter et al., 1990). However, many neurons of the subsequent central auditory pathway can be intensity selective (Brugge and Merzenich, 1973, Phillips and Irvine, 1981, Semple and Kitzes, 1985, Rhode and Smith, 1986, Ehret and Merzenich, 1988, Irvine and Gago, 1990, Phillips, 1990), so the origins, distribution, and mechanisms that shape the response of these neurons is important in studying how sensory systems can generate selectivity at different levels. Research into intensity selectivity can give insight into the specific functioning of the auditory cortex but also provides information that elucidates the general function and organization of sensory cortices.

1.2 Pallid Bat as a Model for Studying Properties of the Auditory Cortex

Bats represent an excellent model for studying auditory processing. In many species of bats, hearing has evolved to include the ability to detect the echoes of active vocalizations with the purpose of prey detection and obstacle avoidance (see review in

(Popper and Fay, 1995). The pallid bat, *Antrozous pallidus*, a bat of the western United States, is what is referred to as a "gleaner", listening for prey-generated noise transients to capture arthropods on the floor of their hunting grounds. The pallid bat uses a 60-30kHz frequency-modulated (FM) echolocation call to avoid obstacles while in flight (Brown, 1976, Brown, 1978, Bell, 1982, Fuzessery et al., 1993). Pallid bats also have an audible FM call of lower frequencies than the echolocation call that is used to attract conspecifics (Arnold and Wilkinson, 2011). These three different types of auditory stimuli play an important role in the pallid bat's sensory experience; however it is the use and detection of an echolocation call that makes the pallid bat an auditory specialist. It also makes the pallid bat a useful model in studying the properties of the auditory cortex because the echolocation call, being specific to the pallid bat, can be used to probe the responses of individual neurons to a sound that would have meaning and that they would encounter in their natural world. In this case the behaviorally relevant stimuli, the high-frequency FM sweep, which neurons in different levels of the auditory pathway have shown selectivity for, also represents a stimulus that is more complex than single-frequency tones with respect to its spectrotemporal properties (Fuzessery, 1994, Razak et al., 1999, Razak and Fuzessery, 2002). By using this stimuli rather than a simple tone, we can gain a better understanding on how the cortex processes more complex sounds, in which different frequencies interact with each other to create unique responses.

The pallid bat is also an excellent model to examine how parallel streams of information can be processed in the cortex at the same time. The auditory cortex of the pallid bat is parceled into two different areas. The dorsal region of the cortex is selective

for high frequency sounds (35-70 kHz) while the ventral region processes low frequency sounds (5-40 k Hz). These two regions together form one continuous tonotopic area. The dorsal region, of the high frequency region (HFR), is comprised mainly of neurons that are selective for the downward sweeps that the pallid bat produces, while the neurons in the ventral region are predominately selective for broadband noise (Razak and Fuzessery, 2002). These two areas represent parallel pathways that are well suited for processing echolocation calls, and prey generated noise transients, respectively. Comparisons in the responses between the two areas can be used to explain how cortical subfields: can function; be generated; and how they might interact with each other.

1.3 Intensity Selectivity in the Auditory Pathway

At the periphery of the auditory pathway neuronal responses in the auditory nerve are monotonic to increasing levels of intensity (Winter et al., 1990). The increase in response in the auditory nerve is non-linear in nature owing to non-linearities that exist with the basilar membrane of the cochlea itself (Yates, 1990, Yates et al., 1990). Different fibers within the auditory nerve exhibit different thresholds of response to intensity and some of these neurons have a gradual increase to certain intensity ranges while others will show a more rapid increase. These neurons can also saturate their responses at different levels of intensity. The variety of these responses will become important to shape the perception of intensity and the complex responses to intensity that will be seen as one ascends the auditory pathway (Winter and Palmer, 1991).

Neurons with non-monotonic responses to increasing intensity start appearing in the level of the cochlear nucleus in the auditory pathway (Winter and Palmer, 1990, Zhou et al., 2012). Neurons in the rat dorsal cochlear nucleus (DCN) can be intensity selective and it was revealed by in-vivo whole-cell recordings that they receive fast saturating excitation and slow saturating inhibition as intensity of stimulus increases (Zhou et al., 2012). Cells in the DCN that are not selective for this increase in stimulus intensity have similarly slow saturating inhibition and excitation. This demonstrates that although the response inputs from the auditory nerve to the rest of the pathway may be monotonic in response to increasing intensity, inhibition that is generated as early as the DCN can create intensity selectivity in local and ascending responses.

Early recordings in the inferior colliculus (IC) of two bat species, *Myotis lucifugus* and *Plecotus townsendii*, showed that there were neurons that had non-monotonic responses to increasing intensity (Grinnell, 1963) . This work has been followed up in other species of bats and similar responses have been seen *Molossus ater* (Vater and Schlegel, 1979), *Molossus molossus*, (Vater and Schlegel, 1979) *Tadarida brasiliensis* (Pollak et al., 1978), *Pteronotus parnellii* (O'Neill, 1985) and *Antrozous pallidus* (Fuzessery, 1994). Non-monotonic responding neurons have been also been studied in the cat IC (Rose et al., 1963, Ehret and Merzenich, 1988, Aitkin, 1991). In one such study neurons with non-monotonic responses represented a large percent (61%) of the neurons studied (Aitkin, 1991). Studies have also been conducted in other mammalian species that have uncovered intensity selective neurons in the IC. (rhesus monkey, (Ryan and Miller, 1978), mouse (Willott et al., 1977, Ehret and Moffat, 1985), rat

(Sivaramakrishnan et al., 2004), and rabbit (Sivaramakrishnan et al., 2004)). To date the IC has been the most well studied nucleus in the auditory pathway in regards to intensity selectivity responses in neurons. These comparative studies have shown that the IC is the first nucleus in the auditory pathway where a majority of neurons display a non-monotonic response to neurons. It has yet to be determined why the amount of intensity selectivity is seen in the primary auditory cortex (A1) of some species may be similar to the responses seen in the IC and why some are much lower in comparison. It is possible that in species where there are a majority of neurons that have an intensity selective response in A1, that these responses are inherited from the IC and somehow this response is inhibited in species with less intensity selectivity in A1. Another possible explanation is that the response seen in the IC has to be recreated in the cortex and that some species have lost or lack this mechanism. It is possible that this differential amount of intensity selectivity that is seen in these two areas, the IC and A1, in some species may play a role in explaining the functions of the two regions.

The medial geniculate body (MGB) of the thalamus is the last nucleus of the ascending auditory pathway that inputs directly to the auditory cortex. There have been fewer in-vivo electrophysiological studies of intensity selectivity in the MGB than other major nuclei of the central auditory pathway. Studies that have been performed have shown that in the MGB of the cat that there is high percentage (74%) of non-monotonic responding neurons to increasing intensity (Rouiller et al., 1983a) and that 62% of neurons demonstrated an increase in response latency to intensity, or paradoxical latency

shift (PLS). This would suggest a similar amount of intensity selectivity as has been reported in the IC.

Numerous studies uncovering intensity selectivity have been conducted in the auditory cortex in different species (cat (Phillips and Irvine, 1981, Phillips et al., 1995, Sutter and Schreiner, 1995, Sutter and Loftus, 2003), rat (Phillips and Kelly, 1989, Polley et al., 2004, Tan et al., 2006, Wu et al., 2006), guinea pig (Taniguchi and Nasu, 1993), and marmoset monkey (Watkins and Barbour, 2011a, Watkins and Barbour, 2011b). These studies have looked at both the distribution of intensity selective neurons and/or their topographic organization (see section 1.4 below) within the cortex. An early study looking at non-monotonic responses in the cat primary cortex (A1) reported 23.9% of neurons tested were intensity selective (Phillips and Irvine, 1981). A following study by the same investigators that quantified intensity selectivity using the percent that a response declined at the highest intensity tested as compared to the maximum response (percent turnover) labeled approximately 45% of the neurons in the cat primary cortex as non-monotonic (Phillips et al., 1985a). The discrepancy in the two studies is due to the second study defining a non-monotonic response as at least a 50% turnover in response at the highest intensity tested from the maximum response obtained. This definition of an intensity selective neuron makes it difficult to compare it to other studies that simply defined non-monotonic as a drop in response at high intensities. Subfields in the cat auditory cortex have shown results that deviate from that found in A1. A study in the cat posterior auditory field, which is located outside of A1, showed that there is a large percentage (70%) of intensity selective neurons (Phillips et al., 1995). Other mammals

have shown results that deviate from those originally found in the cat A1. The results in the albino rat, *Rattus norvegicus*, were in sharp contrast to that of the cat in that most of the neurons in the primary auditory cortex were labeled as monotonic using the same percent turnover criteria used previously in the study of the cat (Phillips and Kelly, 1989). A more recent study has identified an area adjacent to the primary auditory cortex in rats in which most neurons are non-monotonic in nature (Wu et al., 2006). The differences seen between the rat and cat A1 suggest that intensity selectivity may not follow a general mammalian plan and that different species have adapted different levels of selectivity.

Investigation into intensity selectivity have also included studies in the auditory cortex of several bat species, *P. parnellii (*Suga, 1977, Suga and Manabe, 1982), *M. molossus (*Macias et al., 2014), *M. waterhousii (*Macias et al., 2014), *Carollia perspicillata* (Hechavarría and Kössl, 2014*), and M. lucifugus (Suga, 1965, SULLIVAN III, 1982).* In the DSCF region of the mustached bat, *P. parnellii*, 96.5% of the neurons investigated had a non-monotonic response-level function (Suga and Manabe, 1982). In the cortex of *C. perspicillata* 80% of neurons displayed non-monotonic responses to increasing intensity (Hechavarría and Kössl, 2014). In *M. Lucifugus* there have been reports of as high as 63% of neurons having non-monotonic responses to increasing intensity (Suga, 1965) to as little as 19.4% of neurons displaying similar responses (SULLIVAN III, 1982). These data suggest that the high levels of intensity selectivity is a common feature found in the bat auditory cortex. The large numbers of selective

neurons as compared to other species may be a result of the importance that intensity selectivity plays in the echolocation behavior.

The plasticity of intensity selectivity has also been studied in the auditory cortex. In rats, selectivity for intensity ranges can change by associative learning processes, which means that the intensity selectivity may be more closely linked to the relevance of the stimulus (Polley et al., 2004). This study suggests that not only is intensity selectivity better studied using behaviorally relevant sounds, but also researchers should be examining the development and the plasticity of this selectivity by manipulations of these relevant stimuli.

1.4 Topographical Organization of Intensity Tuning

The sensory cortices have a high degree of organization of response properties whether orthogonal to the surface of the cortex as in columnar organization or in parallel to the surface of the cortex as in cortical maps. Cortical maps can be as simple as a representation of the sensory epithelium or as complex as organization of computed properties. The topographic nature of cortical maps has been suggested to help facilitate spatiotemporal computations for neural circuits thus allowing for some of the functional roles that they may play in perception (Kaas, 1997). Cortical mapping of the auditory cortex has been accomplished in many different species (mouse(Stiebler et al., 1997), rat (Kelly and Sally, 1988), (Doron et al., 2002), (Rutkowski et al., 2003), macaque monkey (Merzenich and Brugge, 1973), (Morel et al., 1993), owl monkey (Imig et al., 1977), cat

(Merzenich et al., 1975), (Reale and Imig, 1980), guinea-pig (Hellweg et al., 1977) , ferret (Phillips et al., 1988, Bizley et al., 2005), chinchilla (Harrison et al., 1996). It is important that repeated studies are performed in different mammals to determine what level of homology is there between the auditory fields of different species (Kaas, 2005). This may be particularly important for shedding light on how the human auditory cortex may function and how animal studies can inform us about the possible organization.

Comparative studies of organization in the auditory cortex of bats represent an important tool in understanding the differences that may arise in cortical topography between closely related species which have developed subtle changes in auditory behaviors. Cortical maps of frequency have been studied in a number of bat species namely, *C. perspicillata* (Esser and Eiermann, 1999), *Eptesicus fuscus* (Dear et al., 1993), *Rhinolophus ferrumequinum* (Ostwald, 1984), *Rhinolophus rouxii* (Radtke-Schuller and Schuller, 1995), *P. parnellii* (Suga and Jen, 1976), *Phyllostomus discolor* (Hoffmann et al., 2008), *M. molossus* (Macias et al., 2014), *M. waterhousii* (Macias et al., 2014), and *A. pallidus* (Razak and Fuzessery, 2002). In this dissertation (Chapter 2) we mapped the high frequency region (HFR) of the pallid bat auditory cortex to determine if there is a topography based on properties of sound intensity. Different studies have produced cortical maps in the auditory cortex of mammalian species based on intensity response properties. Studies have shown that there is an organization in the cat cortex with regards to properties of intensity coding (Heil et al., 1992, Schreiner et al., 1992, Heil et al., 1994). It has been further observed in the cat that groups of neurons with monotonic and non-monotonic responses to intensity can be found spatially

segregated from each other (Phillips et al., 1985b, Imig et al., 1990, Phillips et al., 1994, Sutter and Schreiner, 1995). Differences in rate-level function distributions in the auditory fields of primates have also been seen in awake behaving macaques (Recanzone et al., 2000).

In the area of the mustache bat cortex that is responsible for processing the Doppler shifted second harmonic of the characteristic frequency (DSCF) neurons predominately have a non-monotonic rate-level response to intensity while the peripheral areas show a monotonic response. This area displays an organization around the best amplitude of neuronal responses, referred to as ampliotopic (Suga, 1977, Suga and Manabe, 1982). This data is in juxtaposition to what has been seen in the cat with regards to primary vs. secondary fields. The distribution of the non-monotonic responding neurons in the posterior auditory field of cats is larger than in that of A1 (Phillips et al., 1995). The question remains if another bat species shows an organization in the cortex similar to the mustached bat, as a recent study in two bat species, *M. molossus* and *M. waterhousii*, failed to show an ampliotopic representation (Macias et al., 2014). This would suggest that this type of organization around best intensities is not a common feature of the bat auditory cortex.

1.5 Mechanisms of Intensity Selectivity

1.5.1 Neural mechanisms

The balance of excitation and inhibition is a feature of the central nervous system that is responsible for the ability to process many different features of stimuli. This is particularly true in the auditory pathway (Pollak and Park, 1993, Casseday et al., 1994, Fuzessery and Hall, 1996, Casseday et al., 2000, Tan et al., 2004, Tan et al., 2006, Wu et al., 2006, Tan et al., 2007, Razak and Fuzessery, 2009, Fuzessery et al., 2011, Williams and Fuzessery, 2011). Sometimes inhibition from two or more levels in the auditory pathway converge to create neuronal responses to certain stimuli. Inhibition from the thalamus seems to be responsible for shaping the response of the neuron at the characteristic frequency (CF) while inhibition in the cortex is responsible for tuning bandwidth and responses to non-CF stimuli (Kaur et al., 2004). Studies have shown that inhibition plays a role in shaping the response to many properties in the pallid bat auditory pathway. Inhibitory sidebands seem to be responsible for determining FM direction selectivity, which originates mostly in the IC, as well as shaping FM rate selectivity that originates at lower levels in the auditory track (Williams and Fuzessery, 2011). The development of the high-frequency inhibition responsible for rate selectivity can be seen in pallid bats that are two weeks old before the animals experience their echolocation calls (Razak and Fuzessery, 2007, Razak et al., 2008) and thus is present throughout much of the animals life.

Inhibition also plays an important role in shaping intensity selectivity (Pollak and Park, 1993, Sutter and Loftus, 2003, Sivaramakrishnan et al., 2004, Wu et al., 2006, Tan et al., 2007). In-vivo slice preparations from the IC show that blockade of $GABA_A$ synapses are responsible for shaping non-monotonic responses (Sivaramakrishnan et al., 2004). In-vivo patch recording have shown that auditory cortical neurons with non-monotonic response-level functions to increasing intensity have imbalanced tone-evoked excitatory and inhibitory synaptic input (Tan et al., 2006, Wu et al., 2006).

γ-aminobutyric acid (GABA) mediated inhibition has been shown to affect the response to excitatory tones in the IC and the auditory cortex of the pallid bat (Fuzessery and Hall, 1996, Razak and Fuzessery, 2009). In this same study non-monotonic responding neurons in the IC became more monotonic in their rate-level function response when bicuculline, a GABA antagonist was applied (Fuzessery and Hall, 1996). In the mustached bat bicuculline applied to the IC also caused neurons with non-monotonic rate-level functions to change their response to a monotonic rate-level function (Pollak and Park, 1993), thus suggesting a role of inhibition in shaping responses to intensity in two bat species studied. In the big brown bat inhibition in cortical neurons leads to a lower response to sounds at all intensities (Chen and Jen, 2000).

1.5.2 Biochemical Mechanisms

As was just previously mentioned, inhibition plays an important role in shaping intensity selectivity. It is unknown which inhibitory neurons contribute to intensity

selectivity and what intrinsic properties may make them well adapted to this task. Inhibitory interneurons can have numerous different physiological and morphological differences. One way that these neurons can differ from each other is by the calcium-binding proteins that are present. Of the calcium-binding proteins that are present in the auditory cortex and MGB, the distributions of parvalbumin and calbindin have been studied extensively (Zettel et al., 1991, Alcantara et al., 1993, Vater and Braun, 1994, de Venecia et al., 1995, De Venecia et al., 1998, Gao et al., 2000, Cruikshank et al., 2001, Desgent et al., 2005, Martin del Campo et al., 2012). The roles that these proteins play in shaping response selectivity are beginning to be studied (Wu et al., 2008, Sohal et al., 2009). Fast-spiking PV+ neurons have shown to contribute to the inhibition that sharpens frequency tuning in the cortex (Wu et al., 2008). One thing that has been discovered is that PV+ interneurons have a monotonic rate-level function of increasing intensity(Moore and Wehr, 2013). This non-selective nature of the PV+ neurons has been proposed as necessary for an inhibitory input to shape intensity selective neurons (Wu et al., 2006). It is unknown whether PV+ neurons provide the inhibitory input to intensity selective neurons; however studies conducted on the distribution of calcium binding proteins in areas of intensity selectivity may provide insight into their role in shaping this response.

1.6 Thalamocortical Organization of Intensity Selectivity

Cortical responses to sound stimuli may often represent earlier processing at various levels of the auditory pathway. The auditory cortex of mammals receives most of its auditory information from the medial geniculate body (MGB) of the thalamus (see review in (Webster, 1992)). The information transformed in this thalamocortical interface and the local circuits involved in the processing are a crucial component for how the animal begins to process more complex sounds (see review by (Winer et al., 2005). The MGB has three primary divisions, being the ventral (vMGB), dorsal (dMGB), and the medial (mMGB) which serve to send parallel information about different auditory response properties to the cortex. The vMGB, which sends strictly auditory information, is tonotopically organized while the dMGB is atonotopic (mMGB is a multisensory pathway that may be important for learning). Previous studies in the pallid bat have shown that the two distinct subregions of the auditory cortex, the high-frequency region, HFR and the low frequency region, LFR, receive inputs from different divisions of the MGB (Razak et al., 2007). The FM responding HFR receives input predominately from the suprageniculate (SG) nucleus of the dMGB while the noise responding LFR receives inputs from vMGB. In early development of the pallid bat, the SG contributes major inputs to both the HFR and LFR but these connections are refined in the adult so that the SG contributes mainly only to the HFR (Razak et al., 2009)

Mechanisms of selectivity seen in the cortex, like that of rate tuning, have been seen in lower areas of the auditory pathway, which suggests that neuronal response selectivity

can be inherited from earlier processing centers (Fuzessery et al., 2006, Fuzessery et al., 2011), or they can be refined or recreated at the level of the cortex (Razak and Fuzessery, 2009, 2010). Intensity selectivity has been studied in the mustached bat in the MGB (Olsen and Suga, 1991a, b) and the inferior colliculus (O'Neill, 1985). Intensity selectivity that is observed in the MGB of the mustached bat has been shown to be created in subthalamic nuclei in the inferior colliculus (Kuwabara and Suga, 1993). In the cat MGB, 74% of neurons were determined to have a non-monotonic rate-level function (Rouiller et al., 1983b), although this amount of selectivity was not seen in the cat auditory cortex (Phillips and Cynader, 1985). It is unclear to what extent this distribution and the response of the MGB to intensity contributes to the creation of intensity selectivity in the cortex because this data would suggest at least a portion of the intensity selectivity has been lost en route to the cortex.

1.7 Conclusion

Adaptations that allow for greater discrimination of low intensity sounds may be important in the echolocation behavior of bats. In this dissertation, we used the pallid bat, *Antrozous pallidus*, a gleaning bat of the western United States, as a model to study the adaptations for intensity selectivity present in the auditory cortex. In Chapter 2 we performed in-vivo extracellular recordings in the high frequency region (HFR) of the pallid bat auditory cortex to determine the cortical organization and mechanisms of intensity selectivity. We tested several hypotheses in this study: (1) that cortical neurons

will be largely intensity selective to frequency modulated (FM) sweeps; (2) there is a topographic organization to intensity selectivity in the cortex; and (3) high frequency inhibition in the echolocation call enhances intensity selectivity. In Chapter 3 we examined the distribution of parvalbumin (PV) and calbindin (CB) expressing cells in cortical regions and thalamic nuclei in the auditory pathway. Immunohistochemical staining was used to test the hypothesis that there is a differential distribution of these calcium binding proteins in cortical and thalamic regions that have been implicated in processing of echolocation calls compared to non-echolocation associated regions. In Chapter 4 we examined the thalamocortical projections to intensity selective neurons in the HFR of the auditory cortex. Retrograde tracing from intensity selective and non-selective neurons was used to identify the thalamic nuclei that provided input to those neurons. We tested the hypothesis that there is a topographic organization to these thalamocortical projections in relation to the intensity selectivity of the cortical neurons. The results from this dissertation highlight the special adaptations that are present in the auditory cortex of the pallid bat that may be important for processing the low-intensity returning echoes of their call. Deviations from a general mammalian plan or even from other bat species in the properties that lead to these adaptations may strengthen the notion that general patterns of cortical processing and organization may be altered through evolution to support the unique behavioral needs of a species.

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Chapter 2: Intensity Selectivity in the Echolocation Call Selective Region of the Pallid Bat Auditory Cortex

Abstract

Adaptations that allow for greater discrimination of low intensity sounds may be important in the echolocation behavior of bats. In this study, we performed in-vivo extracellular recordings in the auditory cortex of the pallid bat, *Antrozous pallidus*, to determine the cortical organization and mechanisms of intensity selectivity. Downward frequency modulated (DFM) sweeps that approximated echolocation calls were used to study intensity selectivity using a behaviorally relevant sound. We show the region of the auditory cortex that is selective for echolocation calls contains a majority of neurons with a non-monotonic intensity selectivity function. That is, the response first increases and then decreases with increasing intensity. The vast majority (82.2%) of neurons were selective for low intensity DFM sweeps in the range of 10-50 dB SPL, effectively forming an 'acoustic fovea" in the intensity domain for returning echoes. A comparison of neuronal response to DFM sweeps and characteristic frequency (CF) tone bursts showed that many neurons had stronger intensity selectivity to DFM sweeps. Previous studies in the cat and mustached bat have found systematic organizations of intensity selectivity. In this study, cortical mapping also reveal a systematic organization of the intensity selectivity measures used. Mapping also revealed an ampliotopic organization of best intensities. In the response-timing domain, a large percentage (46%) of neurons displayed paradoxical-latency shift (PLS) in which the latency of first spike increased with high intensities. A comparison of neuronal response to DFM sweeps and CF tone bursts showed that many neurons had stronger intensity selectivity to DFM sweeps. This suggested a spectrotemporal integration mechanism that can shape intensity selectivity.

We examined this mechanism using the two-tone inhibition paradigm in which the intensities of both excitatory and inhibitory tones were concurrently increased. We show that the high frequency inhibition (HFI) arrived faster for increasing intensity in neurons that were more tuned for intensity to DFM sweeps than to CF tone bursts. These data suggest that a fast HFI inhibition in neurons with a more non-monotonic response to DFM sweeps to tones may inhibit the early response of a neuron leading to (1) a reduction in the response magnitude at high intensities and causing a non-monotonic intensity selectivity and (2) eliminate action potentials generated early in the response thus increasing the latency of the first spike resulting in a PLS. These results highlight the neural adaptations for low-intensity echolocation behavior in the pallid bat. The implications of these adaptations within the context of the gleaning foraging behavior of the pallid bat are discussed.

2.1 Introduction

Stimulus magnitude is an important parameter in distinguishing and generating meaning of sensory input in nature. In the auditory system, sound intensity can be used to help an animal determine location, distance and size of a sound source. It is important for the brain to detect and discriminate sounds of relevance so that an animal can respond appropriately to avoid predation, find food sources, and mate with conspecifics. In animals like bats that use an active bio-sonar signal this is particularly important as they need to appropriately discriminate the returning echoes from other sounds in their

environment. Spectral cues may not be sufficient in detecting a returning echo especially in an environment where bats of the same species are present and utilizing calls of a similar spectrotemporal structure. The intensity of the sound may be helpful in determining which sounds received at the periphery constitute a returning echo. Intensity selectivity is a neural adaptation that may allow behavioral discrimination of intensities. Neurons that are intensity selective have a non-monotonic response to increasing stimulus intensities, characterized by a cessation or reduction of response to high sound levels (Greenwood and Maruyama, 1965).

Studies on intensity selectivity have most often been conducted using responses to pure-tones and noise (Rouiller et al., 1983, Ehret and Moffat, 1985, Ehret and Merzenich, 1988, Phillips and Kelly, 1989, Aitkin, 1991, Schreiner et al., 1992, Phillips et al., 1994, Sutter and Schreiner, 1995, Watkins and Barbour, 2011, Zhou et al., 2012) with far fewer studies focusing on frequency-modulated (FM) sweeps (SULLIVAN III, 1982, Fuzessery, 1994, Wang et al., 2007). FM sweeps may help illuminate the spectrotemporal mechanisms that are not present in pure tones that can contribute to intensity selectivity. FM sweeps also have the advantage of mimicking some of the components of behaviorally relevant sounds like vocalizations in many mammalian species, including humans (Stevens and Klatt, 1974, Brown, 1976, Zeng et al., 2005, Portfors, 2007). In this study, we investigated intensity selectivity in the auditory cortex of the pallid bat (*Antrozous pallidus*) using as a probe the broadband FM sweep this species uses to echolocate. The pallid bat is a gleaning bat that uses echolocation for obstacle avoidance while listening passively for prey-generated noise transients to hunt

terrestrial prey. To echolocate, it uses ~60-30 kHz, 2-5 msec downward FM sweeps (Bell, 1982, Barber et al., 2003). Prey-generated noise is in the relatively lower frequency range of ~5-35 kHz. The pallid bat processes these two different streams of information in an auditory cortex that is parceled into two continuously tonotopic areas that selectively respond to the respective stimulus: a region that responds to high frequencies (30-75 kHz) selective for downward FM sweeps and a region that responds to low frequencies (5-35kHz) selective for broadband noise stimuli (Razak and Fuzessery, 2002). In this study, we examined intensity selectivity in the high frequency region using FM sweeps that are similar to the bat's echolocation call. The hypothesis tested in this study is that there are neural adaptations that increase intensity selectivity for behaviorally relevant sounds and that in the pallid bat auditory cortex we would see increased intensity selectivity to sounds that closely reflect, in both spectrotemporal and in intensity, the returning echoes of their call.

Sensory cortical maps that have systematic organization of stimulus parameters are important to conserve space and energy and to facilitate easier communication between neurons representing adjacent areas of the sensory periphery (Kaas, 1997). The cortical organization of amplitude representation and intensity selectivity has been studied in bats (Suga, 1977, Macias et al., 2014) and cats (Schreiner et al., 1992, Heil et al., 1994, Phillips et al., 1994, Sutter and Schreiner, 1995). These studies have included several bat species including the mustached bat, *Pteronotus parnellii*, in which a systematic representation of best amplitudes (ampliotopy) has been seen in the Doppler-shifted constant frequency area (DSCF) (Suga, 1977). Recent studies in the bat species

Macrotus waterhousii and *Molosuss molosuss* found no such ampliotopic organization suggesting that this feature is not common to all bats (Macias et al., 2014). Although the topographic organization of intensity selectivity has not been studied in bats, systematic organization of this feature along the iso-frequency axis is present in the cat cortex (Sutter and Schreiner, 1995). A recent study in the rat cortex also found a region outside of the primary cortex where intensity selective neurons are clustered (Wu et al., 2006). These studies bring into discussion whether topographic organization is part of a common mammalian plan or is it particular to certain species. This study looks at whether a topographic organization of best intensities and intensity selectivity is present in the pallid bat auditory cortex.

Responses in the auditory nerve to increasing intensity are monotonic in nature (Winter and Palmer, 1991). Intensity-tuning is first observed in the cochlear nucleus where inhibition helps shape the response to increasing intensity (Winter and Palmer, 1990). Inhibitory mechanisms responsible for the creation of intensity selectivity have been studied in the auditory cortex (Wu et al 2006, Tan et al 2007, Sutter and Loftus 2003). A combination of fast saturating excitation and slow saturating inhibition shape intensity selectivity in both earlier nuclei of the auditory pathway and also in the cortex. (Wu et al., 2006, Zhou et al., 2012). In this study we used the two-tone inhibition over time paradigm (TTI) to study the spectrotemporal mechanisms underlying intensity selectivity in the auditory cortex. It has been shown that early stage inhibition using a similar two-tone paradigm contributed to the intensity selectivity of the excitatory domain of cortical neurons (Sutter and Loftus, 2003). Studies also show that the relative

timing of the delay between synaptic excitatory and inhibitory inputs shape selectivity in cortical neurons (Wu et al., 2006). We hypothesized that: 1) neurons are more intensity tuned for FM sweeps than tones; and that 2) the increased intensity tuning for FM sweeps over tones is a result of faster arrival time of inhibition from an inhibitory tone outside of the excitatory tuning domain with increasing stimulus intensity. Because this study examines the timing of inhibition and the role it plays in shaping intensity tuning we also examined the relationship between the latency of response and the intensity tuning of cortical neurons. Neural recordings in bat IC showed that not only was there the typical decrease in latency seen during an increase in intensity but there were also neurons that increase their latency to an increasing intensity (Grinnell, 1963). This paradoxical-latency shift (PLS) has been hypothesized to be an important mechanism in range detection of combination sensitive neurons in the both of the bats species, *Myotis lucifigus,* and the mustached bat, *P. parnellii* (SULLIVAN III, 1982, Olsen and Suga, 1991). Recently FM sweeps were shown to be more effective at eliciting PLS than tones in the IC of *M. lucifigus* (Wang et al., 2007). In this study we will look at the distribution of neurons showing PLS with increasing intensity, the relationship of PLS to intensity selectivity, and the difference of PLS elicited by FM sweep compared to tones.

The role of intensity selectivity among the auditory system neurons in processing behaviorally relevant sounds is poorly understood. However, it has been show that intensity selectivity in the primary auditory cortex of rats is plastic and can be shaped in associative learning paradigms (Polley et al., 2004). This finding highlights the importance of intensity selectivity behaviorally and strengthens the argument for the need

to study intensity selectivity using behaviorally relevant stimuli across a number of species. Finding the appropriate stimuli without using a learning paradigm can be difficult for many species, however the pallid bat represents a strong model because the relevance of its echolocation call and the large area of the cortex devoted to processing these calls have already been well studied.

2.2 Methods

2.2.1 Animals

Pallid were netted in Arizona, New Mexico, Texas, and California and housed in a 11 x 14 ft room. The bats were able to fly in this room and were provided with crickets/mealworms and water *ad libitum*. The room was maintained on a reversed 12:12 light cycle. All procedures followed the animal welfare guidelines required by the National Institutes of Health and the Institutional Animal Care and Use Committee at the University of California, Riverside

2.2.2 Surgical Procedures

Recordings were obtained from the right auditory cortex of bats (both males and females, n =20 bats) anesthetized with isoflurane inhalation, followed by an i.p. injection of pentobarbital sodium (Nembutal, 30 μg/g). To expose the auditory cortex, the head was held in a bite bar, a midline incision was made in the scalp, and the muscles over the dorsal surface of the skull were reflected to the sides. The front of the skull was scraped

clean and a layer of glass microbeads applied, followed by a layer of dental cement. The bat was then placed in a custom-made Plexiglass holder. A cylindrical aluminum head pin was inserted through a cross-bar over the bat's head and cemented to the previously prepared region of the skull. This pin served to hold the head secure during the recording session. The crossbar holding the head pin was secured behind the bat, leaving no interference between the speaker and the ear. The location of the auditory cortex was determined relative to the rostrocaudal extent of the midsagittal sinus, the distance laterally from the midsagittal sinus, and the location of a prominent lateral blood vessel that lies parallel to the midsagittal sinus (Razak and Fuzessery, 2002). The size of the exposure was ~2mm^2. Exposed muscle was covered with petroleum jelly, and exposed brain surface was covered with silicone oil to prevent desiccation. At the end of the recording session the incision was sutured and lidocaine and a topical antibiotic were applied to the wound. The animal was allowed to recover in isolation before the next surgery. Following 1-3 recording sessions the bat was euthanized with an overdose of pentobarbital solution and trans-cardially perfused with 4% paraformaldehyde with subsequent removal of the brain for histological processing.

2.2.3 Recording Procedures

Experiments were conducted in a warm (~80°F), sound-proof chamber lined with anechoic foam (Gretch-Ken Industries, Oregon). Bats were kept anesthetized throughout the course of the experiments with additional pentobarbital sodium (one-third of pre-surgical dose) injections. Acoustic stimulation and data acquisition were driven by

custom software (Batlab, Dr. Don Gans, Kent State University) and Microstar DSP board based hardware. Programmable attenuators (PA5, Tucker-Davis Technologies, Florida) allowed control of sound intensities before amplification by an integrated amplifier (Yamaha AX430). Stimuli were delivered using an LCY- K100 ribbon tweeter (Madisound, Wisconsin) placed 38 cm directly in front of the bat at an elevation aligned with the snout. Because a major goal of this study was to examine intensity tuning to the FM sweeps used in echolocation, the 0^o elevation/azimuth speaker location was chosen as it approximates echoes coming back along the flight path. In addition, most FM sweep selective neurons tuned between 30–60 kHz in the pallid bat auditory cortex are binaurally insensitive (EO/O type neurons) when tested with interaural intensity differences (Razak and Fuzessery, 2002). Therefore, except for changes in absolute thresholds, the results presented below are likely to be similar at least for a narrow range of azimuths around 0^o from which echoes return.

The frequency response curve of the sound delivery system, measured with a 1/4-in microphone (Bruel and Kjaer, Denmark) had a roll-off from 30 to 80 kHz that was gradual at a rate ~20 dB/octave. Sound intensity levels for FM sweeps were calculated as the average intensity across the range of frequencies in the sweep. Recordings were obtained using glass electrodes (1M NaCl, 2 – 10 MΩ impedance) at depths between 200 and 600μm. Penetrations were made orthogonal to the surface of the cortex. Action potentials were amplified by a Dagan extracellular preamplifier (2400A) and a spike signal enhancer (FHC, Maine) and band-pass filtered (0.3–3 kHz, Krohn-Hite, MA). Extracellular single-unit recordings were identified based on window discriminator

threshold-crossing and consistency of action potential amplitude and waveform displayed on an oscilloscope. Waveforms and peri-stimulus time histograms were stored. Responses were quantified as the total number (20 stimulus repetitions, 1 Hz repetition rate) of action potentials occurring within 200 ms of stimulus onset. Adjustments for spontaneous activity were not necessary because there was no spontaneous activity in these recordings.

2.2.4 Data Acquisition

The focus of this study was on the high-frequency, FM sweep-selective region of the pallid bat A1 (Razak and Fuzessery, 2002). This region is likely to be involved in echolocation behavior. The FM sweep-selective region contains neurons tuned between 25 – 70 kHz and is located rostral and medial to the lower frequency neurons (tuning 5 – 35 kHz) that are noise-selective (Razak and Fuzessery, 2002). The FM sweep-selective neurons respond better to downward sweeps than to noise or upward sweeps with energy in the same spectral band. Using tones, noise, and downward sweeps as search stimuli, neurons with characteristic frequency (CF) >30 kHz, and with stronger response to downward FM sweeps than noise and upward FM sweeps were isolated.

Once single units were isolated various response properties were obtained. Pure tones (25–70kHz, 5 ms duration, 1 ms rise/fall times,1 Hz repetition rate) were used to determine the CF and minimum threshold (MT) for tones. CF was defined as the frequency that elicited action potentials to at least four of five successive stimulus repetitions at the lowest intensity, this intensity being the MT.

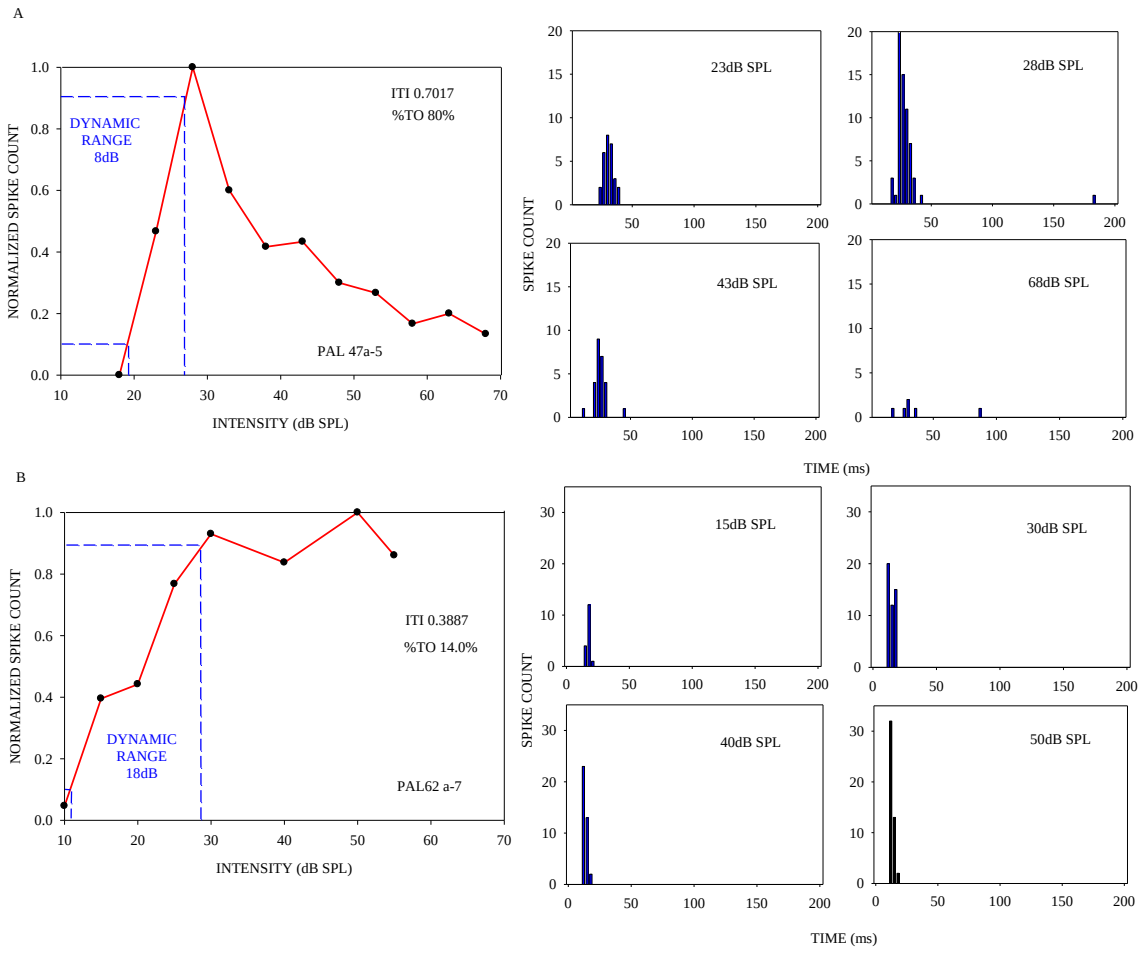


Figure 2.1: Measures of intensity selectivity.

Example of a non-monotonic or intensity selective response from a neuron in response to a downward FM sweep (60-30 KHz) (A). (left) Response-intensity function with %TO, ITI, and dynamic range values. (right) Post-stimulus time histograms (PSTHs) denoting responses throughout the range of intensities tested. The PSTHs show an initial increase in response with increasing intensity. This response begins to decline after reaching the maximum intensity. Example of a monotonic or non-intensity tuned response shown in (B). (right) PSTHs shows a response that continues to increase throughout the range of intensities. %TO values represent the response for 40dB above threshold. Dynamic range is a calculation of the range of intensity starting at 10% of the maximum response to 90% of the maximum response. Spike counts were normalized to maximum response.

2.2.5 Intensity Selectivity to FM Sweeps

Downward FM sweeps (5 ms duration, 1 ms rise/fall times, 1 Hz repetition rate) were presented at different intensities (in 5 dB steps increasing from MT). Responses to 20 stimulus presentations were recorded and an intensity selectivity was plotted. The best intensity (BI) was defined as the intensity at which the maximum number of spikes was elicited. The dynamic range (DR) was defined as the intensity range over which spikes counts increase from 10% to 90% of maximum response. Two measures were used to quantify intensity tuning. The percent turnover (%TO) indicates the monotonicity of the intensity selectivity function. %TO was calculated as:

$$\%TO = \frac{\text{Max. Response} - \text{Response at highest intensity}}{\text{Max. Response}} \times 100\%$$

(Phillips and Kelly, 1989). Because of the varying values of MT not all neurons were tested with the same number of intensities, so the %TO uses the highest intensity tested or 40 dB above threshold, whichever value is lower. %TO ranges between 0 and 100, with a higher number indicating stronger non-monotonicity. An intensity tuning index (ITI) was used to quantify the sharpness of intensity selectivity, where mean is the average response at all of the intensities tested and max is the maximum response elicited:

$$ITI = \left(\frac{N}{N-1}\right)\left(1 - \frac{\text{mean}}{\text{max}}\right)$$

ITI ranges between 0 and 1, with a higher number indicating sharper intensity selectivity. Latencies measures were derived from the median first-spike latency of the responses to 20 stimulus presentations.

2.2.6 Cortical Maps and Quantification

Cortical maps of %TO, ITI, and BI were created. Mapping studies were performed such that each penetration was 100–200 μm from a neighboring penetration in the rostral/caudal and medial/lateral directions. Deviations from this were typically caused by vasculature in a given local area. For each penetration, a two-dimensional set of coordinates were determined either using the scales on the Kopf electrode positioner, which were measured to the nearest 100 and 10 μm in the rostral/caudal and medial/lateral directions, respectively or recreated from a visually drawn map of vasculature landmarks and penetration markings. The coordinates were then plotted using the Voronoi function in Matlab (Kilgard et al. 2001). Null points in which there were no penetrations were used around the borders to preserve the relative shape and area of the polygons. Only one recording was made per penetration to create the maps. Compass plots were generated using the coordinates for the cortical maps and the appropriate tuning parameter (CF, BI, %TO, or ITI). The data (anatomical coordinates and tuning parameter values) were first normalized to the zero mean and unit standard deviation and a plane was fit to the tuning parameter as a function of x and y using a 2-D least-squares linear regression with the Matlab "fit" function. The gradients of the planar fit (direction and magnitude) were plotted as a compass plot and then R^2 value returned

by the “fit” function was represented using continuous color scale applied to vectors. The quantification of maps was performed by our collaborator, Dr. Stuart Yarrow from the University of Edinburg, Scotland.

2.2.7 Two-tone Inhibition Paradigm

To examine the contribution of spectral-temporal integration to intensity tuning for FM sweeps, a two-tone inhibition over time (TTI) method was used (Calford and Semple, 1995, Brosch and Schreiner, 1997, Gordon and O'Neill, 1998, Fuzessery et al., 2006, Razak and Fuzessery, 2006, Razak, 2013). Two tones were presented with different delays between them. The frequency of one tone was at the CF (excitatory tone) and was presented at an intensity of 10 – 20 dB above threshold and duration of 5 –10ms. The second tone was presented at the same intensity and duration of 5 –10ms. The frequency of the second tone was varied between 25 – 70 (1 kHz steps kHz and its onset time was varied with respect to that of the excitatory tone. Therefore, a resolution of 1 kHz was used to search for the appropriate inhibitory tone. When a frequency was discovered that inhibited the response to the excitatory tone by 50%, it was selected for the remainder of the TTI recordings. In the study, we focused only on the high-frequency inhibitory sideband because this inhibition shapes selectivity for DFMs that mimic echolocation calls in direction and rate. The arrival time of inhibition was defined as the shortest delay between the two tones that produced a 50% reduction in response than that obtained from the CF tone alone. Negative delays denote that the onset of the excitatory tone occurred before that of the inhibitory tone. Positive delays indicate that the onset of

the excitatory tone occurred after that of the inhibitory tone. Inhibition at negative arrival times mean inhibition occurred even when the inhibitory tone was delayed relative to the excitatory tone. Therefore, negative arrival times are interpreted as fast arriving inhibition relative to excitation. Positive arrival times mean inhibition occurred only when the inhibitory tone was advanced relative to excitatory tone. Positive arrival times are interpreted as slow inhibition relative to excitation. To explore how intensity affected the arrival time of inhibition, the excitatory and inhibitory tones were co-varied in intensity. Three to five different intensities were tested for each neuron. The intensities that were selected were determined by the minimum threshold of the neuron but usually represented a range of 10 - 40 dB above MT with steps of 5 -10dB. This paradigm allows an investigation of how timing of inhibition relative to excitation changes with increasing intensity.

2.3 Results

2.3.1 Neurons in the echolocation region of the pallid bat cortex are selective for low intensity.

The aim of this study was to investigate the intensity selectivity and mechanisms of high-frequency (>30 kHz) cortical neurons putatively involved in echolocation behavior. Downward FM (DFM) sweeps that were similar in bandwidth and duration to the bat's emitted echolocation call were used to characterize the response of 287 neurons in the high frequency region of the cortex of 20 bats . Many of the neurons that were

sampled in the auditory cortex showed at least some intensity tuning to DFM sweeps with 92.3% having a %TO of 20% or higher. Figure 2A shows the distribution of neurons across 4 different ranges of %TO values. A value of 100% means the neurons are highly selective for a range of intensities as the response of the neuron will be completely eliminated for higher intensities. A value of 0% means that a neuron is non-selective for intensity in ranges tested as the largest response was seen at the highest intensity tested. The majority of neurons displayed a high degree of intensity selectivity having a %TO of above 50%.

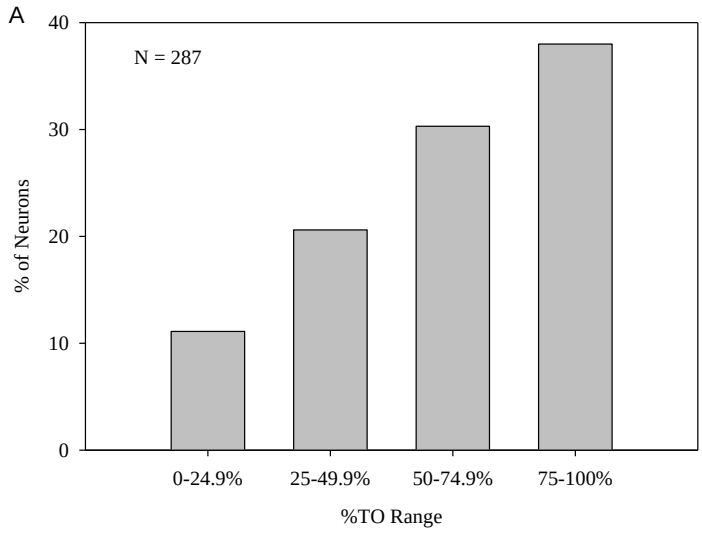
The majority of neurons had an ITI value above 0.2500. This means that intensity selective neurons are sharply tuned and have a narrow range of intensities that they respond best to (Figure 2B). Figure 2C shows the distribution of neurons across different ranges (dB) of dynamic range (DR). The DR is a measurement of the range of intensities, from threshold (measured as 10% of maximum response for the purposes of dynamic range) to 90% response of maximum. Neurons in the echolocation region had a DR that fell predominately in the range of 4-15dB meaning that most neurons arrived at their best intensity (BI) within a small change of intensities. Neurons in which the response at the lowest intensity never got below 10% of maximum were excluded from this measurement (N = 50).

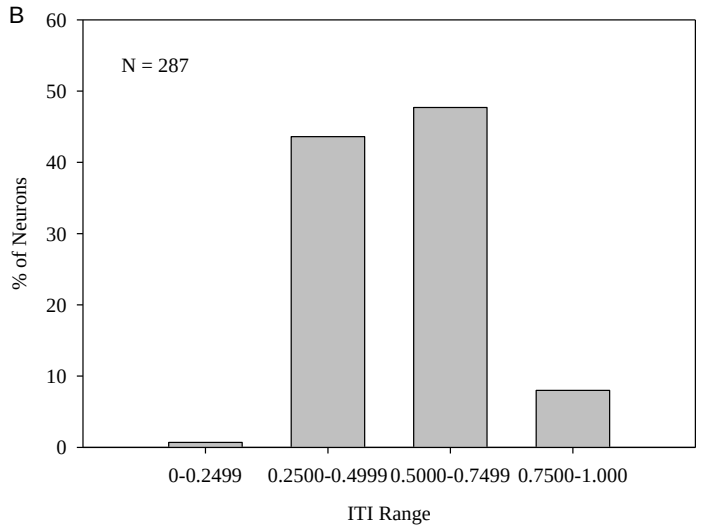
After discovering that many neurons had a high level of intensity selectivity we wanted to determine if the measures of intensity selectivity and sharpness correlate with each other. Data from 284 neurons showed a strong correlation (Linear regression, R^2 =

0.446, $p<0.001$) between the values of %TO and ITI when responding to DFM sweeps (Figure 3A). This means that neurons that are highly tuned for intensity also are more likely to be selective for a narrower range of intensities than neurons that are less selective. There were weaker relationships seen when comparing both %TO and ITI to dynamic range values (Linear regression, $R^2 = 0.222$, $p<0.001$ and $R^2 = 0.078$, $p<0.001$, respectively) (Figure 3B,C). These weaker correlations could be attributed to subtle differences in dynamic range values and that these small differences have no functional significance other than to show that most neurons have a best intensity (BI) close to threshold.

The large percentage of intensity selective neurons provided an opportunity to record reliable BIs and determine the range of this selectivity in the cortex. The majority of neurons that were tested with DFM sweeps were tuned to low intensities. Response-level functions revealed BI values that ranged from 0.4 dB SPL to 80.8 dB SPL. Of 271 neurons in which a BI could be determined only ~15% of neurons had a BI>50dB SPL (Figure 4A). The vast majority was selective for intensities below 40 dB. This selectivity for low intensities and the narrow range of DR values for most neurons means that majority of neurons should also be sensitive to low intensities. Sensitivity can be seen as the lowest intensity that still elicits a response in the neuron. Response-level functions revealed MT values that ranged from -5.1 dB SPL to 61.3 dB SPL. Of 285 neurons in which an MT was determined, ~50% of neurons had MT<20 db SPL. Less than 1% of neurons had MT>50.o dB SPL (Figure 4B). The pallid bat can produce an echolocation call that is approximately 80 db SPL (unpublished data) and receive

returning echoes that are approximately 30-40 dB SPL from a target that is 1 meter away. The sensitivity and selectivity for low intensities means that the auditory cortex is well adapted to process the low intensity returning echoes that it receives for its call.


A
N = 287
% of Neurons
0
10
20
30
40
0-24.9%
25-49.9%
50-74.9%
75-100%
%TO Range


B
N = 287
% of Neurons
0
10
20
30
40
50
60
0-0.2499
0.2500-0.4999
0.5000-0.7499
0.7500-1.000
ITI Range

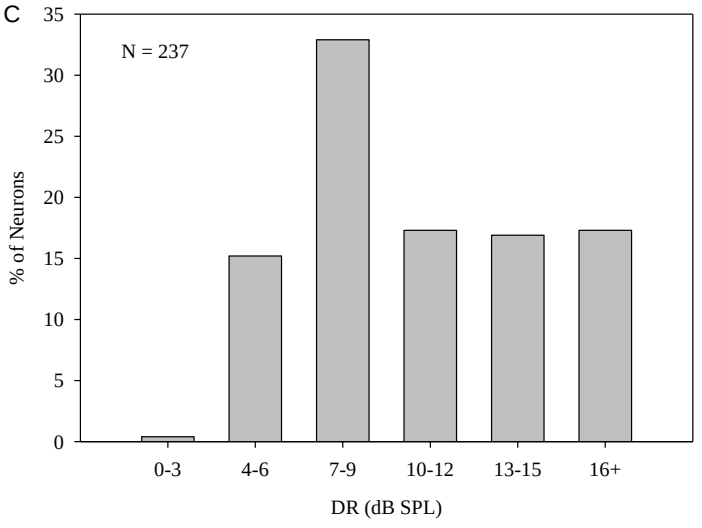

C
N = 237
% of Neurons
0
5
10
15
20
25
30
35
0-3
4-6
7-9
10-12
13-15
16+
DR (dB SPL)

Figure 2.2: Intensity selectivity distribution in the auditory cortex

(A) %TO distributions over four ranges from least selective (0-24.9%) to most selective (75-100%). (B) ITI distributions over four ranges from least sharpest tuning (0-0.2499) to sharpest tuning (0.7500-1.000). (C) DR distributions over six ranges. All responses were recorded to DFM stimuli.

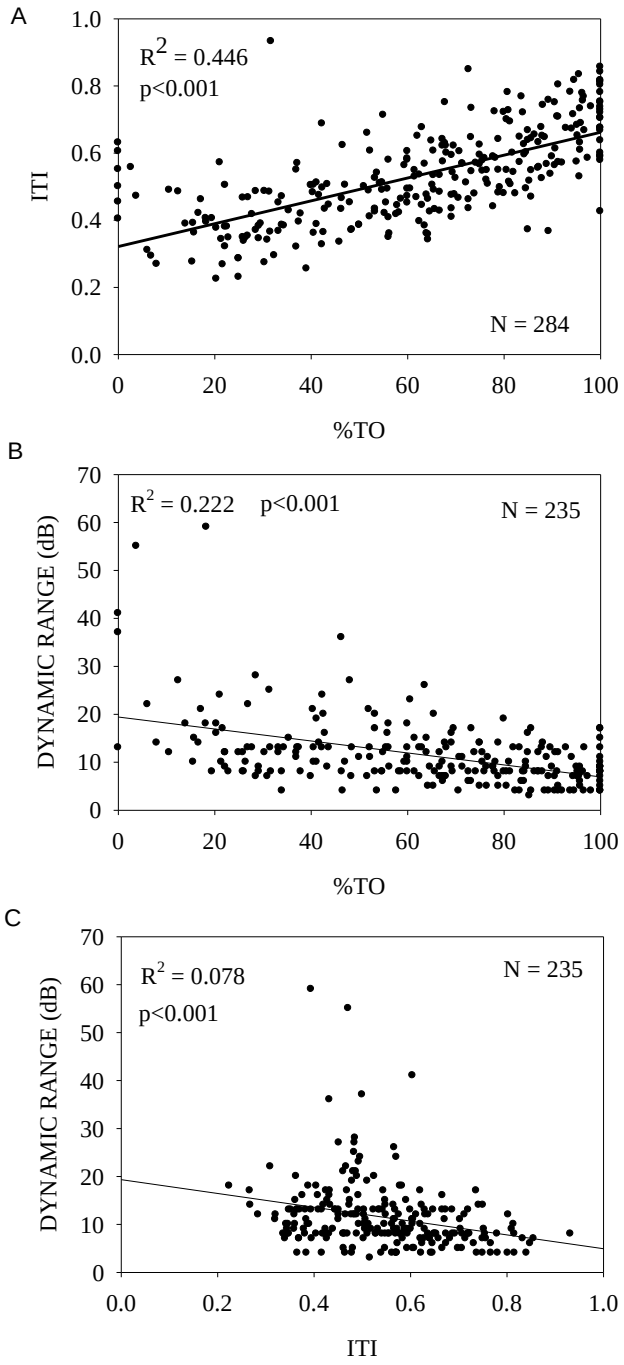

A
1.0
0.8
0.6
0.4
0.2
0.0
ITI
R2 = 0.446
p<0.001
N = 284
0
20
40
60
80
100
%TO
B
70
60
50
40
30
20
10
0
DYNAMIC RANGE (dB)
R2 = 0.222 p<0.001
N = 235
0
20
40
60
80
100
%TO
C
70
60
50
40
30
20
10
0
DYNAMIC RANGE (dB)
R2 = 0.078
p<0.001
N = 235
0.0
0.2
0.4
0.6
0.8
1.0
ITI

Figure 2.3: Correlation of measures of intensity selectivity.

(A) There is a high correlation (Linear regression analysis, $R^2 = 0.446$, $p<0.001$) between values of %TO and ITI. This means that neurons that are tuned for intensity (high %TO) are usually sharply tuned for a small range of intensities (high ITI value). Weaker correlations were seen for dynamic range vs. %TO (Linear regression analysis, $R^2 = 0.222$, $p<0.001$) (B), and ITI (Linear regression analysis, $R^2 = 0.078$, $p<0.001$)(C). All response were recorded to DFM stimuli.

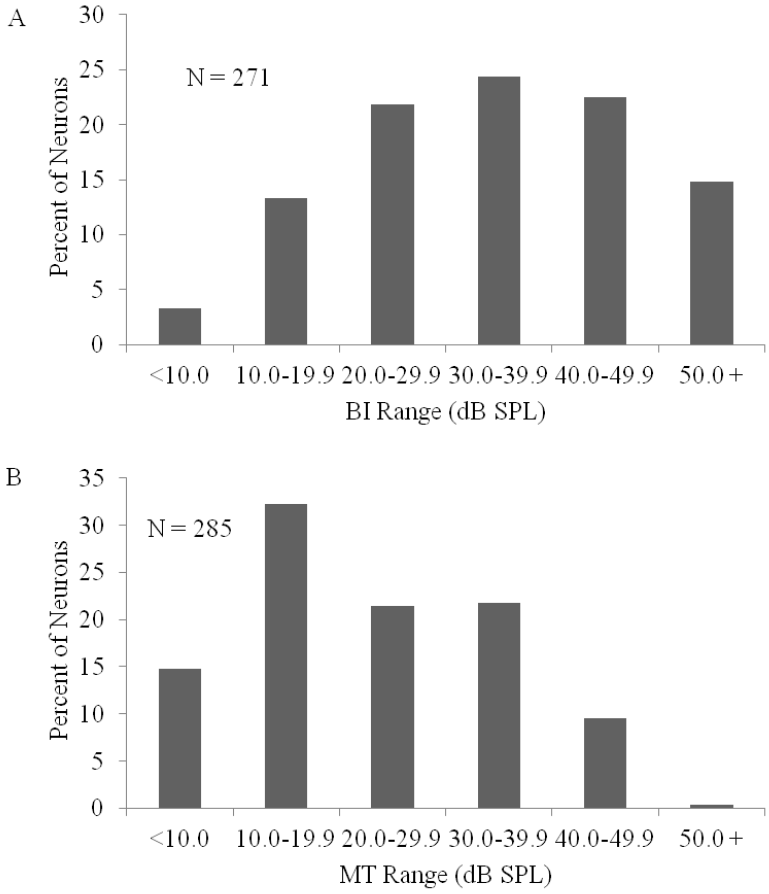


Figure 2.4: Cortical neurons are selective for low intensities.

(A) The majority of neurons showed a range of best intensities between 10-50 dB SPL. (B) Most neurons also had a minimum threshold that was lower than 50 dB SPL. All responses were recorded to DFM stimuli.

2.3.2 Neurons with higher characteristic frequency values are less selective for intensity.

The echolocation region of the pallid bat auditory cortex is selective for frequencies between 30-70 kHz. These frequencies represent the lower to higher end of the bat's echolocation call. A single echolocation call sweeping downward from

approximately 60 kHz to 30 kHz will activate neurons that have CF s within this range. We wanted to determine if the neuron's CF is related to its intensity selectivity. 280 (235 for DR) neurons were divided into three groups by their CF: 30-39 kHz, 40-49 kHz, and 50+ kHz. The average %TO of the 30-39 kHz group was 63.2% (N = 57), the 40-49 kHz was 67.9% (N = 125), and the 50+ kHz group was 54.4% (N = 98). There was a significant difference across the CF groups (1-way ANOVA, $P < 0.01$). A Tukey pair-wise comparison showed a significant difference between the 40-49 kHz and 50+ kHz groups ($P < 0.05$), although no significant difference was shown between the 30-39 kHz group and the other two groups (Table 1). The average ITI of the 30-39 kHz group was 0.535 (N = 57), the 40-49 kHz group was 0.568 (N = 125), and the 50+ kHz group was 0.535 (N = 98). The ITI values for the three groups followed a similar pattern across CF groups as did %TO with a significant difference between the groups (1-way ANOVA, $P < 0.001$). A Tukey pair-wise comparison showed only a significant difference between the 40-49 kHz group and the 50+ kHz group ($P < 0.001$) (Table 1). The median DR of the 30-39 kHz group was 11.3 dB (N = 49), the 40-49 kHz was 7.6 dB (N = 108), and the 50+ kHz group was 13.0 dB (N = 78). Neurons whose response did not go down below10% of maximum response were not included the analysis. There was no significant difference across the CF groups (1-way ANOVA Table 1). The average BI of the 30-39 kHz group was 35.1 dB SPL (N = 56), the 40-49 kHz group was 33.0 dB SPL (N = 125), and the 50+ kHz group was 36.8 dB SPL (N = 94). There was no difference in the average BIs between the three groups (1-way ANOVA Table 1). Neurons that had a bi-modal response (2 BIs) were excluded from the analysis (N = 15). The average MT of

the 30-39 kHz group was 25.0 dB SPL (N = 56), the 40-49 kHz group was 23.2 dB SPL (N = 125), and the 50+ kHz group was 21.9 dB SPL (N = 94). There was no difference in the average MTs between the three groups as determined by 1-way ANOVA (Table 1). These data suggest that neurons that are selective for frequencies in the lower and middle frequency portions of the echolocation call are more intensity selective than those selective for higher frequencies.

Measure	**30-39 kHz**	**40-49 kHz**	**50+ kHz**
%TO	63.18 +/- 3.50 SEM *a,b*	67.89 +/- 2.17 SEM *b*	54.39 +/- 3.00 SEM *a*
ITI	0.535 +/- 0.02 SEM *a,b*	0.568 +/- 0.01 SEM *b*	0.535 +/- 0.02 SEM *a*
DR (dB SPL)	11.27 +/- 1.07 SEM	7.642 +/- 0.74 SEM	12.95 +/- 0.70 SEM
BI (dB SPL)	35.08 +/- 1.90 SEM	32.99 +/- 1.35 SEM	36.80 +/- 1.46 SEM
MT (dB SPL)	25.04 +/- 1.42 SEM	23.19 +/- 1.17 SEM	21.93 +/- 1.17 SEM

Table 2.1: Intensity selectivity across CF ranges.

Mean values of %TO, ITI, DR, BI, and MT divided by CF ranges. A significant difference between groups was found for %TO and ITI (1-way ANOVA). Different lower case letters represent significant differences as determined by Tukey pair-wise comparison. DR, BI and MT have been determined by 1-way ANOVA to have no significant difference between the CF groups. SEM = Standard Error of the Mean.

2.3.3 Intensity selectivity and best intensities are organized in the HFR of the cortex

The organizations of three intensity selectivity measures across the auditory cortex were investigated. Cortical maps using Voronoi tessellation were generated for 9 bats using three response properties related to intensity tuning: %TO, ITI, and BI (Figure

5,6,7). Because of the stereotyped tonotopic gradient that is seen in high-frequency region of the pallid bat cortex the values for CF have been overlaid on top of each response property map to give the reader a sense of the relative location within the auditory cortex (Razak and Fuzessery, 2002). Points where no responses could be obtained to DFM sweeps were also recorded on the map and were left white as to represent areas that may be on the border of the auditory cortex. For maps of %TO and ITI a clustering analysis using the Pearson distance correlation (see Yarrow et al 2014) was performed. 6 out of 9 bats showed a significant organization with respect to %TO using this measure (Table 2.): PAL 44, 45, 46, 54, 55,and 64 with p-values of 0.006, 0.010, 0.002, 0.052, 0.024, and 0.021 respectively. For maps of ITI, only 2 maps showed a significant relationship (Table 3): PAL 45 and 46 with p-values of 0.012 and <0.001, respectively. This analysis revealed that there is a topography that is based off the values of %TO, however, it does not let us say anything about the precise organization of the topography except that there is clustering.

To determine if there was a systematic topography to the organization of different intensity selective measures a gradient analysis was performed for CF, %TO, ITI, and BI. Compass plots were also constructed from the same points used in the maps to visualize trends in increasing values for CF, %TO, ITI, and BI (Figure 8A,B,C,D). The compass plots show the direction of the increasing trend (arrow) with the magnitude of the arrow equaling the slope on the linear regression fit (see methods) and the color depicting the R^2 value for goodness of fit. Box and whisker plots have been prepared for all 4 parameters (Figure 8E,F) showing the median gradient angle, α (Figure 8E) and the

median magnitude of the vector (Figure 8F). Table 4 shows the p-values for the F-statistic of the linear correlation of each individual map. As expected all of the bats showed a significant organization with respect to increasing CF with increasing values in the medial direction. For %TO, 6 out of 9 bats showed a significant organization: PAL 44, 45, 46, 47, 54, and 55. The results are similar to that found with the Pearson distance correlation in that PAL 44, 45, 46, 54, and 55 had significant measures for both analyses. The increasing values of %TO for those bats with a significant organization are oriented in the lateral direction, opposite of the tonotopic gradient. This is consistent with what was shown earlier in that lower CF neurons had a significantly higher %TO value and those of a higher CF were less selective. For ITI, only 3 out of 9 bats showed a significant organization: PAL 45, 46, and 47. Only PAL 45 and 46 had significant organization with both this analysis and the Pearson distance correlation. For BI, 6 out of 9 bats showed a significant organization: PAL 44, 45, 46, 47, 54, and 55. The increasing values of BI for bats with a significant organization were all in the medial direction parallel to that of the tonotopic gradient, with the exception of PAL 55 which had the opposite orientation.

Although more precise characterization of the topographic nature of intensity selectivity cannot be made using these two measures, it is clear that a systematic representation of BIs and %TO values exists and unlike other response properties that have been described in the auditory cortex they do not seem to run orthogonal to the tonotopic gradient but are in line with it. Although it is difficult to speculate with the limited number of samples, the nature of local organization, it could be that with intensity

selectivity in the bat, the full range of best intensities and selectivity may not be available to every CF.

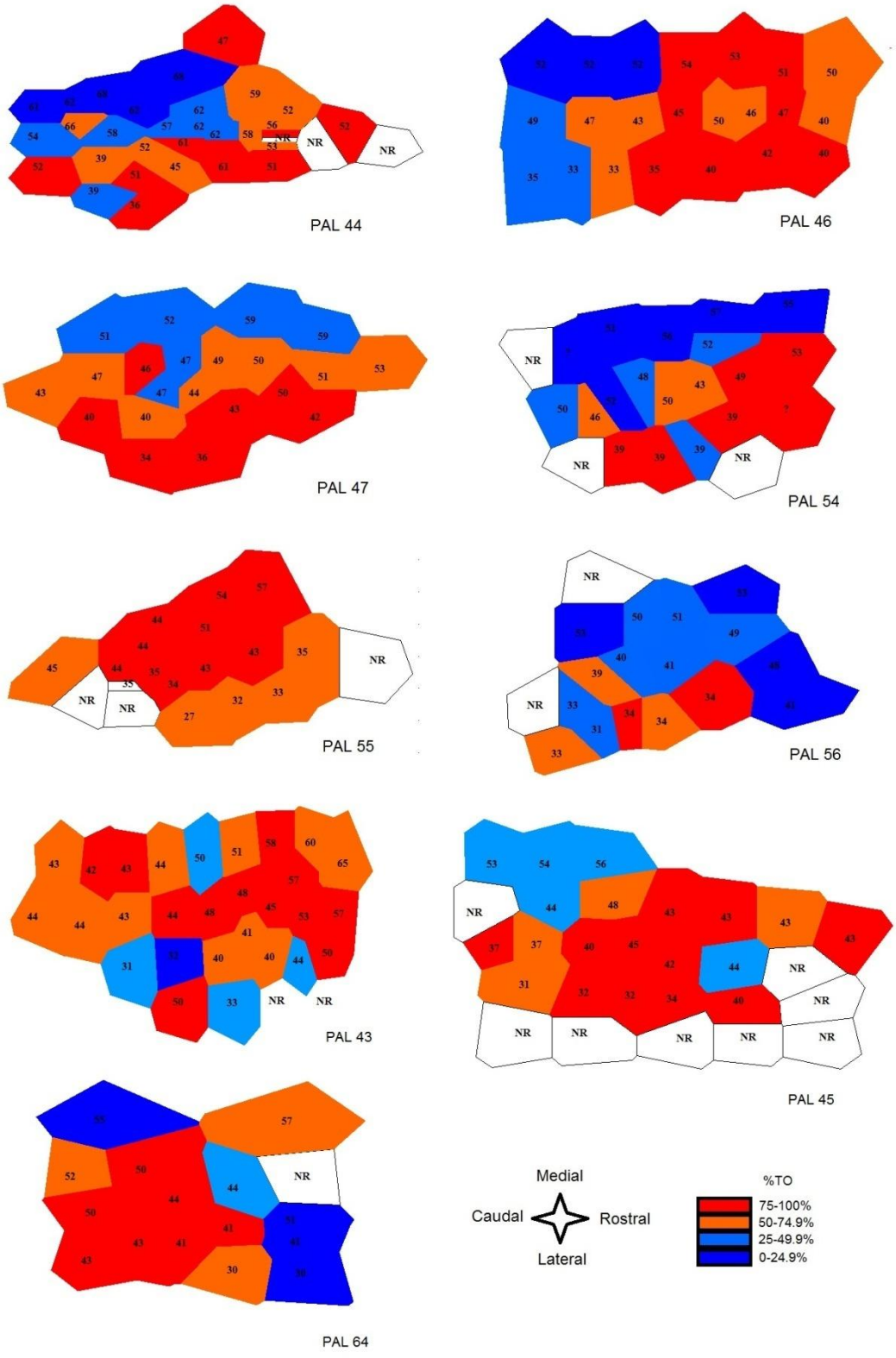

PAL 44
PAL 46
PAL 47
PAL 54
PAL 55
PAL 56
PAL 43
PAL 45
PAL 64
Medial
Caudal
Rostral
Lateral
%TO
75-100%
50-74.9%
25-49.9%
0-24.9%

Figure 2.5: Cortical maps of %TO.

These maps represent the spatial distribution of the %TO values seen in the cortex. Recordings were mapped using Voronoi tessellation, where the centers of the polygons represent the electrode placement and are approximately between 100 to 200 mm from the adjacent recording site. The number within each polygon represents the neuron's CF. Colors that fill the polygons represent the %TO value range of that neuron: (D. Blue) 0-24.9%, (L.Blue) 25-49.9%, (Orange) 50-74.9%, (Red) 75-100%. All responses recorded to DFM stimuli.

%TO	Pearson distance correlation	
Bat	C_{PC}	p
PAL43	0.0047	0.44
PAL44	0.20	0.0062*
PAL45	0.26	0.0095*
PAL46	0.33	0.0015*
PAL47	0.078	0.16
PAL54	0.17	0.052*
PAL55	0.29	0.024*
PAL56	0.16	0.071
PAL64	0.26	0.021*

Table 2.2. Pearson distance correlation of %TO maps

For each %TO map a Pearson distance correlation was performed to determine if neurons with similar %TO values were organized in a clustered fashion. C_{PC} = Pearson distance correlation value. Six out nine bats were determined to have a significant organization based on this measure (PAL 44, 45,46,54,55,64).

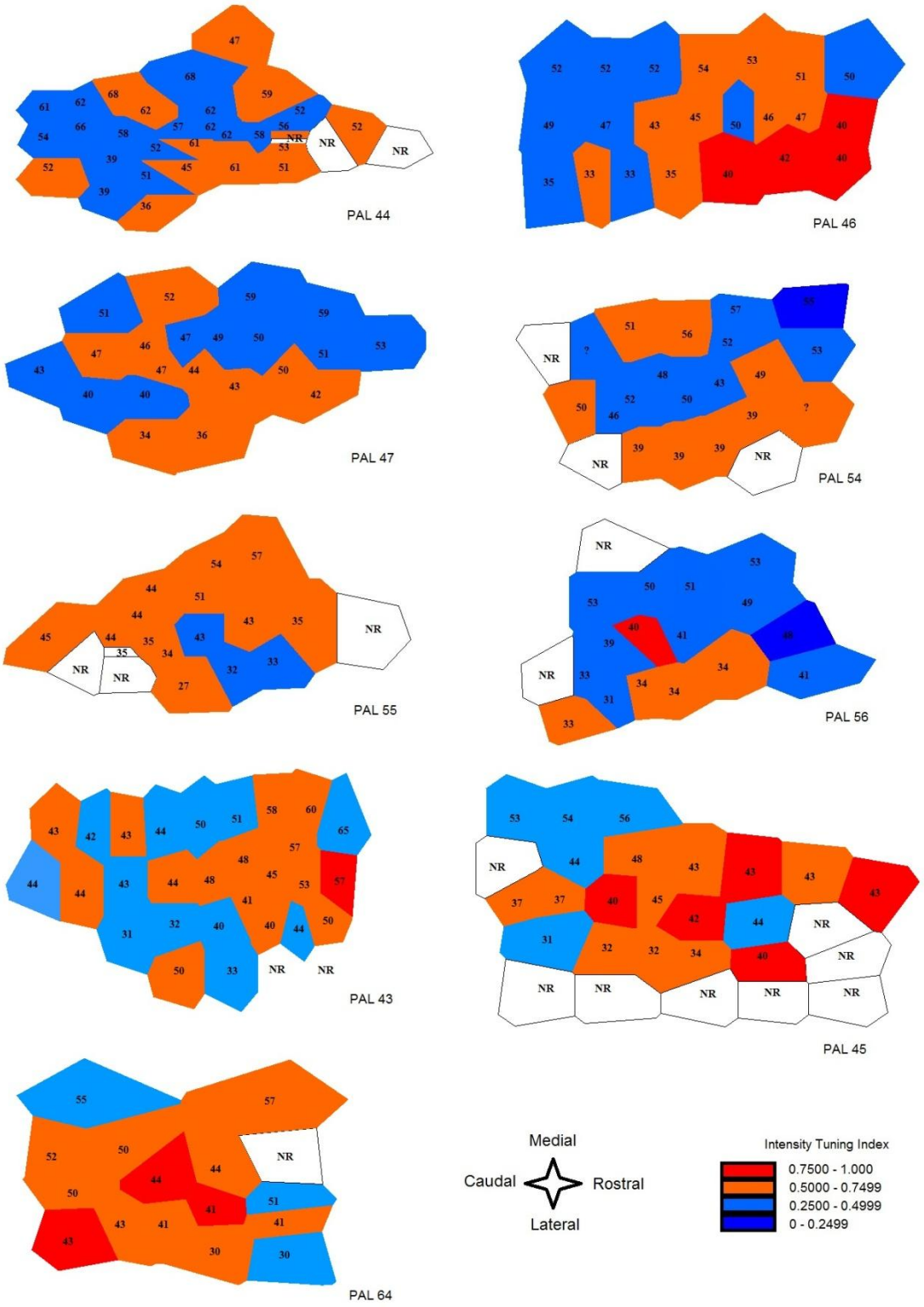

PAL 44
PAL 46
PAL 47
PAL 54
PAL 55
PAL 56
PAL 43
PAL 45
PAL 64
Medial
Caudal
Rostral
Lateral
Intensity Tuning Index
0.7500 - 1.000
0.5000 - 0.7499
0.2500 - 0.4999
0 - 0.2499

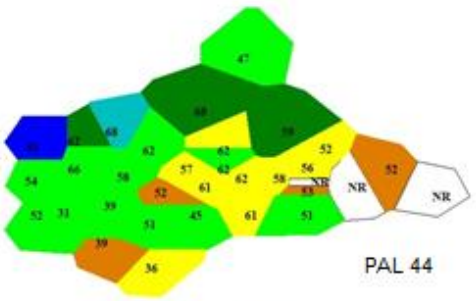
Figure 2.6: Cortical maps of intensity tuning index.

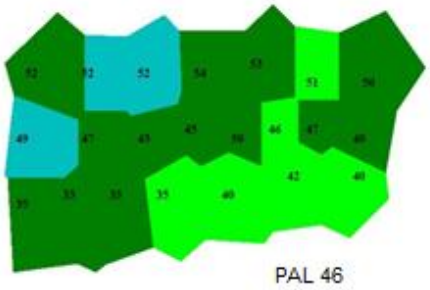
These maps represent the spatial distribution of the ITI values seen in the cortex. Recordings were mapped using Voronoi tessellation, where the centers of the polygons represent the electrode placement and are approximately between 100 to 200 mm from the adjacent recording site. The number within each polygon represents the neuron's CF. Colors that fill the polygons represent the ITI value range of that neuron: (D. Blue) 0-0.2499, (L.Blue), 0.2500-0.4999, (Orange) 0.5000-0.7499, (Red) 0.7500-1.000. All responses recorded to DFM stimuli.

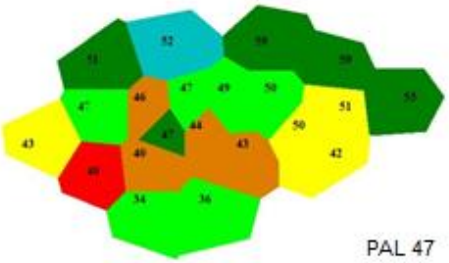
ITI	Pearson distance correlation	
Bat	C_{PC}	p
PAL43	0.034	0.30
PAL44	0.083	0.16
PAL45	0.23	0.012*
PAL46	0.50	< 0.001*
PAL47	-0.027	0.59
PAL54	0.13	0.14
PAL55	-0.025	0.54
PAL56	-0.046	0.58
PAL64	0.11	0.22

Table 2.3. Pearson distance correlation of ITI maps

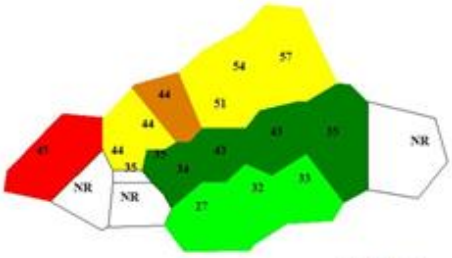
For each ITI map a Pearson distance correlation was performed to determine if neurons with similar ITI values were organized in a clustered fashion. C_{PC} = Pearson distance correlation value. Only two bats were determined to have a significant organization based on this measure (PAL 45, 46).

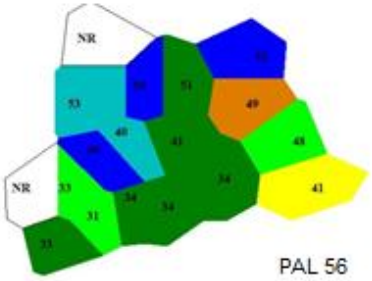

PAL 44

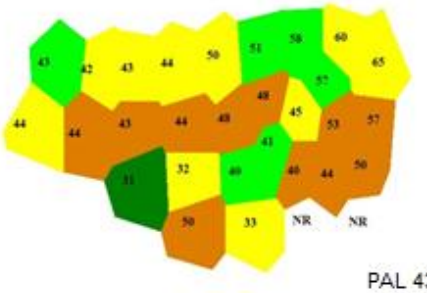

PAL 46

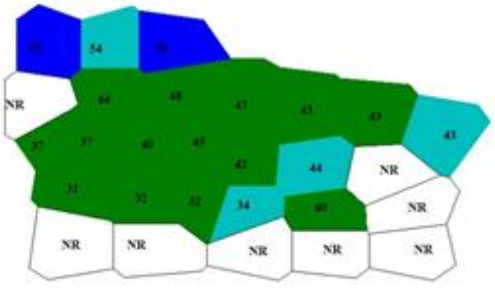

PAL 47

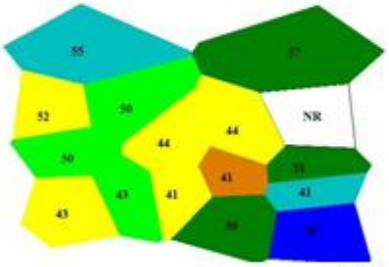

PAL 54

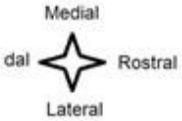

PAL 55


PAL 56


PAL 43


PAL 45


PAL 64


Medial
dal
Rostral
Lateral

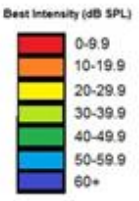

Best Intensity (dB SPL)
0-9.9
10-19.9
20-29.9
30-39.9
40-49.9
50-59.9
60+

Figure 2.7: Cortical maps of best intensity.

These maps represent the spatial distribution of the BI values seen in the cortex. Recordings were mapped using Voronoi tessellation, where the centers of the polygons represent the electrode placement and are approximately between 100 to 200 mm from the adjacent recording site. The number within each polygon represents the neuron's CF. Colors that fill the polygons represent the BI values with values of low intensities being towards red and higher intensities being towards blue.

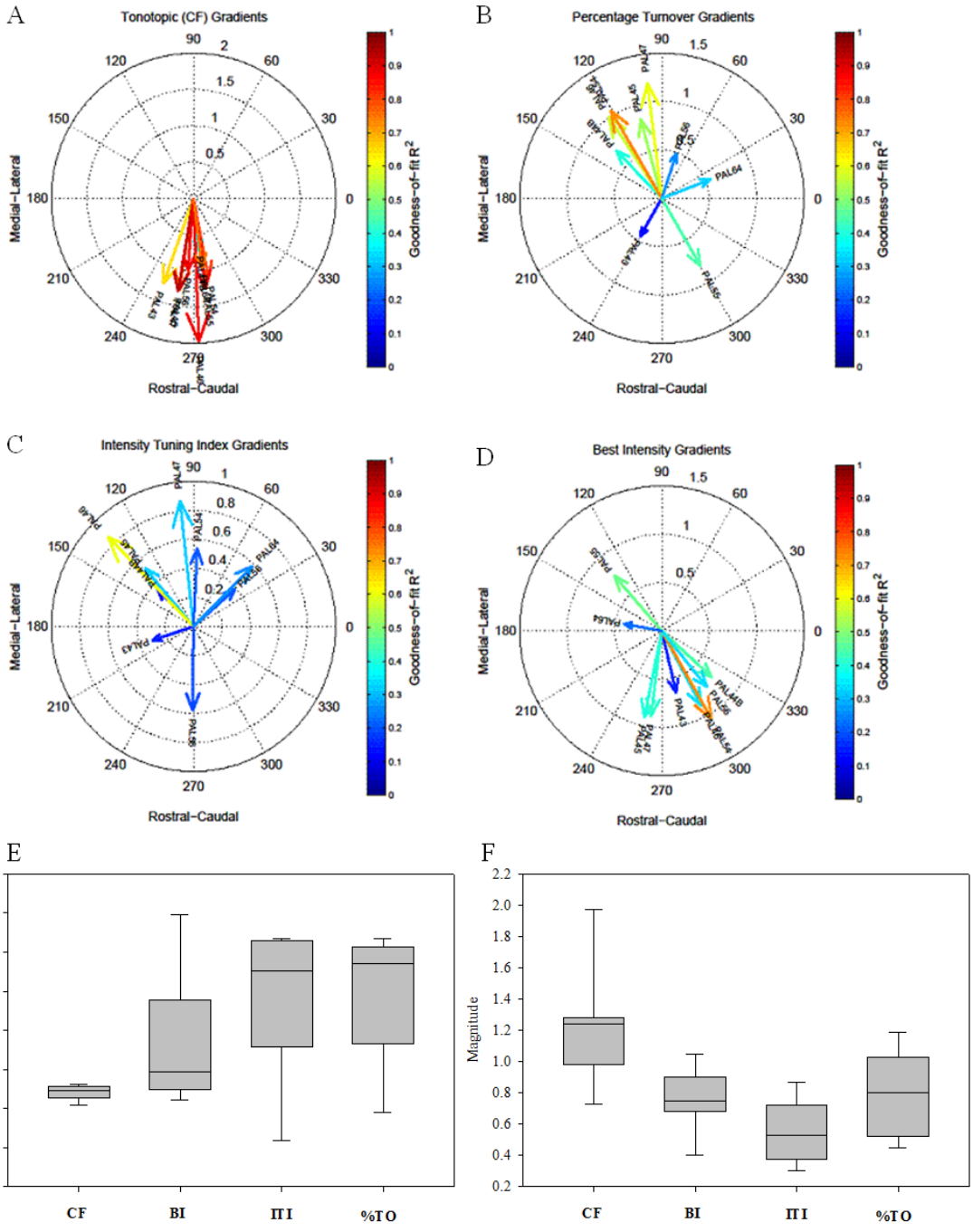

A
Tonotopic (CF) Gradients
B
Percentage Turnover Gradients
C
Intensity Tuning Index Gradients
D
Best Intensity Gradients
Medial–Lateral
Rostral–Caudal
Goodness–of–fit R2
E
α
CF
BI
ITI
%TO
F
Magnitude
CF
BI
ITI
%TO

Figure 2.8: Gradient analysis of intensity selectivity measures.

Circular plots characterizing the angular orientation of CF (A), %TO (B), ITI (C), and BI (D) of individual maps presented in Figures 5, 6, and 7. The arrows point to the mean angular direction of the circular distribution and its magnitude represents the slope of the linear regression while the color represents the R^2 value. (E,F) Box and whisker plots showing median values (line), $25^{th} – 75^{th}$ percentile (box), and max/min (whiskers). (E). Values for median gradient angles. (F) Values for median gradient magnitudes.

	PAL43	*PAL44*	*PAL45*	*PAL46*	*PAL47*	*PAL54*	*PAL55*	*PAL56*	*PAL64*
CF	*<.0.001**	*0.003**	*<.0.001**	*<.0.001**	*<.0.001**	*<.0.001**	*<.0.001**	*<.0.001**	*<.0.001**
%TO	*0.143*	*<.0.001**	*0.011**	*0.018**	*0.007**	*<.0.001**	*0.020**	*0.071*	*0.216*
ITI	*0.133*	*0.219*	*0.035**	*<.0.001**	*0.02**	*0.136*	*0.268*	*0.199*	*0.159*
BI	*0.175*	*0.001**	*0.002**	*<.0.001**	*<.0.001**	*<.0.001**	*0.027**	*0.132*	*0.093*

Table 2.4. P-Values for F-tests on the linear fits for mapping studies

P-values for F-tests on the linear fits versus a constant model. The p-values are calculated from the mapping studies. 9 out 9 bats had a significant value meaning that all the bats had a significant correlation between location in the cortex and the increasing value of CF. 6 out of 9 bats had significant value for linear fits of the %TO correlation. 3 out of 9 bats had a significant value for the linear fits of the ITI correlation. 6 out of 9 bats had significant value for linear fits of the BI correlation.

2.3.4 Neurons have a higher level of intensity selectivity for behaviorally relevant stimuli.

The purpose of this study was to examine the intensity-tuned responses to DFM sweeps that are similar to the bats natural echolocation call. For a smaller subset of neurons (N = 94), we were able to also look at the responses to a 5ms tone burst at the CF of that neuron to compare a simple sound to this more complex behaviorally relevant one. We compared intensity selectivity measures on a population level and found that all of the neurons (N=288) that were tested with DFM sweeps had a mean %TO of 61.8% (+/- 2.8% SEM) which was significantly higher (t-test, $p < 0.001$) than the neurons which were also tested with CF tone burst (N=94) which had a mean %TO of 48.7% (+/-2.8%

SEM) (Figure 9A). This means that as whole neurons in the auditory cortex had a higher intensity selectivity for DFM sweeps than CF tone burst. A similar result was seen when comparing ITI values across the same groups. The mean ITI value for neurons probed with DFM sweeps was 0.532 (+/-0.011 SEM) while neurons that were tested with CF tone bursts had a significantly lower (t-test, $p < 0.001$) ITI value with the mean being 0.468 (+/- 0.008 SEM) (Figure 9B). The results show that neurons in the HFR of the auditory cortex are more intensity selective and have a sharper selectivity when being stimulated with a behaviorally relevant sound rather than a simple tone.

To investigate further where this difference in selectivity in the population may arise from we examined the differences that occur across CF ranges. In comparing the population data of responses to DFM and CF tone bursts, neurons were more sharply tuned to intensity (ITI values) at the CFs in the 40-49 KHz range (Figure 10A). There was a significant difference between median values for ITI for the 40-49 kHz range (Mann-Whitney rank sum test, $p < 0.001$). There was no significance difference for the 30-39 kHz and 50+ kHz ranges (Figure 10A). In comparing the population data of responses to DFM and CF tone bursts, neurons had greater amount of intensity tuning (%TO values) at the CFs below the 50+ KHz range (Figure 10B). There was a significant difference in %TO median values between DFM and CF tone bursts for the 30-39 kHz and 40-49 kHz ranges (Mann-Whitney rank sum test, $p = 0.008$ and $p<0.001$ respectively) and no significant difference was seen in the 50+ kHz range (Figure 10B). The difference of intensity selectivity in the population of neurons in the HFR between DFM and CF stimuli seem to be driven predominately by the significant differences seen

only in the lower CF ranges of the neurons. This is possible evidence that the bat's echolocation call which would include the entire range of frequencies interacting over time as opposed to stimulation with a simple tone might elicit more intensity selectivity because of its spectrotemporal properties.

Although we saw population differences intensity selectivity we were also interested if individual neurons probed with both stimuli showed differences in intensity selectivity. Individual neurons (N = 93) were tested for both DFM sweeps and CF sweeps. What we found was that neurons that had a high %TO for DFM sweeps had a larger difference in their response to the two stimuli (%TO DFM - % TO CF) (Figure 11A.). This means that the differences in intensity selectivity that was seen by using the two stimuli in the population data was not created by individual neurons always having a constant difference in selectivity between the two stimuli, but rather there are a large group of neurons that are more selective for DFM sweeps alone. Figure 10B bears out this trend by showing a graph of four different %TO ranges to DFM sweeps and the mean difference of the %TO value for DFM to the value for CF tones (%TO DFM - %TO CF). For neurons in the highest selectivity group for DFM (75-100%TO) the mean difference was 24.3% (+/-4.1%SEM), for the 50-75%TO range the mean difference was 15.4% (+/-4.2%SEM), for the 25-50%TO range the mean difference was 5.9% (+/-5.1%SEM), and for the 0-25% range the mean difference was -16.0% (+/-10.2%SEM). A one-way ANOVA showed that there was a significant difference in the mean values among the %TO ranges ($p < 0.001$) and Tukey pairwise comparison showed significant differences in 75-100% vs. 0-25% ($p < 0.001$), 75-100% vs. 25-50% ($p = 0.049$), and 50-75% vs. 0-

25% ($p = 0.005$) and no significant differences in 75-100% vs. 50-75% ($p = 0.461$), 50-75% vs. 0-25% ($p = 0.530$), and 25-50% vs. 0-25% ($p = 0.120$) (Figure 11B). This data suggest that there are neurons that have more intensity selectivity for the stimuli that mimics the spectrotemporal pattern of their echolocation call but this increased selectivity is not due to an increased intensity selectivity to all sounds. This suggests that in a population of neurons the spectrotemporal components provide for a mechanism of greater intensity selectivity for the echolocation call.

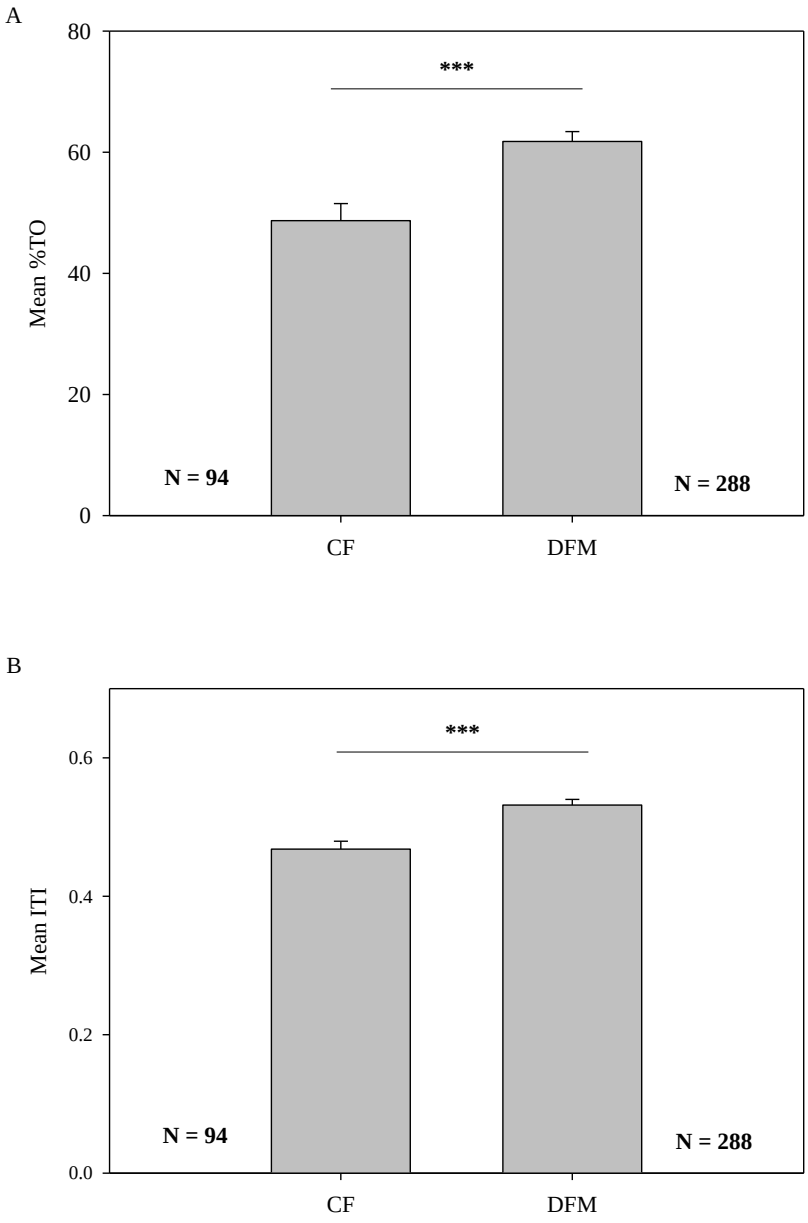


Figure 2.9: Comparison of intensity selectivity between FM sweeps and CF tone bursts.

Intensity selectivity measures were compared in population representing responses to DFM and CF tone burst. (A) There is a significant difference in the %TO values between response to DFM and CF tone bursts in neurons (t-test, $p<0.001$). (B) There is a significant difference in the ITI values between response to DFM and CF tone bursts in neurons. (t- test, $p<0.001$).

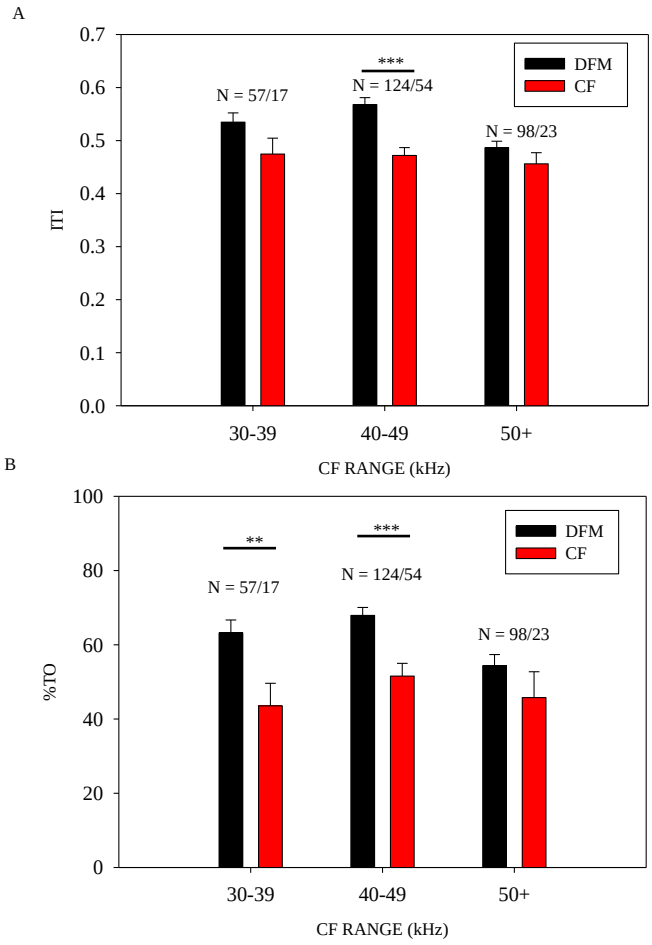


Figure 2.10: Neurons with higher CFs have less intensity selectivity.

Intensity tuning measures were compared against CF values in population representing responses to DFM and CF tone burst. *Top:* (A) There is a significant difference in the ITI values between response to DFM and CF tone bursts in neurons that have a CF value of 40-49.9 KHz (Mann-Whitney rank sum test, $p<0.001$). (B) There is a significant difference in the %TO values between response to DFM and CF tone bursts in neurons that have a CF value of 30-39.9 and 40-49.9 KHz (Mann-Whitney rank sum test, $p=0.008$ and $p<0.001$, respectively).

	DFM		CF Tone	
CF Range	**N**	**Median ITI**	**N**	**Median ITI**
30-39 kHz	57	0.507	17	0.437
40-49 kHz	125	0.574	54	0.465
50 kHz	98	0.486	23	0.456
CF Range	**N**	**Median %TO**	**N**	**Median %TO**
30-39 kHz	57	63.2%	17	43.5%
40-49 kHz	125	67.9%	54	51.6%
50 kHz	98	55.5%	23	36.7%

Table 2.5. Median ITI and %TO values for CF ranges: DFM vs. CF Tones

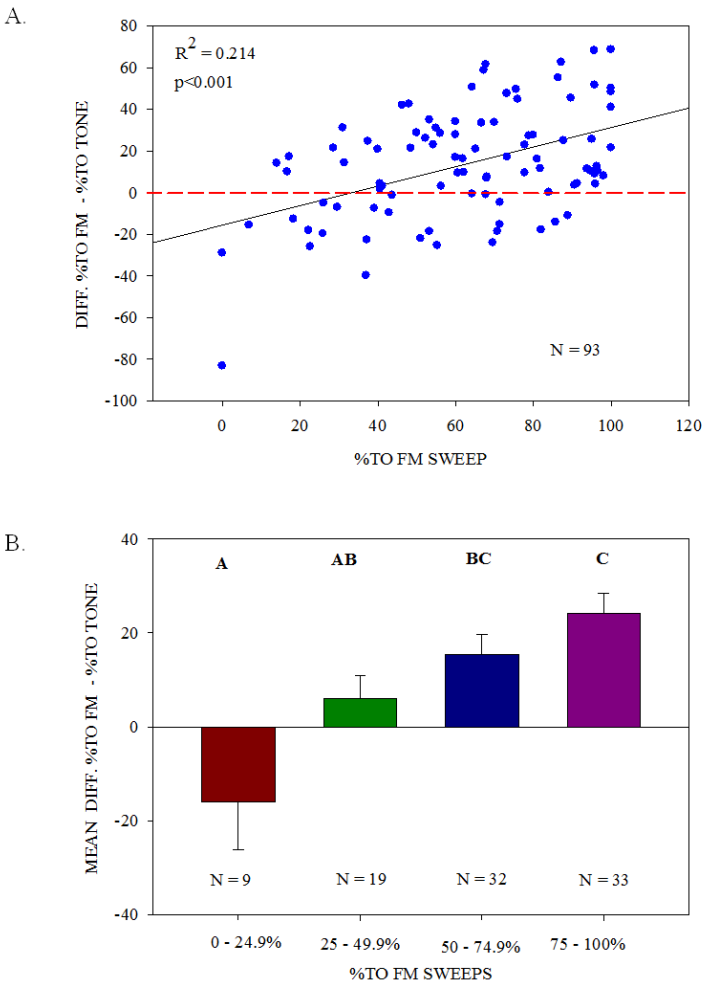


Figure 2.11: Individual neurons have high level of intensity selectivity for FM sweeps than for CF tone bursts.

Data collected from neurons tested to both DFM sweeps and CF tone bursts. (A) There is a correlation between the amount of intensity tuning to DFM and the difference between intensity tuning to DFM seeps and CF tone bursts (Linear regression, R2 = 0.214, p<0.001). (B) Neurons with a high %TO to DFM sweeps have a higher difference in intensity tuning of DFM sweeps to CF tones (One-way ANOVA, p<0.001. Similar letters represent non-significant differences (p>0.05) between %TO ranges (Tukey pairwise multiple comparison)

2.3.5 High frequency inhibition is responsible for increased intensity selectivity for DFM sweeps.

If a population of neurons in the HFR are more selective to DFM sweeps than CF tone bursts it is most likely because of spectrotemporal interactions that would only be present in the more complex DFM sweep. As previously mention inhibition has been shown to play a role in shaping intensity selectivity so we explored the possibility that high frequency inhibition outside of the excitatory CF of the neuron could be involved. 46 neurons were investigated using a two-tone inhibition paradigm over different intensities to determine the role that high frequency inhibition plays in shaping intensity selectivity. Of these neurons only 37 (78.7%) showed inhibition that lead to a reduction of 50% of the response to the CF tone alone to at least 3 different intensities (neurons were excluded from the analysis if they showed inhibition to less than three intensities). 17 (45.9%) of these neurons had a %TO for DFM sweeps that was at least 10% larger than that for CF tone stimuli, meaning these neurons were more intensity tuned for DFM sweeps. 7 (18.9%) neurons had an opposite response where %TO for CF tone stimuli was at least 10% larger than it was for DFM sweep stimuli. 10 (27.0%) neurons showed only a small difference in %TO values (less than 10%) for DFM sweeps and tone stimuli. An example of a neuron with a higher %TO in response to a DFM sweep than to a tone stimuli at CF is shown in Figure 12A. This neuron shows that when the response in spike count is graphed against delay of excitatory tone relative to the inhibitory tone that the arrival time of inhibition, the delay at which the maximum response is reduced 50%, becomes shorter as the intensity of the stimuli is increased (Figure 12B).

All 37 neurons that had inhibition for at least three intensities were graphed in a similar manner as Figure 12B and their arrival times of inhibition were subsequently graphed against the intensity of the stimuli. A linear regression between arrival time of inhibition and intensity was performed for each neuron. A negative value for the slope represents an arrival time of inhibition that becomes faster as intensity increases, while a negative slope reflects an arrival time that becomes slower with increasing intensity. The mean slope of these regressions was -0.209 (SE+/- 0.06) for neurons who had a greater than a +10% difference in %TO for DFM sweeps than CF tones. The mean slope of these regressions was 0.024 (SE+/- 0.06) for neurons who had a greater than a +10% difference in %TO for CF tones than DFM sweeps. A t-test ($p=0.044$) showed a significant difference between these two values demonstrating that neurons that are more tuned to DFM sweeps than CF tones will have greater increase in the arrival time of inhibition with increasing intensity (Figure 13). This means that the greater intensity selectivity that is seen for the DFM stimuli has been created in part by the temporal interactions of the high frequency inhibition present in the sweep and the excitatory characteristic frequency of the neuron. The interaction changes with intensity so that an increase in intensity means that inhibition arrives earlier and thus can reduce the excitatory response.

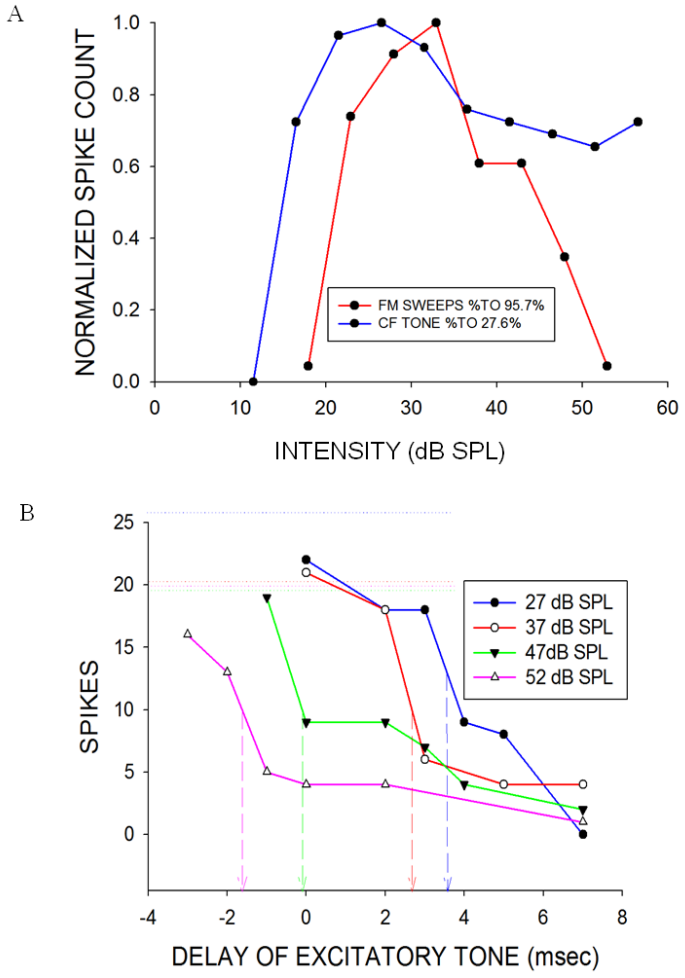


Figure 2.12: Two-tone inhibition can show intensity differences in the arrival times of inhibition

(A) Example of response-intensity functions of a single neuron in response to both DFM sweep and CF tone burst. This neuron is more tuned to intensity for DFM sweeps than CF tone bursts. (B) Response function of two-tone inhibition at different intensities for the same neuron. The dotted vertical line represents the arrival time of inhibition, the delay at which the response to the two tones has dropped by 50% of that by just the excitatory tone alone. The arrival time of inhibition gets faster as the intensity of the stimulus increases.

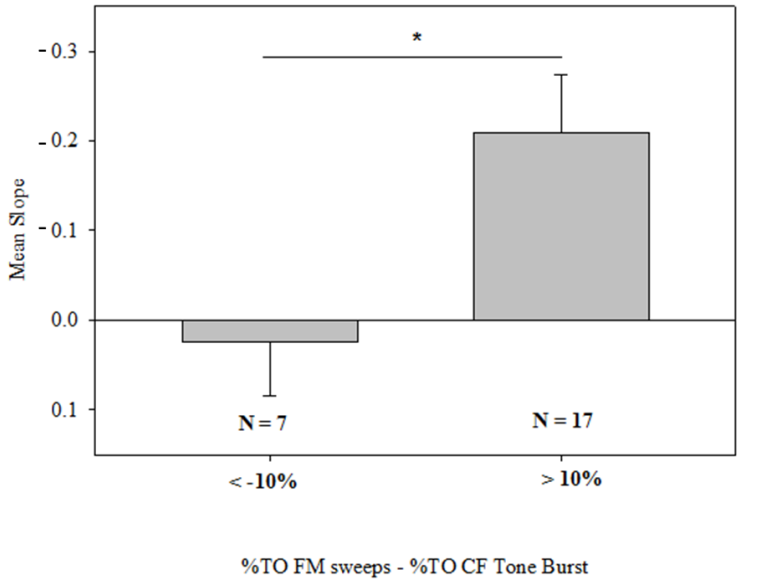


Figure 2.13: Faster arrival time of inhibition with increasing intensity shapes intensity selectivity response to DFM sweeps.

Neurons that were more intensity selective for DFM sweeps (%TO difference > +10%) than to CF tones had a significantly faster arrival time of inhibition with increasing intensity than neurons that were more intensity selective for CF tones (%TO difference < -10%) (t-test, p=0.044). Mean values determined by taking the slope of arrival time vs. intensity for each neuron.

2.3.6 Paradoxical latency shifts are seen in intensity selective neurons

Intensity selectivity leads to changes in the amount of response due to increasing intensity. Changes in other response parameters like first-spike latency may also be affected in an intensity dependent manner. As mentioned previously some neurons may show a paradoxical latency shift (PLS) in which the latency increases at higher intensities

as compared to the typical shortening of latency seen with increased intensity. As the latency of response may be important in the spectrotemporal interactions seen in an FM sweep we wanted to determine how many neurons displayed PLS in the HFR of the cortex along with the relationship of PLS to intensity selectivity measures. The median first spike latency (MFSL) was determined for different intensities of 287 neurons in response to DFM sweeps and 94 neurons in response to CF tones. A neuron was determined to have PLS if it showed an increase in the latency for two consecutive increasing intensities tested following the intensity at which the neuron displayed its shortest latency with the last intensity tested having to be at minimum 2 ms longer than the shortest latency. If the shortest latency was at the highest intensity tested or if the latencies for increasing intensities following the shortest latency did no increase more than 2ms, then it was determined that the neuron was defined by a non-paradoxical latency shift (NPLS). If a neuron had only one intensity tested higher than the intensity at which the shortest latency was seen, the neuron was excluded from the analysis because it would be difficult to determine the trend based on a single data point. If the latencies at intensities higher than that of the shortest latency were highly variable then the neuron was also excluded from analysis. Figure 14 shows example response-intensity and latency-intensity functions of neurons with a high %TO and PLS (A), a low %TO and PLS (B), a high %TO and NPLS (C), and a low %TO and NPLS (D). Of the 287 neurons tested with DFM sweeps 104 (36.2%) had NPLS, 132 (46.0%) had PLS, and 51 (17.8%) were undefined. If undefined neurons are excluded then 44.1% of the neurons had NPLS and 55.9% had PLS for DFM sweeps. Of the 94 neurons tested with CF tone

bursts 36 (38.3%) had NPLS, 39 (41.5%) had PLS, and 19 (20.2%) were undefined. If undefined neurons are excluded then 48.0% had NPLS and 52.0% had PLS. This data shows that large number of neurons in the HFR tested with DFM as well as CF tone bursts of the cortex have PLS. This could suggest that spectrotemporal components that shaped intensity selectivity may not play a role in shaping response latencies, although the actual change in latencies was not quantified in this study and may yet be affected by these contributions. We also wanted to examine the relationship between intensity selectivity and the latency of response. Neurons that display PLS are significantly more intensity selective when presented DFM stimuli than NPLS neurons. PLS neurons have a mean %TO of 70.9% while NPLS neurons have a mean %TO of 49.2% (t-test, $p<0.001$) (Figure 15A). PLS neurons also have sharper intensity selectivity when presented DFM stimuli than NPLS neurons. PLS neurons have a mean ITI of 0.552 while NPLS neurons have a mean of 0.480 (t-test, $p<0.001$) (Figure 15B). This data suggests that the latency of response, whether a neuron has PLS or NPLS, is related to intensity selectivity.

Although there is no difference in the percent of neurons with PLS for CF tone bursts and DFM sweeps, we wanted to examine if neurons that a higher level of intensity selectivity for DFM were more likely to have PLS than neurons that had a similar selectivity for CF tones. DFM sweeps were more efficient at generating PLS for neurons with high %TO than CF tones are (Figure 16A for DFM and 16B for CF). Out of 132 neurons with PLS to DFM sweeps 1 (0.8%) has a %TO between 0-24.9%, 18 (13.6%) have a %TO between 25-49.9%, 51 (38.6%) have a %TO between 50-74.9%, and 62 (47.0%) have a %TO between 74.9%-100%. Out of 104 neurons with NPLS to DFM

sweeps 27 (26.0%) have a %TO between 0-24.9%, 32 (30.8%) have a %TO between 25-49.9%, 15 (14.4%) have a %TO between 50-74.9%, and 30 (28.8%) have a %TO between 74.9%-100%. This means that the percentage of neurons that have PLS increases the more intensity selective the neurons are. This is different than what is seen when neurons are probed with CF tone bursts. Neurons presented with CF stimuli did not have higher percentages of PLS neurons with increasing intensity selectivity. Out of 39 neurons with PLS to CF tone bursts 5 (12.8%) have a %TO between 0-24.9%, 13 (33.3%) have a %TO between 25-49.9%, 11 (28.3%) have a %TO between 50-74.9%, and 10 (25.6%) have a %TO between 74.9%-100%. Out of 36 neurons with NPLS to CF tone bursts 9 (25.0%) have a %TO between 0-24.9%, 9 (25.0%) have a %TO between 25-49.9%, 10 (22.2%) have a %TO between 50-74.9%, and 8 (22.2%) have a %TO between 74.9%-100%. As the previous data represents the population, we also wanted to look at individual neurons to see if their responses to DFM and CF were different. Figure 17 shows 4 examples of the response: PLS for both DFM and CF, PLS for DFM only, and PLS, for CF only, and NPLS for both. The data shows that the majority of neurons either have PLS for both stimuli or have NPLS for both stimuli.

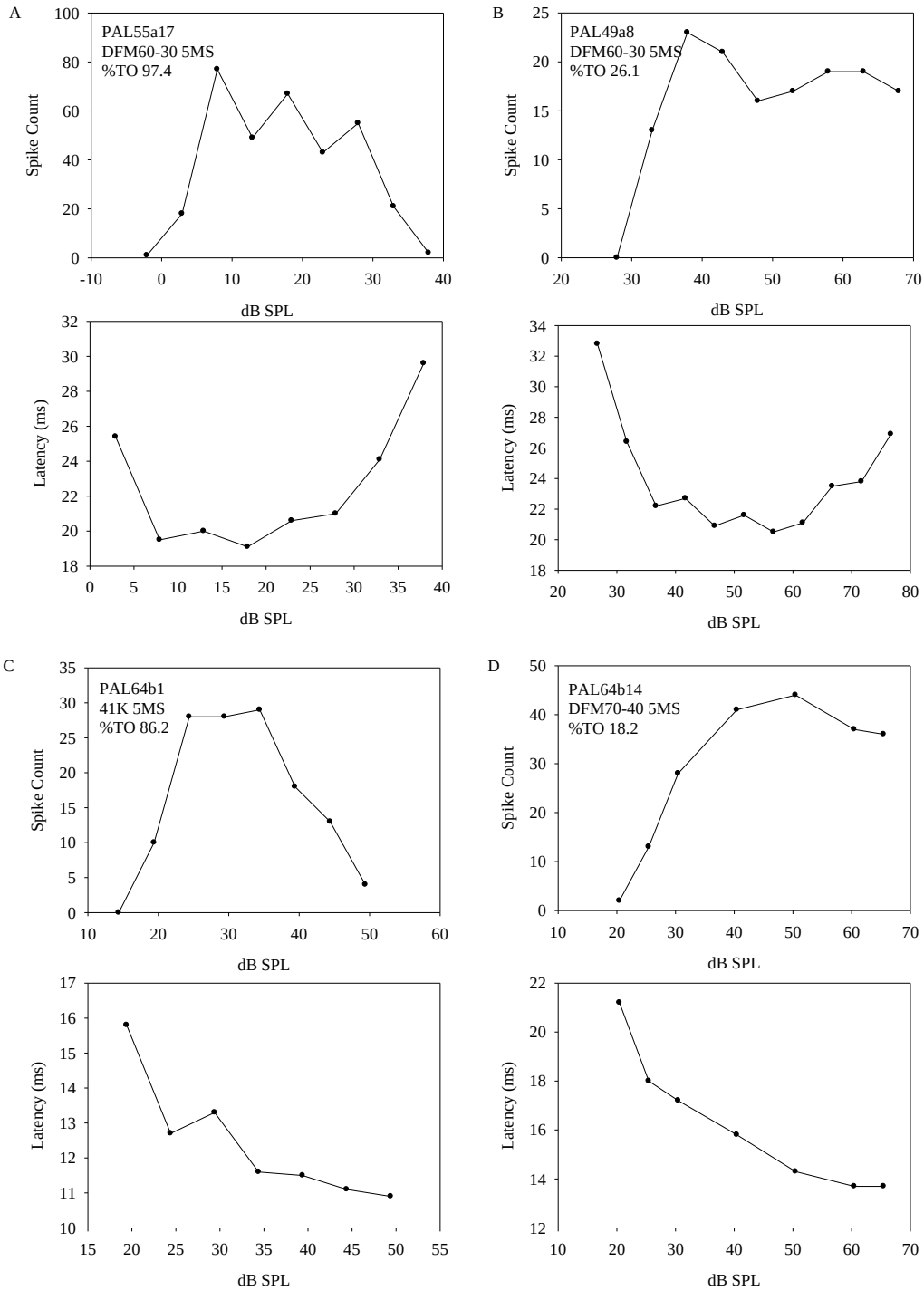

A
PAL55a17
DFM60-30 5MS
%TO 97.4
Spike Count
dB SPL
Latency (ms)
dB SPL
B
PAL49a8
DFM60-30 5MS
%TO 26.1
Spike Count
dB SPL
Latency (ms)
dB SPL
C
PAL64b1
41K 5MS
%TO 86.2
Spike Count
dB SPL
Latency (ms)
dB SPL
D
PAL64b14
DFM70-40 5MS
%TO 18.2
Spike Count
dB SPL
Latency (ms)
dB SPL

Figure 2.14: Examples of neurons displaying PLS and NPLS.

(top A,B,C,D) Response-intensity functions and (bottom A,B,C,D) Latency-intensity functions to DFM sweeps (A,B,D) and CF tone (C). (A) Non-monotonic response with PLS. (B) Monotonic response with PLS. (C) non-monotonic response with NPLS. (D) Monotonic response with NPLS.

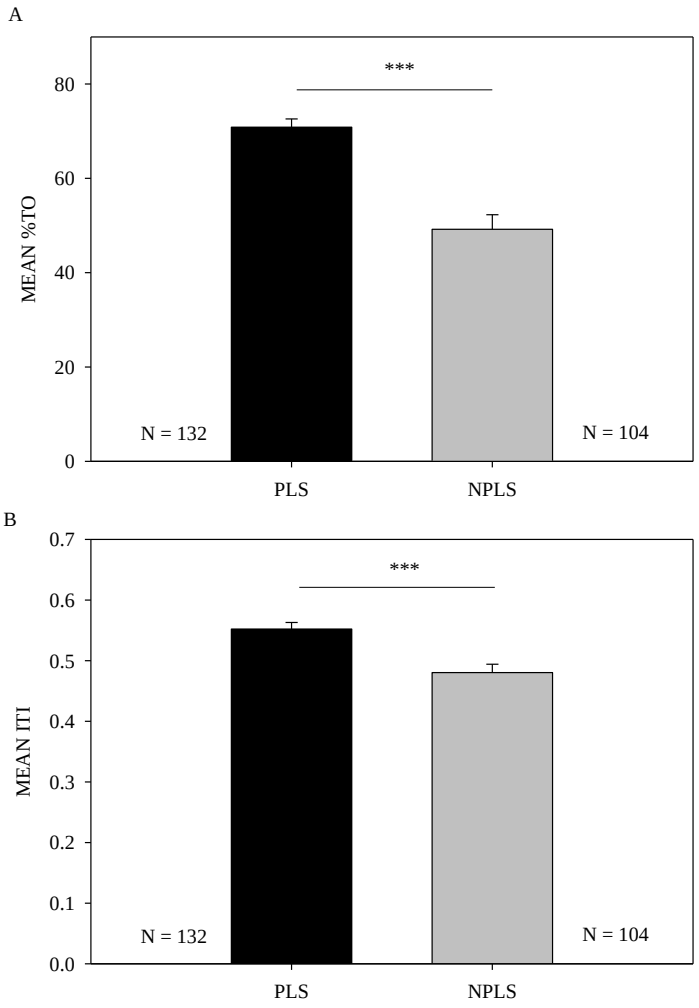


Figure 2.15: PLS neurons are more intensity selective

A. Mean %TO for neurons with PLS and NPLS. There is a significant difference between the two populations (t-test, $p<0.001$). B. Mean ITI for neurons with PLS and NPLS. There is a significant difference between the two populations (t-test, $p<0.001$). All responses were to DFM stimuli.

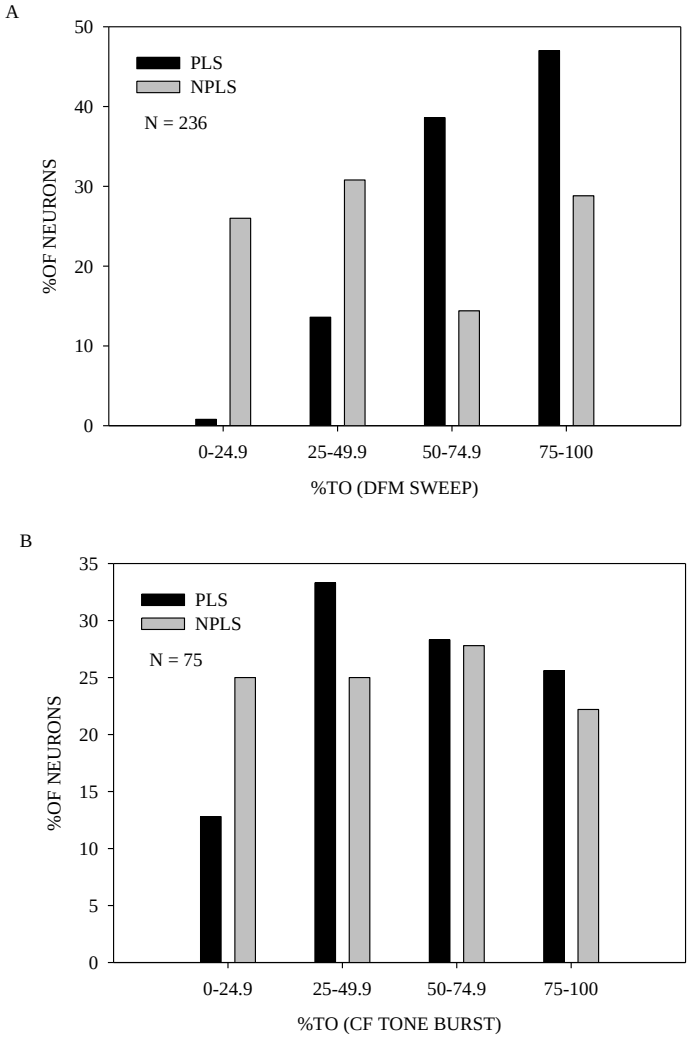


Figure 2.16: FM sweeps are more likely to produce responses that are intensity selective and have PLS.

Responses to DFM sweeps. The majority of PLS neurons have higher %TO values. (B) Response to CF tone bursts. Both PLS and NPLS responses show an equal distribution across %TO ranges.

	PLS		NPLS	
	Total	**%**	**Total**	**%**
DFM	132	-	104	-
%TO				
0-24.9	1	0.8	27	26.0
25.9-49.9	18	13.6	32	30.8
50.0-74.9	51	38.6	15	14.4
74.9-100	62	47.0	30	28.8
CF	39	-	36	-
%TO				
0-24.9	5	12.8	9	25.0
25.9-49.9	13	33.3	9	25.0
50.0-74.9	11	28.3	10	27.8
74.9-100	10	25.6	8	22.2

Table 2.6. %TO values for PLS and NPLS neurons.

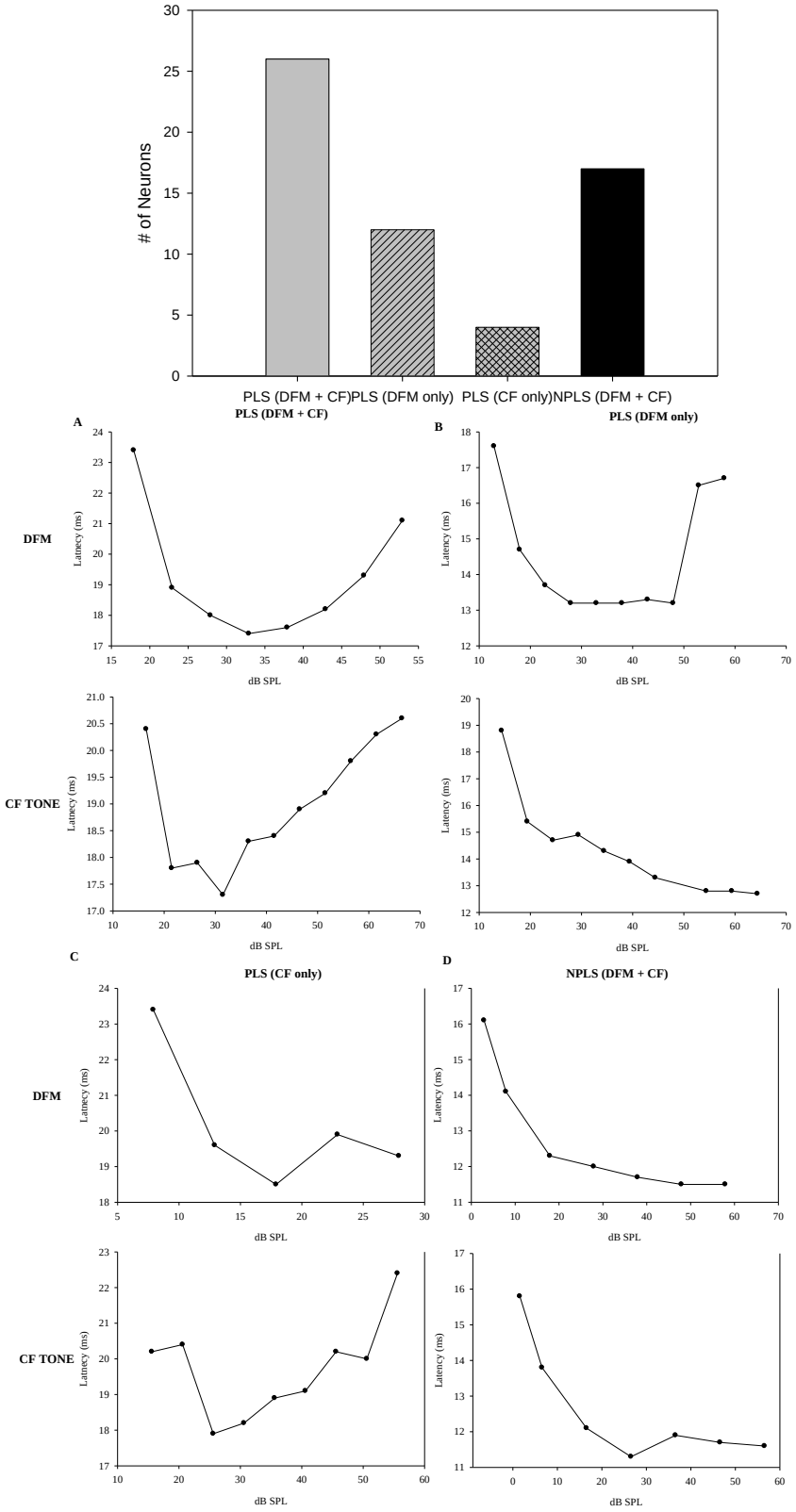

30
25
20
15
10
5
0
# of Neurons
PLS (DFM + CF)
PLS (DFM only)
PLS (CF only)
NPLS (DFM + CF)
A
B
C
D
DFM
CF TONE
Latency (ms)
dB SPL

Figure 2.17: Most individual neurons show PLS to both DFM and CF.

(upper) The number of neurons who showed PLS to both DFM and CF, only to DFM, only to CF, or showed NPLS for both. (bottom) Examples neurons where (A) PLS for both CF and DFM, (B) PLS for DFM only, (C) PLS for CF only, and (D) NPLS for both.

2.3.7 Neurons with PLS are selective for low intensities

If PLS is involved in the processing of the returning echoes of echolocation calls then neurons with PLS may be selective for lower intensities. We wanted to examine the best intensities of neurons and see if there was a difference between those with PLS and those with NPLS. PLS neurons have a lower BI than neurons that have NPLS to DFM stimuli. The mean BI for PLS neurons is 29.8 db SPL (+/- 1.1 SE), while the mean BI for NPLS neurons is 41.2 dB SPL (+/- 15.1 SE). A t-test ($p<0.001$) confirms that this represents a significant difference between PLS and NPLS neurons (Figure 18). We also wanted to examine whether there was a relationship to the BI of the neuron and the intensity at which it had its shortest latency. There is a significant correlation (Linear regression, $R^2 = 0.632$, $p < 0.001$) found between the BI (dB SPL) of a neuron and the intensity (dB SPL) at which the neuron responded to DFM sweeps with the shortest first-spike latency (Figure 19A). This means that the intensity that has the peak response may often be the intensity which also produces the shortest latency. This suggests that the shortness of the latency could be related to the amount of response from the neuron. However, no such correlation was found when comparing the BI (dB SPL) and the shortest first-spike latency (ms) (Linear regression, $R^2 <0.001$, $p < 0.001$) (Figure 19B). This means neurons with different BIs may have different first MFSLs. This could allow for a population of neurons to represent a wide range of possible combinations of timing of response and intensity at which the neuron responds.

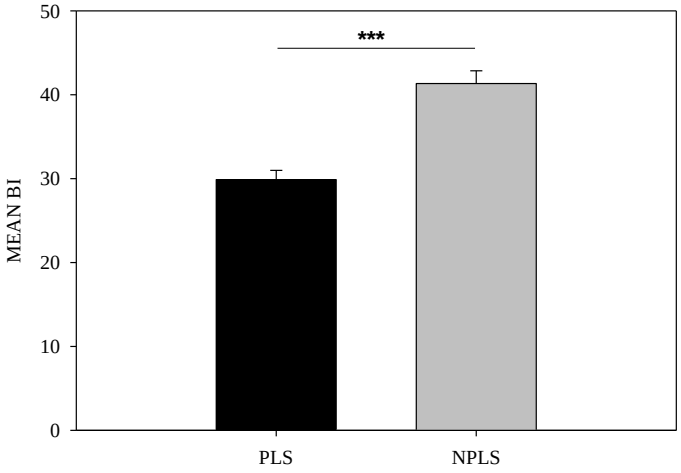


Figure 2.18: PLS neurons are selective for lower intensities.

Mean BI for neurons determined to show PLS and NPLS. There is a significant difference between the two populations (t-test, $p<0.001$). All responses were to DFM stimuli.

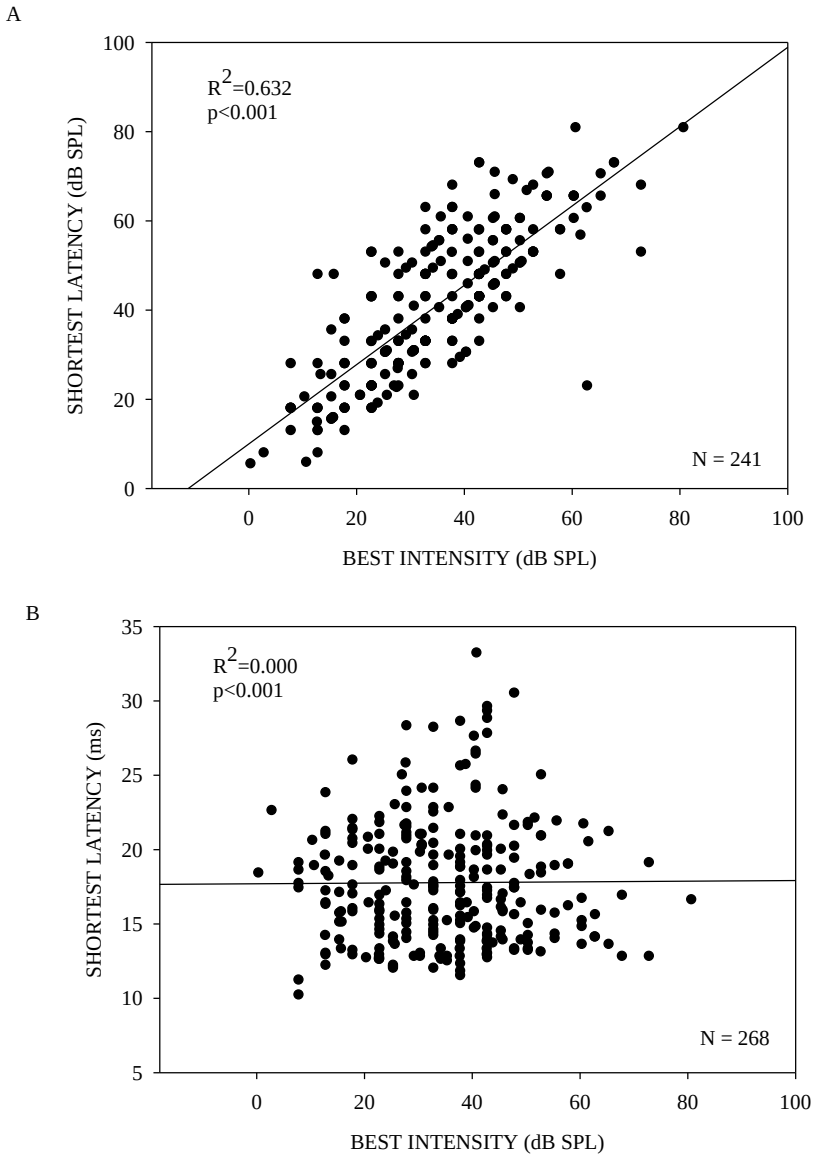


Figure 2.19: Neuron's best intensities are likely to produce the shortest latency response.

(A) There is a significant correlation between the BI of a neuron and the intensity at which produces the shortest latency of response. (Linear regression, $R^2 = 0.632$, $p<0.001$).(B) There is no correlation between the BI of a neuron and the timing of the first spike (Linear regression, $R^2 = 0.000$, $p<0.001$).

2.4 Discussion

The role that intensity selectivity plays in processing complex sounds is just beginning to be understood. This study took the examination of this phenomena one step further by using a behaviorally relevant complex sound. The echolocation call of the pallid bat represents an opportunity to look at the spectrotemporal contributions to intensity tuning while using a stimuli in which we know the relevance to the animal being studied. In this study we proposed that intensity selectivity has an important role in processing the low intensity, returning echoes in the bats call and that the region of the auditory cortex that is selective for the echolocation call will have neural adaptations that facilitate this**.** Our main findings are as follows: 1) that is response to downward frequency modulated sweeps (DFM) sweeps that mimic the echolocation call, most of the neurons in the high-frequency region of the auditory cortex are selective for low intensities; 2) that there is a systematic representation of intensity selectivity and best intensities (BI) in the cortex; 3) that neurons are more intensity selective for the behaviorally relevant sweeps than for tones; 4) high-frequency inhibition is a mechanism for the increased selectivity for the DFM sweeps; and 5) paradoxical latency shifts (PLS) are a common feature of intensity selective neurons and may also play a role in processing the low intensity returning echoes.

2.4.1 Intensity selectivity across species

Intensity tuning in the auditory cortex has been studied in many species of mammals including cat (Phillips et al., 1985, Schreiner et al., 1992, Sutter and Schreiner,

1995), rat (Phillips and Kelly, 1989, Wu et al., 2006), marmosets (Watkins and Barbour, 2011), and both mustached (Suga, 1977) and short-tailed fruit bats (Hechavarría and Kössl, 2014). Non-monotonic, or intensity selective neurons have been discovered in all of the species studied. Our study examined the intensity selectivity found in the auditory cortex of the pallid bat and found that the majority of neurons studied showed some level of intensity selectivity (%TO>20%). Although different measures have been used in some studies, this large number of intensity selective neurons found within the cortex is consistent with these previous studies in the cat, the marmoset and the two bat species. In delay-tuned neurons in the dorsal cortex of the short-tailed fruit bat, *Carollia perspicillata*, 80% of the neurons had some level (equivalent to %TO above 10%) of intensity selectivity (Hechavarría and Kössl, 2014). In that study the authors saw that over 50% of the intensity selective neurons had a "notched" response-level function where response peaked again at a significantly higher intensity (60-90 dB SPL). This "notched" response was not seen in our study but the possibility cannot be excluded as many of neurons where not tested at significantly higher intensities than their BI and the neurons in our study were not delay-tuned.

The rat is the only species in which it has been shown to have a low number of neurons in the cortex with intensity selectivity, although there has been an area in the rat cortex adjacent to the primary auditory cortex that has a higher percentage of non-monotonic neurons (Phillips and Kelly, 1989, Wu et al., 2006). An interesting finding is that as compared with anesthetized cats (Phillips et al., 1985), which have a large number of intensity selective neurons, the pallid bat have a large range of %TO values

and do not always represent a complete reduction in response, whereas the neurons the cat cortex seem to be all or none in terms of their selectivity (Figure 20). The reasons why some mammals have higher levels of intensity selectivity is unclear although it may have to do with the difference between animals that use sounds to detect prey items as in the cat or to listen for the attenuated returning echoes as in bats.

2.4.2 Cortical Organization of Intensity Tuning

Topographic organization of auditory response properties provide a substrate for the efficient wiring of similar parameters. The simplest form of organization that any sensory cortex can display is one that is directly representative of the stimulus parameters that can be encoded by the sensory epithelium (see review in (Kaas, 1997). In the auditory cortex of most mammals there are usually well organized maps of frequency on which other topographic parameters can be mapped in relation to (mouse(Stiebler et al., 1997), rat (Kelly and Sally, 1988), (Doron et al., 2002), (Rutkowski et al., 2003), macaque monkey (Merzenich and Brugge, 1973), (Morel et al., 1993), owl monkey (Imig et al., 1977), cat (Merzenich et al., 1975), (Reale and Imig, 1980), guinea-pig (Hellweg et al., 1977) , ferret (Phillips et al., 1988, Bizley et al., 2005), chinchilla (Harrison et al., 1996), mustache bat (Suga and Jen, 1976b), and pallid bat (Razak and Fuzessery, 2002). It is reasonable to assume that a fundamental property of sound that is extracted by the cochlea, as in intensity and the parameters associated with it like best intensity and intensity selectivity is also mapped in systematic way. In this study we looked for topographic representation of three response parameters: how tuned the neuron is to a

specific intensity range (%TO), how sharp is that tuning (ITI), and what is the amplitude that provides the maximum response (BI). Similar mapping of responses to either noise or tones have been studied in cat(Schreiner et al., 1992, Heil et al., 1994, Phillips et al., 1994, Sutter and Schreiner, 1995), guinea pig (Taniguchi and Nasu, 1993), rat(Wu et al., 2006), and bat(Suga, 1977, Macias et al., 2014). These studies have included several bat species including the mustached bat, *P. parnellii*, in which a systematic representation of best intensities has been seen in the Doppler-shifted constant frequency area (DSCF)(Suga, 1977). Recent studies in *M. waterhousii* and *M. molosuss* found no such ampliotopic organization suggesting that this feature is not common to all bats(Macias et al., 2014). The differences in the two studies may have resulted from the particular set of neurons that were examined. The ampliotopy that was seen in the mustached bat was discovered in the DSCF area that has a particular relevance to the bats ability to compensate for the Doppler shift in the returning echoes as it over represents frequencies in the bats most intense second harmonic. In the cat neurons with non-monotonic intensity selectivity functions are clustered together, spatially segregated from neurons with monotonic intensity selectivity functions (Schreiner et al., 1992, Phillips et al., 1994, Sutter and Schreiner, 1995) with neurons best intensities having a systematic representation mapped along the iso-frequency contour(Heil et al., 1994, Sutter and Schreiner, 1995). These studies were all performed using responses to tone stimuli and although the cat auditory cortex has been mapped using FM stimuli for direction and rate selectivity (Mendelson et al., 1993) it has yet, to our knowledge, been mapped for intensity selectivity or best intensities using FM sweeps. Our data also show, similar to

the cat studies, a clustering of neurons with similar intensity selectivity with a systematic representation of both this measure and that of the best intensities that are associated with it. It is possible that each species will have evolved independent systems of intensity selectivity organization to best fit its own particular need and that the organization seen in the cat is different from the bat due to the specialized needs of echolocation.

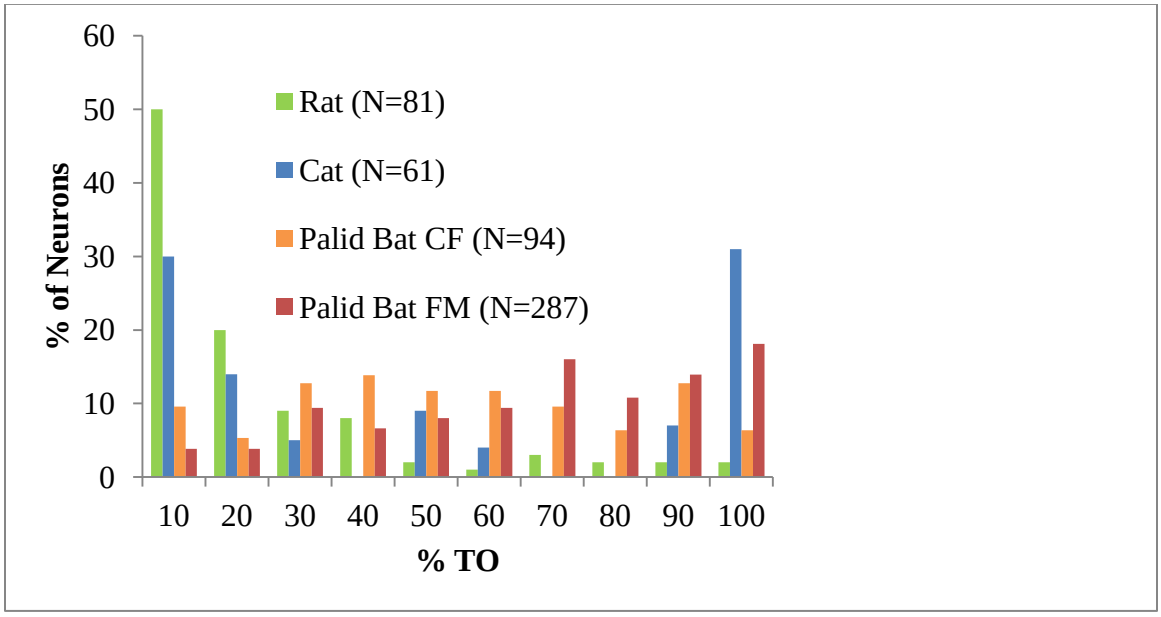


Figure 2.20: Comparison of mammalian intensity selectivity in the auditory cortex.

This is the distribution of %TO values that have been compiled for three different mammalian species in three studies. The majority of rat neurons have low values of %TO while the cat shows a bimodal distribution. In the pallid bat the majority of neurons have %TO value of 50% or higher for FM sweep stimulus whereas the values for the CF tone burst stimulus are slightly lower. Data adapted from (Phillips et al., 1985, Phillips and Kelly, 1989)

2.4.3 High frequency inhibition is responsible for increasing the intensity selectivity to echolocation call stimuli

In this study we wanted to use a stimulus that was more complex than a single tone and integrated information in a spectrotemporal manner while also being a known behaviorally relevant stimuli. In comparing intensity selectivity generated for CF tone bursts and DFM stimuli we saw that there was greater intensity selectivity to the DFM. This type of comparative study using a behaviorally relevant stimuli has not been performed before and is difficult to assess in other species because it is difficult to understand what are behaviorally relevant features of sounds for many non-chiropteran mammals. The use of the DFM sweep allows us to investigate the spectrotemporal contributions that are not present in a tone. One of these contributions is that of inhibition found outside of the excitatory response area. In the DFM used to probe the pallid bat cortical neurons there is high frequency inhibition present. This inhibition is a mechanism to explain rate selectivity in the pallid bat cortex (Razak and Fuzessery, 2006, 2007). In this study, we investigated whether the same high frequency inhibition may also contribute to the selectivity seen for intensity.

To investigate the contribution of this inhibition we used a two-tone inhibition (TTI) over time paradigm, which has been used to study sideband inhibition in the pallid bat (Fuzessery et al., 2006, Razak and Fuzessery, 2006), the mustached bat (Gordon and O'Neill, 1998) and the mouse (Trujillo et al., 2013). We examined neurons in which the intensity selectivity for DFM sweeps was greater than it was for CF tones and saw that

the arrival time of inhibition changed in an intensity dependent manner. This is similar to a recent study that also saw intensity dependent changes in the arrival time of inhibition in the pallid bat cortex (Razak, 2013). That study observed changing the relative intensities of the excitatory and inhibitory tones, however, in our study the intensities were maintained the same for both tones. The intensity dependent changes were observed in two groups: 1) neurons whose intensity selectivity was greater for DFM sweeps than tones, and 2) neurons whose intensity selectivity was greater for CF tones than DFM sweeps. Although for both groups the arrival time of inhibition became faster with an increase in intensity, the neurons that were more selective when DFM sweeps were used, had a significantly faster change in the arrival time with increasing intensity. A study that examined the properties of the inhibitory and excitatory inputs to intensity selective neurons suggested that inhibitory inputs in the cortex may inherit a shorter integration time with increases in intensity (Wu et al., 2006). That study, taken together with our data suggest that intensity selectivity can be increased by inhibitory inputs from the high-frequency inhibition in DFM sweeps that have an intensity dependent shortening of latencies. It has also been shown that the inhibitory inputs to intensity selective neurons have monotonic response-level, non-selective function while the excitatory inputs are intensity selective in nature (Wu et al., 2006). In this study the latency data (see below) suggest that many intensity selective neurons have a lengthening of their first spike response to increasing intensities and that neurons that are less intensity selective are more likely to have a decreased latency to increasing intensities. This is consistent with what has recently been shown in the bat, *C. perspicillata* in which there was large

amount of intensity selective neurons that show a similar change in latencies with increasing intensity (Hechavarría and Kössl, 2014). Also we have already seen that neurons with a CF in the higher range of the echolocations call (50kHz+) have significantly lower levels of intensity selectivity. Both of these facts suggest that the high frequency inhibition in the DFM sweep most likely has a monotonic response and a shortening of response to increasing intensities. This inhibition is combining with a non-monotonic excitatory response that has longer latencies to increasing intensity. This combination creates more intensity selectivity than would be produced by the excitatory tone alone.

A possible methodological confound was that during the two-tone paradigm the same inhibitory tone was used throughout the intensity ranges. This could represent a problem if the inhibitory tone that was discovered originally outside of the excitatory tuning curve was at some intensities closer to or fell inside the excitatory tuning curve. This type of change in response should present itself as an inability to create an inhibition that reduced response by 50% in which then that data point would not have been included in the analysis. However, we cannot exclude the possibility that that this may have biased the results.

2.4.4 Inhibitory input

It is clear that synaptic inhibition is shaping the response of the cortical neuron to intensity. The origin of this input is yet to be determined. There are models to suggest that monotonic responses could be inherited from thalamic inputs while non-monotonic

responses may be created de novo in the cortex (Wu et al., 2006). Recent studies show that in mouse auditory cortex, that PV+, presumptive inhibitory neurons have a monotonic rate-level function in response to increasing intensity (Moore and Wehr, 2013). This monotonic response of inhibition has been shown to be important for shaping the non-monotonic response of other neurons (Wu et al., 2006). In this study only a small number of neurons that have a largely monotonic response to increases in intensity were seen. This would suggest that PV+ neurons in the cortex either are few or do not have the same response to intensity as seen in the mouse. Either suggestion might point to the fact that it is not cortical inhibition that is shaping this response, but that the response is inherited from sub-cortical structures like the medial geniculate body (MGB). Data from a recent study shows that there is a small percentage of PV+ cells in the high-frequency region of the pallid bat cortex (Campo et al., 2014). It is difficult to determine if this small number of presumptive inhibitory cells can provide enough inhibitory inputs to shape the large amount of non-monotonic responses seen in the cortex. The small number of PV+ neurons could mean that a sampling bias may be present and that the responses gathered were mostly from non-PV+ neurons. There is a possibility that the inhibitory inputs in the cortex could also be coming from other cells than PV+ cells. In the same study there was an absence of PV+ cells in the MGB of the thalamus that provides input to this region (Campo et al., 2014). However, there was staining of PV that was observed mainly in the neuropil in the SG, which has been shown to be the major input to the high frequency region of the pallid bat cortex (Razak et al., 2007). This could also suggest that perhaps the monotonic inhibitory response needed to shape

the non-monotonic excitatory response in the cortex be coming from a subthalamic nucleus like the IC which has been shown to have both monotonic and non-monotonic responses to DFM sweeps (Fuzessery, 1994, Fuzessery and Hall, 1996).

2.4.5 Latency of response

Typically when presented with increasing stimulus intensity, many neurons in the auditory system display what is called an intensity-dependent latency change. This means that latency decreases as the intensity increases. Paradoxical latency shift (PLS) is when a neuron's response latency to a stimulus becomes greater with an increase in intensity. There is evidence that as you ascend the auditory pathway a larger percentage of cells exhibit this phenomenon (Klug et al., 2000). In the short-tailed fruit bat, *Carollia perspicillata*, 47% of cortical neurons tested showed PLS (Hechavarría and Kössl, 2014) which is almost exactly what was seen in this study with the pallid bat (46%). As in our study FM sweeps were used as a stimuli. It has been shown that in the IC of little brown bats, *Myotis lucifugus*, FM sweeps are more effective at eliciting responses that display paradoxical latency shift than tone bursts are (Wang et al., 2007). The data in this study suggest a similar finding in the pallid bat auditory cortex. There is behavioral evidence that shows that a broadband sound such as FM sweeps are better suited for temporal processing than tones (Simmons, 1979). This means that FM sweeps that are present in many bat species echolocation calls are able to be used more effectively for target range discrimination. The PLS that is present in some neurons has been proposed as a mechanism to help facilitate the coincidence detection needed in the combination

sensitive neurons that are needed for range detection. (SULLIVAN III, 1982, Olsen and Suga, 1991). Combination sensitive neurons have not yet been discovered in the pallid bat auditory cortex but this study shows that there are a larger number of neurons that display PLS and may have a hand in creating the low-intensity selective responses of these neurons. Future studies will have to be dedicated to searching for combination sensitive neurons used to detect target range and determining the role the PLS neurons play in the pallid bat auditory cortex.

At the periphery PLS is not present and is seen at higher numbers as you ascend the auditory pathway (Klug et al., 2000). This means that PLS is a modification of the intensity dependent latency change seen in the auditory nerve. Inhibition has been shown to play an important role in shaping this modification in two bat species *Talidarida brasiliensis* and *P. parnellii*. Bicuculline was applied to neurons in the IC and PLS disappears (Klug et al., 2000). In the pallid bat cortex, high frequency inhibition present in the downward FM sweeps of the echolocation call has already been implicated in the rate and direction selectivity of cortical neurons (Razak and Fuzessery, 2006, 2009, Razak, 2012). This study also showed that high-frequency inhibition can increase the amount intensity selectivity seen in neuronal responses. As the neurons with PLS tend to show high levels of intensity selectivity it is reasonable to assume that this inhibition is also shaping that response as well. The amount of PLS that is seen in the cortex could be a direct result that the increase in the speed of inhibition as intensity increases could be cutting off the beginning of the response, thus accomplishing two things; lowering the response output to a high intensity and also lengthening the latency at a higher intensity.

This suggests that both intensity tuning and the PLS, that often accompanies it, are consequences of the high frequency inhibition seen in the spectral temporal contributions of the FM sweep.

2.4.6 Intensity compensation

Echolocation is an active process in which the bat is listening for a returning echo of its own call, or pulse. This means that the bat has control over not only the call itself but some of the properties of the returning echo. It has been shown in CF-FM bats that they are able to compensate for Doppler-shifts in the frequencies of their returning calls by increasing the frequencies of their outgoing pulses (Schnitzler, 1973, Schuller, 1980, Hiryu et al., 2005). Studies have shown that both the mustached bat, *P. parnellii*, and greater horseshoe bat, *Rhinolophus ferrumequinum*, will lower the frequency of their outgoing call so that the returning frequency falls within an 'acoustic fovea' that represents the frequencies present in the constant frequency (CF) portion its dominant harmonics (Suga and Jen, 1976a, Schuller and Pollak, 1979, Keating et al., 1994). These 'acoustic fovea' have a disproportionate number of neurons tuned to a small range of frequencies allowing for better resolution of signal content. Along with compensating for changes in frequency, it has also been shown that bats can compensate for intensity changes as they are approaching a target (Kobler et al., 1985, Hartley, 1992, Boonman and Jones, 2002, Hiryu et al., 2007, Hiryu et al., 2008). In a study similar to the classic pendulum study in which a bat was shown to compensate for Doppler shifts as it swings on a pendulum towards a target, researchers showed that bats simultaneously can

compensate for fluctuations due to Doppler shifts and changes in intensity (Schnitzler, 1968, Kobler et al., 1985). The intensity compensation in that study mimicked the changes in amplitude that the bat received as the pendulum swung towards and away from the target. Another study shows evidence that the intensity compensation seen in at least Daubenton's bat, *Myotis daubentonii* does not reflect an active feedback in which the bat is constantly updating its call intensity based on the target or returning echo's strength, rather is based on a stereotyped change in the intensity when approaching a target (Boonman and Jones, 2002).

Our data suggests that the narrow range of intensities that the neurons in the pallid bat's auditory cortex is selective for may represent an 'acoustic fovea' for echo intensity. This small range of intensities (~30 dB) that make up the majority of best intensities may be something that is unique to gleaning bats as a recent study showed that another gleaning bat, *M. waterhousii* also had a similar small range of best intensities seen in the auditory cortex, while a bat that catches it prey in the air, *M. molossus*, had a larger range of best intensities similar to the distribution previously seen in another aerial hawking bat, *P. parnellii* in the Doppler-shift compensated frequency (DSCF) region of the cortex (Suga, 1977, Suga and Manabe, 1982, Macias et al., 2014). In both species, *M. waterhousii* and *M. molossus* the majority of their neurons have BI's less than 50dB. This representation of low level sounds in non-monotonic neurons has also been seen in a study of the cat auditory cortex (Phillips et al., 1994), whereas other studies have shown BIs that have higher intensities in the rat (Polley et al., 2007) and macaque (Recanzone et al., 2000). This could mean that species have intensity selective neurons that respond to

different ranges of intensity based the behaviorally relevant sounds (i.e. predator or prey generated noises, vocalizations, etc.). Future studies will have to measure the exact nature of this intensity compensation to determine if they align with the values of best intensity seen within this study and whether they follow a stereotyped pattern or show more of a dynamic change based echo strength.

2.4.7 Future studies in the role of intensity selectivity in echolocation

Although it is still unclear the role that intensity selectivity plays in the processing of many behaviorally relevant sounds it is clear that it plays an important role in echolocation behavior of bats. Data in the study suggests that neurons in the cortex of the pallid bat: 1) have a stronger response to the DFM sweeps that mimic the soft returning echoes of the bats call; and 2) are selective for the lower intensities that would be present in the echoes. The increased intensity selectivity that is seen for DFM sweeps is produced by high frequency inhibition, however future studies will have to examine whether blocking of cortical inhibition can create an altered, non-selective response and whether this reduction in inhibition has any behavioral consequences in the pallid bats ability avoid obstacles using its echolocation call. Future studies will also have to determine if the low intensities that these neurons are selective for due form an acoustic fovea by examining the pallid bats ability to compensate for changes in the returning echoes to keep them within this intensity range.

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Chapter 3: Parvalbumin and Calbindin Expression in the Parallel Thalamocortical Pathways in the Pallid Bat

Abstract

The pallid bat (*Antrozous pallidus*) listens to prey-generated noise to localize and hunt terrestrial prey while reserving echolocation for orientation and to avoid obstacles. The thalamocortical connections in the pallid bat are organized as parallel pathways that may serve echolocation and prey localization behaviors. Thalamic inputs to the cortical echolocation call- and noise-selective regions originate primarily in the suprageniculate nucleus (SG) and ventral division of medial geniculate body (MGBv), respectively. Here we examined the distribution of Parvalbumin (PV) and Calbindin (CB) expressing cells in cortical regions and thalamic nuclei of these pathways. Electrophysiology was used to identify cortical regions selective for echolocation calls and noise. Immunohistochemistry was used to stain for PV and CB in the auditory cortex and MGB. A higher percentage (relative to Nissl-stained cells) of PV+ cells compared to CB+ cells was found in both echolocation call- and noise-selective regions. This was due to differences in layers V-VI, but not layers I-IV. In the MGB, CB+ cells were present across all divisions of the MGB. However, there was a higher percentage of CB+ neurons in the MGBv than the SG. Perhaps the most surprising result was the virtual absence of PV staining in the MGBv. PV staining was present primarily in the SG. Even in the SG, the staining was mostly diffuse in the neuropil, with rare cell body staining. These data are supportive of the notion that calcium binding proteins are differentially distributed in different processing streams. Comparative data, however, do not support a general mammalian pattern of PV/CB staining that distinguishes lemniscal and non-lemniscal pathways.

3.1 Introduction

Parvalbumin (PV) and calbindin (CB) are fast cytosolic calcium buffers that have differential distributions in different regions/nuclei of sensory pathways (Rogers et al., 1990, Rausell and Jones, 1991, Baimbridge et al., 1992, Bennett-Clarke et al., 1992, Hof et al., 1999). The functional roles of neurons containing these proteins are only beginning to be identified (Wu et al., 2008, Sohal et al., 2009). These proteins have served as useful markers in neuroanatomical studies because of the differential expression patterns. For example, there is a complementary expression pattern of PV and CB in the auditory thalamocortical system of some mammalian species. In the rabbit and in rodents, the ventral medial geniculate body (MGBv) and dorsal MGB (MGBd) divisions of the auditory thalamus primarily express PV and CB, respectively (de Venecia et al., 1995, de Venecia et al., 1998, Cruikshank et al., 2001). In the macaque monkey as well, MGBv is distinguished by high PV expression and low CB expression (Jones et al., 1995). In the mouse and macaque auditory cortex, PV and CB expression can distinguish primary from secondary auditory fields (Molinari et al., 1995, Kosaki et al., 1997, Cruikshank et al., 2001). These data suggest a mammalian plan of complementary PV/CB expression in the lemniscal/non-lemniscal auditory pathways. However, in the macaque MGBd, both PV and CB are strongly expressed. Studies of bats also show divergence from a general plan. In the mustached bat, PV and CB are strongly expressed in both MGBd and MGBv (Zettel et al., 1991). In the horseshoe bat, both PV and CB are expressed strongly in the MGBv (Vater and Braun, 1994). PV/CB

expression patterns may therefore distinguish species-specific processing streams as opposed to being biochemical markers of lemniscal/non-lemniscal pathways.

The goal of this study was to examine expression patterns of calcium binding proteins in a bat species in which different thalamocortical pathways may serve two different behaviors. PV and CB expression pattern was investigated in the MGB and auditory cortex of the pallid bat (*Antrozous pallidus*). Unlike the mustached and horseshoe bats which primarily use echolocation to hunt aerial prey, the pallid bat belongs to a small group of bats called 'gleaners' which hunt terrestrial prey (Bell, 1982, Fuzessery et al., 1993, Barber et al., 2003). Gleaners depend, at least partly, on listening to prey-generated sounds to hunt prey and are found across families suggesting convergent evolution of this behavior. Pallid bats listen to prey-generated noise to localize and hunt prey such as crickets and scorpions while reserving echolocation for obstacle avoidance.

The auditory cortex of the pallid bat is dominated by two adjacent, and mostly segregated, regions with response selectivity for the sounds used in echolocation (60→30 kHz downward frequency modulated sweeps, 2-6 msec) and prey localization (5-35 kHz noise transients) (Razak and Fuzessery, 2002, 2006, Razak, 2011). Both cortical regions are overlain on a tonotopic map representing the species-specific audible range (~5-70 kHz). In addition, neurons in both regions have short latency responses and narrow tuning suggesting that they are both part of the primary auditory cortex (A1). However, only one half of this tonotopic map receives input from the MGBv (Razak et al., 2007,

Razak et al., 2009, Razak and Fuzessery, 2010). The noise-selective region (tuning between 5-35 kHz) receives input from the MGBv. The echolocation call-selective region (tuning between 30-70 kHz) receives most input from the suprageniculate nucleus (SG) of the MGBd. These data suggest that the thalamocortical connections emphasize segregation of the two pathways in the pallid bat, perhaps to enhance segregated processing of two sound streams (FM and noise) that likely co-occur in natural hunting situations (Barber et al., 2003). This study tested the hypothesis that these two parallel processing streams show different patterns of PV/CB expression.

Data show that the expression of PV/CB in the pallid bat MGB is different from any other species examined, including other bats. PV is expressed only in the SG of the pallid bat MGB. CB is expressed across the MGB, but in a larger percent of cells in the MGBv compared to the SG. In A1, PV is expressed similarly in the noise- and echolocation call-selective region. More PV+ than CB+ cells are present in A1.

3.2 Methods

Adult pallid bats were netted in Arizona, California, New Mexico or Texas and held in a 11 × 14 ft room at the University of California, Riverside. The bats were able to fly in this room and were provided crickets/mealworms and water *ad libitum*. The room was maintained on a reversed 12:12 light:dark cycle. All procedures followed the animal welfare guidelines required by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

3.2.1 General overview of procedure

Single- and multi-unit electrophysiology was used to generate a coarse map of the right auditory cortex in terms of characteristic frequencies (CF). A fluorescent marker was placed in putative FM- and/or noise-selective region identified based on CF. In sections containing the cortical fluorescent marker, PV and CB stained cells were counted on the opposite (left) hemisphere at locations homotopic to the dye location. In the MGB, PV and CB staining was compared between different divisions identified based on Nissl-staining.

3.2.2 Surgical procedures for cortical electrophysiology

Recordings were obtained from bats anesthetized with isoflurane inhalation, followed by an i.p. injection of pentobarbital sodium (30 µg/g body wt) and acepromazine (2 µg/g body wt). Both male and female bats were used. Because the goal of electrophysiology was to simply identify tonotopy to target dye marking, it is unlikely that the pentobarbital anesthesia had a significant impact on the observed results. To expose the auditory cortex, the head was held in a bite bar, a midline incision was made in the scalp, and the muscles over the dorsal surface of the skull were reflected to the sides. The front of the skull was scraped clean and a layer of glass microbeads applied, followed by a layer of dental cement. The bat was then placed in a Plexiglas holder. A cylindrical aluminum head pin was inserted through a cross bar over the bat's head and cemented to the previously prepared region of the skull. This pin served to hold the bat's head secure during the recording session. The location of A1 was determined relative to the

rostrocaudal extent of the midsagittal sinus, the distance laterally from the midsagittal sinus, and the location of a prominent lateral blood vessel that travels parallel to the midsagittal sinus (Razak and Fuzessery, 2002). The size of the exposure was usually ~2 mm^2. Exposed muscle was covered with petroleum jelly, and exposed brain surface was covered with paraffin oil to prevent desiccation.

3.2.3 Recording procedures

Electrophysiological recordings were used to identify and mark putative noise- and/or FM sweep-selective regions of the pallid bat auditory cortex. A number of previous studies have shown that the noise-selective region is tonotopically organized with CF between ~5 and 30 kHz, and robust responses to broadband noise (Razak and Fuzessery, 2002, 2007, Razak, 2011, 2012a). The FM sweep-selective region is also tonotopically organized with CF between ~30 and 60 kHz and direction and rate selective responses to the 60→30 kHz FM sweeps used by the pallid bat to echolocate (Razak and Fuzessery, 2002, 2006, 2009, Razak, 2012b). Therefore, the focus was to obtain a map of CFs and place a dye to mark the high and/or low CF cortex that corresponds to FM- and/or noise-selective region. In a number of sites, the intuition based on CF regarding noise *versus* FM sweep selectivity was verified by recording responses to these sounds as well.

Experiments were conducted in a warm (80°F), sound-proofed chamber lined with anechoic foam (Gretch-Ken Industries, Oregon). All recordings were obtained from the right hemisphere. Bats were kept anesthetized throughout the course of the

experiments with additional pentobarbital sodium (one-third of presurgical dose) injections. Acoustic stimulation and data acquisition were driven by custom written software (Batlab, written by Dr. Don Gans, Kent State University) and a Microstar digital signal processing board. Programmable attenuators (PA5, Tucker-Davis Technologies, Florida) allowed control of sound intensities before amplification by an integrated amplifier (Yamaha AX430) or a power amplifier (Parasound, HCA1100).

Extracellular single- or multi-unit recordings were obtained using glass electrodes (1M NaCl, 2-10 MΩ impedance) at depths between 200 and 600 μm. Penetrations were made orthogonal to the surface of the cortex. Action potentials were amplified by a Dagan extracellular preamplifier (2400A) and a spike signal enhancer (FHC, Maine) and band-pass filtered (0.3-3 kHz, Krohn-Hite, MA). Waveforms and peri-stimulus time histograms were stored using the Microstar DSP board and Batlab software. Single-unit recordings were identified based on window discrimination and the consistency of action potential amplitude and waveform displayed on an oscilloscope.

Stimuli were presented using an LCY-K100 ribbon tweeter (Madisound, Wisconsin) fitted with a funnel that was inserted into the left pinna (contralateral to recorded cortex). The amplifier-speaker-funnel frequency response curve measured with a 1/4-in microphone (Bruel and Kjaer, Denmark) was flat within ±3 dB for frequencies from 8-35 kHz. The decline in response at higher frequencies was smooth up to 70 kHz at a fall-off rate of ~20 dB/octave. In two experiments, the FM sweep-selective region was

identified using a free-field speaker (LCY-K100 ribbon tweeter) placed at 0^{o} azimuth and elevation with respect to the bat's snout at a distance of 40 cm.

Upon penetrating the cortical surface with the electrode, pure tones (5-60 kHz, 1 msec rise/fall time, 5-10 msec duration), downward and upward FM sweeps (30-70 kHz, 20-40 kHz bandwidth, 2msec/rise/fall time, 2-30 msec duration) and noise (5-40 kHz broadband, 1 msec rise/fall time, 5-10 msec) were played at a repetition rate of 1 Hz and at different intensities (10-70 dB) to search for sound driven responses. When robust multi-unit responses or isolated single-unit responses to one or more of these stimuli were obtained, the CF was determined. CF was defined as the tone frequency that elicited action potentials to at least five successive stimulus repetitions at the lowest intensity. This intensity was noted as the minimum threshold (MT) of the neuron. A similar procedure was used to map the CFs across the cortex.

3.2.4 Dye injection

A fluorescent dye was injected to mark the low-CF (5-30 kHz) and/or high-CF (30-60 kHz) cortical regions. Tips of glass electrodes (~10 µm diameter) were capillary-filled with 20mg/mL dextran tetramethyl rhodamine (Invitrogen, Carlsbad, CA, diluted with 0.9% normal saline) and back filled with 1 M NaCl. The dye was injected using a Midgard precision current source (Stoelting, Wood Dale, IL) with 7 seconds on-7 seconds off current stimulus of +4 µA. The duration of injection was 5 minutes at a depth of ~300 -600 µm. Following histological processing (details below), dye injection

sites were viewed with a Nikon Eclipse 80i Microscope with epiflourescent light filters. Images were taken with a Nikon Digital Sight Camera.

3.2.5 Immunohistochemistry

Following a lethal injection of sodium pentobarbital (125 mg/kg), bats were transcardially perfused using a peristaltic pump (Fisher Scientific) with 0.1M PBS followed by 4% paraformaldehyde (0.1M PB, pH 7.4). The brains were immediately removed, post-fixed overnight (~15-20 hours) in 4% parafomaldahyde, and cryoprotected in 30% sucrose until sinking. Brains were coronally sectioned at 40µm on a cryostat. Immunohistochemistry was carried out with free-floating sections at room temperature with agitation unless otherwise indicated. Sections were pretreated in 0.5% H_2O_2 (in 0.1M PBS, pH 7.4, 30min) to reduce endogenous peroxidase activity, then rinsed with 0.3% tween-20 detergent (in 0.1M PBS, 3X10min.), and blocked with 6.7% goat normal serum (s-1000, Vector) (in 0.1M PBS, 2-3 hours). Sections were incubated at 4^{o}C in a solution containing either rabbit anti-PV (1:5000, PV-25, Swant, Bellinzona, Switzerland) or mouse anti-CB (1:5,000, D-28k, Swant, Bellinoza, Switzerland), 2% goat normal serum, 0.3% triton X-100 (in 0.1M PBS, 2-4 nights).

Sections were rinsed in 0.3% tween-20 detergent (in 0.1M PBS, 3X10min), followed by an incubation in a solution containing Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (1:500, Jackson, West Grove, PA), 2% goat normal serum, 0.3% triton X-100 (in 0.1M PBS, 2-3 hours). Sections were rinsed (in 0.1M PBS, 3X10min). Staining was visualized without agitation by first pre-incubating the sections

in 3,3'-diaminobenzidine (DAB) (sk-4100, Vector, Burlingame, CA) solution, followed by incubation with H_2O_2 and DAB. Sections were rinsed in dH_20 (2x10min) transferred to 0.1M PBS, mounted on gelatin-coated slides, air dried, and cover-slipped using DPX mounting medium (Electron Microscopy Sciences).

3.2.6 Antibody characterization

An antibody against calbindin D-28k (300, Swant) was examined for antigen specificity using Western blot analysis (Figure 1). Brain extracts from the pallid bats were separated using SDS-PAGE protein separation, transferred to a nitrocellulose membrane and then probed with the antibody. A single band at 28 kDa was observed confirming a high level of specificity in the pallid bat. An antibody against parvalbumin (PV25, Swant) could not be validated in the pallid bat because according to the manufacturer, the PV 25 antibody does not recognize the antigen after SDS-gel electrophorectic separation of brain extracts (Swant product specification sheet). We have used this antibody in a previous study (Martin del Campo et al., 2012) in the mouse auditory cortex and reported similar cortical staining patterns. The manufacturers report that staining is absent in PV KO mouse brain tissue. As far as authors are aware, there have been no examples of cross reaction with other antigens on record.

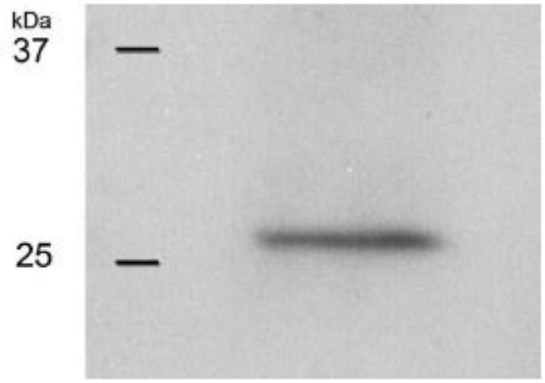

Figure 3.1: Specificity of calbindin D28K antibody.

Western blotting of pallid bat brain tissue after electrophoretic SDS-PAGE protein separation. Position of molecular mass markers in kDa reported is on the left.

3.2.7 Image Analysis, Counting, and Data Representation

Nissl+, CB+ and PV+ cells were counted in the left hemisphere of each cortex. The general location of the auditory cortex was identified based on hippocampus landmarks. More accurate location of the FM sweep- and/or noise-selective regions in the right hemisphere was accomplished based on the dye injection (Figure 2A, B). Homotypic regions on the left hemisphere were chosen for counting (Figure 2C, D). Adjacent sections were counted for CB+, PV+ and Nissl+ cells.

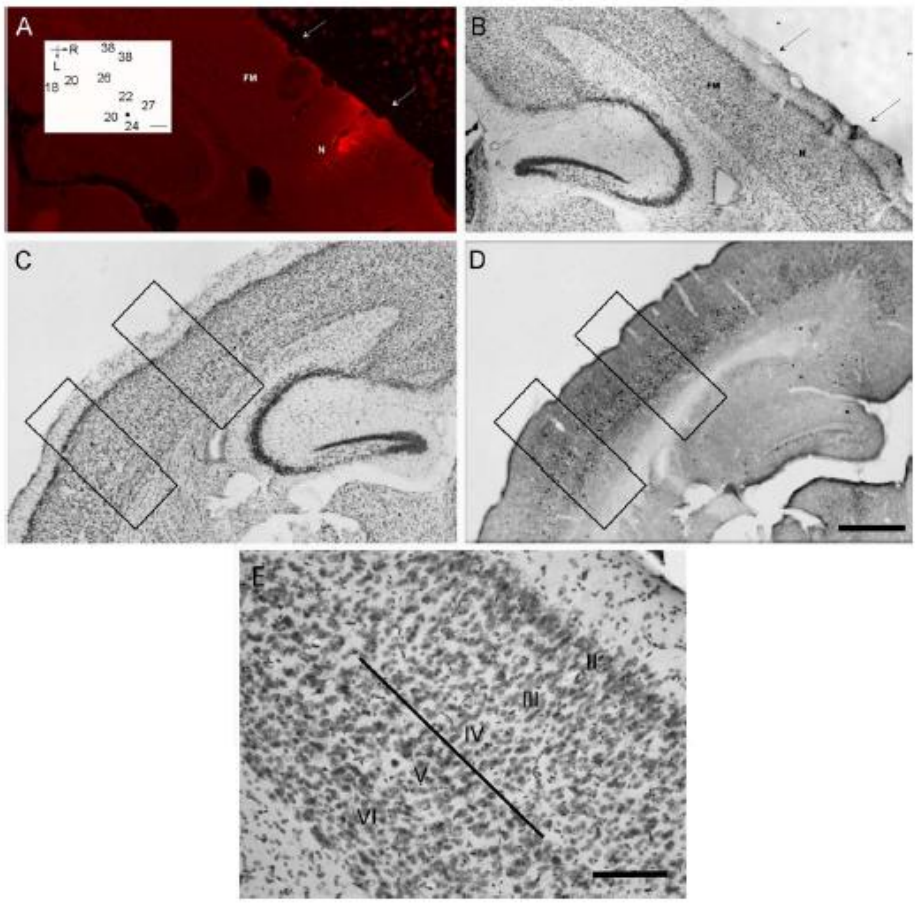
A
B
C
D
E
II
III
IV
V
VI

Figure 3.2: Illustration of the main methods used in this study.

(A) Photomicrograph of a coronal section through the echolocation call (FM) and noise (N) selective regions of the pallid bat auditory cortex. Fluroruby was used to mark the N region. These regions were identified using electrophysiology. The inset shows the map of characteristic frequencies (CF) identified using electrophysiology from the right auditory cortex (pallid bat PAL024). The black circle shows the site of fluroruby injection to mark the noise-selective neurons (CFs ~20-27 kHz). The neurons located more medially with CF ~ 38 kHz are in the FM sweep selective region. R-rostral; L-lateral. Scale bar in inset ~200 µm. (B) Photomicrograph of the section located adjacent to the section shown in A. This section was Nissl stained. (C) The contralateral side of the same section shown in B was used to identify layers and count Nissl stained cells in the putative FM and N region. The assumption that the locations of FM and N selective regions were symmetrical across the midline was made. Nissl-stained cells were counted within the 400 µm wide rectangles spanning the layers of the cortex. (D) Photomicrograph of a PV stained section located adjacent (40 or 80 µm) to the Nissl stained section. The number of PV positive cells was counted within the 400 µm wide rectangles placed over the putative FM and N regions. (E) Nissl-stained section of the primary auditory cortex. The solid line demarcates layer IV and V to facilitate a comparison of cell counts between layers I-IV and layers V-VI. Scale bar in D: 500 µm (applicable to A-D). Scale bar in E: 200 µm.

NIS Elements advanced research software was used to capture 400µm wide images of the auditory cortex in CB+, PV+ and Nissl-stained sections (height from white matter to pia). The auditory cortex was divided into layers I-IV and layers V-VI based on the laminar distribution of cells (solid line, Figure 2E). Layer IV contains small, densely concentrated cells and layer V contains larger, more darkly stained and sparsely spaced pyramidal cells. Based on this difference, the boundary between layers IV and V was placed between 54% and 61% of total section height (pia to white matter) for all bats in this study. This is consistent with a previous paper on the pallid bat cortex (Marsh et al., 2002) which placed the boundary at ~60% from the pia. The boundary between the layers IV and V was then marked on adjacent sections stained for PV or CB to count cells positive for these calcium binding proteins in layers I-IV and layers V-VI. A finer-grained layer analysis was not possible because the boundaries between layers III-IV and layers V-VI were not consistently distinguishable with Nissl staining.

Prior to counting, images were adjusted for brightness and contrast using NIS Elements advanced research software. Nissl-stained cells were counted in one of the eight randomly selected 50µm wide rectangles within the 400µm image of Nissl-stained section. This count was multiplied by a factor of 8. CB+ and PV+ cells were counted across the entire 400µm wide image of CB and PV stained sections. Only those CB+ and PV+ cells that had intensely stained soma were included in the counts (e.g., arrows in Figure 4). Counting bias was avoided by only counting cells that either had their soma contained completely within the image or that fell on the left border of the image. Those cells that fell on the right border of the image were excluded from counting (Gundersen

et al., 1988). Data are represented in terms of percentage of CB+ or PV+ cells relative to Nissl-stained cells.

The different divisions of the MGB were identified based on Nissl-staining (Figure 3) based on the scheme of Morest (1964). Although the SG is considered a part of the MGBd, we analyzed it separately in this study because most of the echolocation pathway appears to be routed through the SG (Razak et al., 2007). In the text, 'MGBd' refers to parts of this division excluding the SG. Figure 3 shows three Nissl-stained sections through the MGB arranged in a rostral to caudal fashion. The SG can be distinguished from the MGBv and MGBd based on darkly stained cells with a larger soma. It is relatively more difficult to distinguish MGBd and MGBv based on Nissl staining. The approximate boundary between these two divisions were placed based on previous studies that used fiber staining procedures and showed that the MGBv is mostly devoid of fibers compared to the MGBd (Razak et al., 2007, Shen 1996). It must be noted that the primary focus was on differentiating the SG and the MGBv, as these nuclei are the main sources of input to the FM sweep- and noise-selective regions of the cortex. Cell counts in the MGB were made in 100 μm^2 squares placed in the center of the dashed contours (Figure 3) outlining each region.

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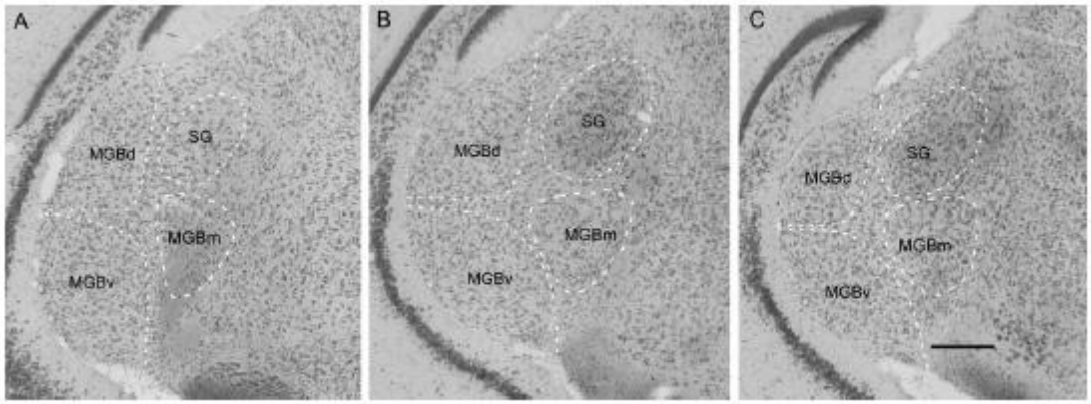

Figure 3: The three panels (A-C) show a rostral to caudal sequence of sections through the MGB of a pallid bat.

The sections were at 70% (A), 40% (B) and 20% (C) rostrocaudal MGB positions (100% being most rostral). The dashed white lines demarcate the approximate boundaries of the various regions of interest in this study. Although the SG is considered a part of the MGBd, they were analyzed separately. The SG is most distinct based on large cell bodies and darker Nissl staining

3.3 Results

PV expression was studied in 7 bats in the MGB. In 6 of these bats, PV expression was also studied in the FM- and noise-selective regions of the auditory cortex. In a separate set of 5 bats, CB expression was studied in the MGB. In 4 of these bats, we also examined CB expression in the auditory cortex.

3.3.1 PV and CB staining in the auditory cortex

Figure 4 shows representative PV and CB staining in the auditory cortex. Intense staining of cell bodies was present for both PV and CB. The percentage of PV+ and CB+ cells relative to Nissl-stained cells was compared between the FM and N areas and analyzed using 2-way ANOVA (cortical area x calcium binding protein) with post-hoc Tukey pair-wise comparisons (Figure 5). Table 2 shows the absolute cell counts in the sections used for the analysis. There was no difference between FM and N areas in terms of the percentage of calcium binding proteins ($p>0.05$). However, within each area, there were more PV+ cells than CB+ cells ($p<0.05$) when the cells were counted across all six cortical layers (Figure 5A). Analyzing granular/supragranular layers I-IV (Figure 5B) and infragranular layers V-VI (Figure 5C) separately indicates the difference between PV and CB expression was mainly in the infragranular layers ($p<0.001$) in both the FM and N regions. There was no difference in the percentage of CB+ and PV+ cells in layers I-IV in both regions ($p>0.05$). It was not possible to conduct a more fine-grained layer-specific analysis because the layers V-VI boundary and layers III-IV boundaries could not be consistently identified using Nissl-staining. These data indicate that the two calcium binding proteins tested were expressed at different levels in the cortex, without a difference between functional cortical areas.

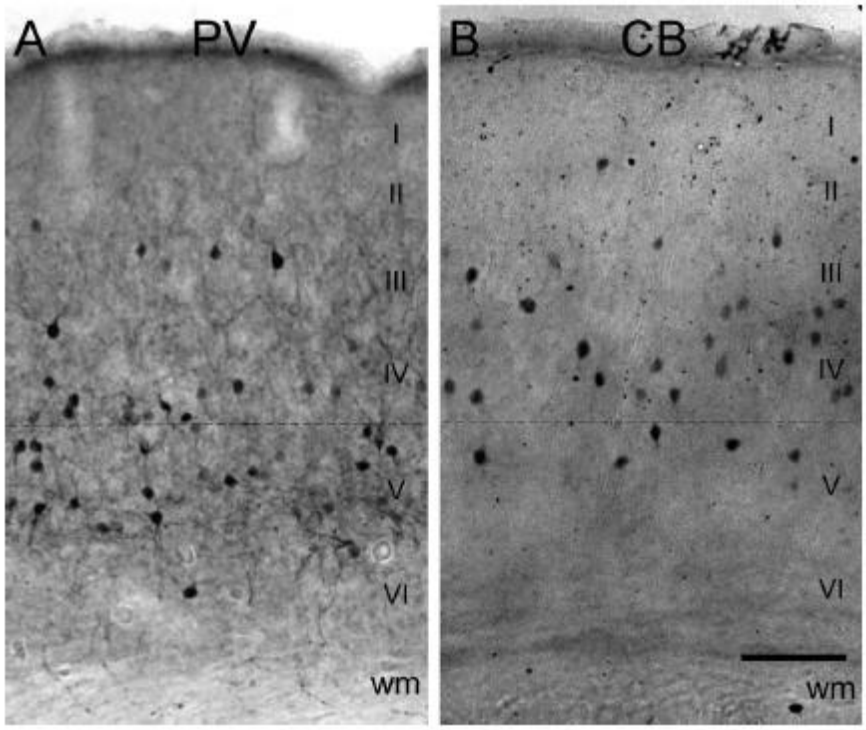

Figure 4: Example photomicrographs of PV (A) and CB (B) immunostaining in the auditory cortex of the pallid bat.

Arrows indicate example cells with intense staining. Dashed line is at the boundary between layers IV and V identified in a Nissl-stained adjacent section. Wm: white matter. Scale bar applicable for both panels: 150 µm.

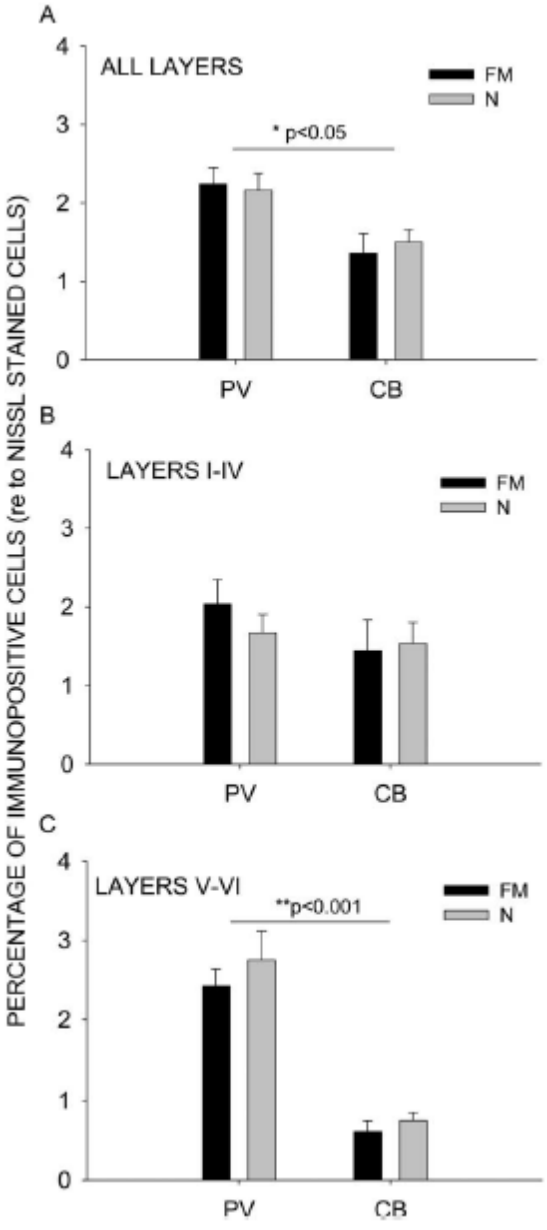
A
ALL LAYERS
FM
N
* p<0.05
PV
CB
B
LAYERS I-IV
FM
N
PV
CB
C
LAYERS V-VI
**p<0.001
FM
N
PV
CB
PERCENTAGE OF IMMUNOPOSITIVE CELLS (re to NISSL STAINED CELLS)
0
1
2
3
4

Figure 3.5: Distribution of Parvalbumin (PV) and Calbindin (CB) immunoreactive (IR) cells in the FM and Noise regions of Pallid Bat auditory cortex.

The data for this figure are shown in Table 2. (A) Percentage of PV+ or CB+ cells expressed as a percentage of Nissl-stained cells across all layers of the cortex. Both FM and Noise regions contain a significantly greater percentage of PV+ than CB+ cells. There was no difference in expression of either calcium binding protein across the two cortical regions. (B) Comparison only within layers I-IV shows no difference in the percentage of PV+ and CB+ cells in either FM or Noise regions. However, in layers V-VI, there was significant difference in the percentage of PV+ and CB+ neurons (C).

			All Layers			Layers I-IV			Layers V-VI		
	Section ID	Region	Nissl cells	IR cells	% IR cells	Nissl cells	IR cells	% IR cells	Nissl cells	IR cells	% IR cells
PV	PAL23-1	FM	1376	36	2.6	736	18	2.4	640	18	2.8
		N	1440	30	2.1	728	12	1.6	712	18	2.5
	PAL23-2	FM	1344[a]	40	3.0	704[a]	19	2.7	640[a]	21	3.3
		N	1418[a]	13	0.9	784[a]	9	1.1	634[a]	4	0.6
	PAL24-1	FM	1200	38	3.2	592	23	3.9	608	15	2.5
		N	1512	44	2.9	800	17	2.1	712	27	3.8
	PAL24-2	FM	1232	38	3.1	560	15	2.7	672	23	3.4
		N	1528	31	2.0	824	24	2.9	704	7	1.0
	PAL27-1	FM	1288	30	2.3	680	22	3.2	608	8	1.3
		N	1520	24	1.6	872	1	0.1	648	23	3.5
	PAL27-2	FM	1336	36	2.7	616	18	2.9	720	18	2.5
		N	1144	35	3.1	568	14	2.5	576	21	3.6
	PAL37-1	FM	1376	16	1.2	728	8	1.1	648	8	1.2
		N	1520	27	1.8	720	15	2.1	800	12	1.5
	PAL37-2	FM	1288	16	1.2	624	3	0.5	664	13	2.0
		N	1232	34	2.8	696	13	1.9	536	21	3.9
	PAL40-1	FM	1136	24	2.1	552	9	1.6	584	15	2.6
		N	1024	36	3.5	544	13	2.4	480	23	4.8
	PAL40-2	FM	1136	16	1.4	528	6	1.1	608	10	1.6
		N	1112	18	1.6	656	7	1.1	456	11	2.4
	PAL53-1	FM	1376	29	2.1	744	12	1.6	632	17	2.7
		N	1560	25	1.6	944	13	1.4	616	14	2.3
	PAL53-2	FM	1328	27	2.0	704	5	0.7	624	20	3.2
		N	1600	30	1.9	880	8	0.9	720	22	3.1
CB	PAL64-1	FM	1376	4	0.4	736	3	0.4	640	1	0.2
		N	1376	11	1.0	712	8	1.1	664	3	0.5
	PAL64-2	FM	1496	6	0.5	904	1	0.1	592	5	0.8
		N	1488	3	2.3	864	1	0.1	624	2	0.3
	PAL67-1	FM	1544	3	2.3	872	2	0.2	672	1	0.1
		N	1496	21	1.6	880	16	1.8	616	5	0.8
	PAL67-2	FM	1608	24	1.7	920	20	2.2	688	4	0.6
		N	1592	17	1.3	912	13	1.4	680	4	0.6
	PAL68-1	FM	1344[a]	11	0.8	704[a]	8	1.1	640[a]	3	0.5
		N	1418[a]	20	1.4	784[a]	13	1.7	634[a]	7	1.1
	PAL68-2	FM	1344[a]	25	1.9	704[a]	20	2.8	640[a]	5	0.8
		N	1418[a]	28	2.0	784[a]	21	2.7	634[a]	7	1.1
	PAL69-1	FM	1408	24	1.7	768	16	2.1	640	8	1.3
		N	1536	22	1.4	944	16	1.7	592	6	1.0
	PAL69-2	FM	1344[a]	22	1.6	704[a]	18	2.6	640[a]	4	0.6
		N	1418[a]	18	1.3	784[a]	14	1.8	634[a]	4	0.6

[a] A few sections that would be used for Nissl-counting were damaged. An average Nissl count of all sections corresponding to the same FM/N region and layers of the cortex were used in place of the missing counts.

Table 3.1: Quantification of CB+ and PV+ Cells in the Auditory Cortex

3.3.2 Differential staining patterns of PV and CB in the MGB

The MGBv, MGBd and the SG were analyzed for expression of CB and PV. Although the SG is generally considered a part of the MGBd, we analyzed the data separately for these two regions because the echolocation pathway is routed to a larger extent through the SG. In the text below, 'MGBd' refers to the parts of the dorsal division excluding the SG.

Expression patterns of CB and PV in the MGB were region-specific. Perhaps the most surprising finding was the virtual absence of PV+ cells in the MGB (Figures 6A-C, 7A-D). Figure 6A-C shows PV staining in three sections through the MGB. Figure 7 shows PV expression in two other bats. Only the SG showed evidence of PV staining, and even there, the staining was diffuse and limited to the neuropil with rare somata staining (1-5 cells per brain, n=7 brains). MGBv and MGBd were devoid of PV staining. Figure 8 shows both MGB and cortex (note this not auditory cortex) in the same section. Staining is visible in cortical cells and the SG, but not in the MGBv or MGBd, indicating that the weak PV immunoreactivity in the MGB is not a result of potential differences in staining protocol. Given that only the rare cell body in the SG showed PV staining, percentage of PV stained cells was not quantified.

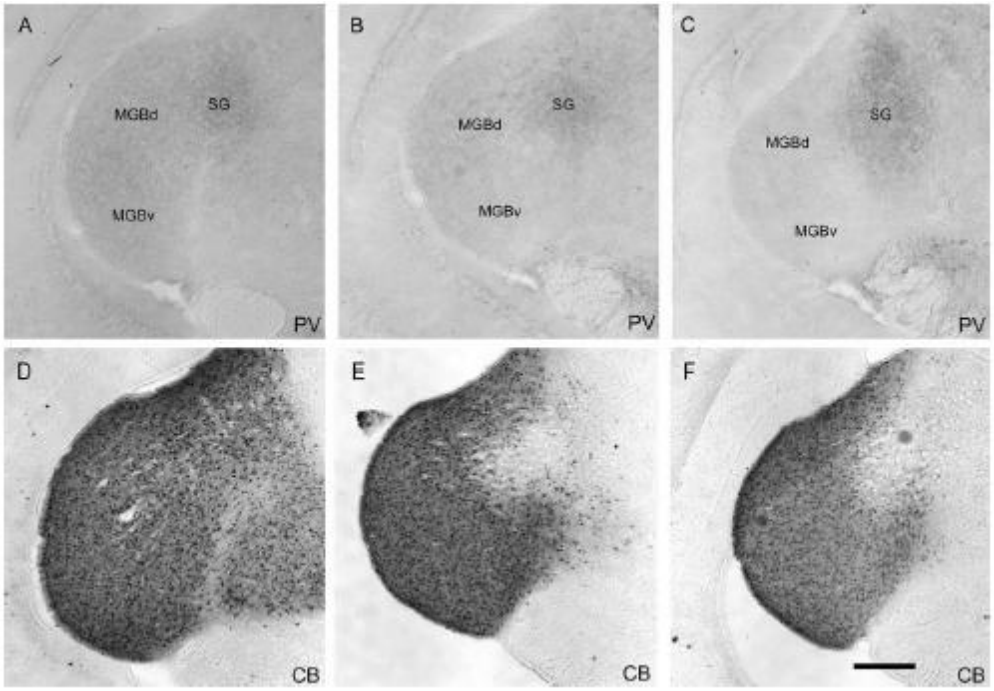

Figure 3.6: Parvalbumin (A-C) and calbindin (D-F) staining in the MGB suggestive of complementary expression patterns.

The left to right (e.g., A→C) progression of sections for each animal is in a rostral to caudal direction. The PV and CB stained sections are from two different bats taken at approximately similar rostrocaudal locations of the MGB (70%, 55% and 40% rostral to caudal). The labels indicate approximately the center of each MGB region demarcated using adjacent Nissl-stained sections. PV staining was limited to the SG in which it was diffuse and limited mostly to the neuropil. PV staining was not discernible in the MGBd and MGBv. CB+ cells, in contrast, were found in all three regions analyzed. However, more caudally, there was a reduction in CB+ cells in the SG essentially appearing in a complementary fashion to PV staining. The scale bar applicable for all panels is 250 μm.

In contrast to PV, CB immunoreactivity was present in all divisions of the MGB, albeit at different levels and in a pattern that appears complementary to PV expression. Figure 6D-F shows CB staining in the MGB. Comparison of PV and CB staining in

Figure 6 indicates the complementary patterns with more CB+ cells in the MGBd and MGBv than the SG, and PV staining only in the SG. Figure 7 shows CB staining in the MGB of two other bats. Quantification of CB+ cell counts relative to Nissl-stained cells supports the observation of more CB+ cells in the MGBd and MGBv compared to the SG (Table 3, Figure 9). A one-way ANOVA analysis revealed that there was no difference between the MGBv and the MGBd in the percentage of CB+ cells. However, the percentage of CB+ neurons in the SG was significantly lower ($p<0.01$) than the other two regions. Together these data indicate differential staining patterns of PV and CB in the different regions of the MGB of the pallid bat.

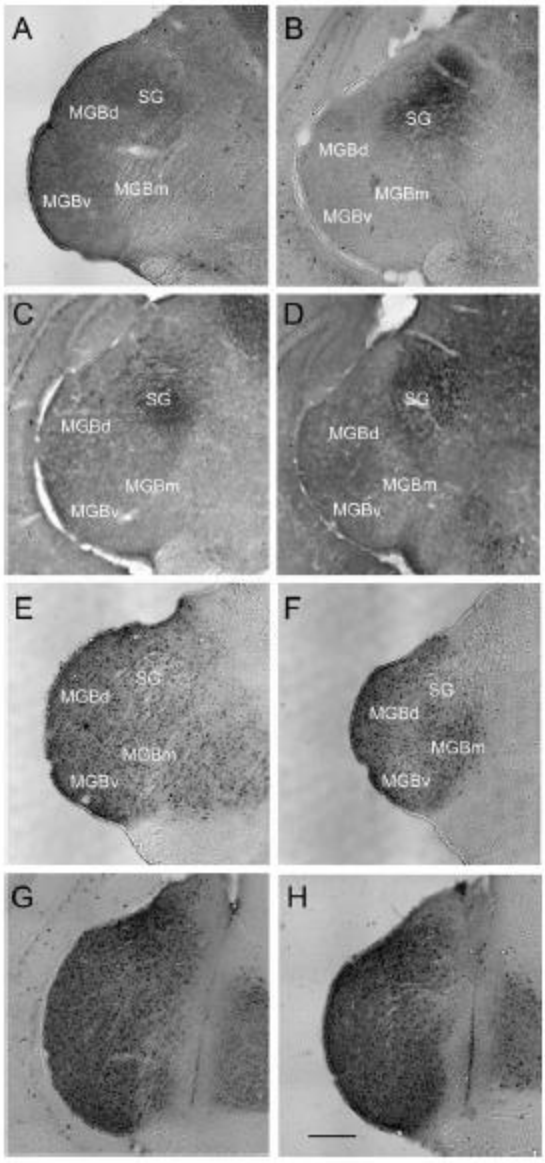

Figure 3.7: Additional examples of PV (A-D) and CB (E-H) staining in the MGB.

Each row corresponds to two sections from the same bat with the left column being more rostral than the right. The sections are approximately at the same rostrocaudal location in the MGB. The scale bar applicable to all panels is 250 µm.

Region	Section ID	% Location[1]	Nissl Cells	CB+ Cells	% CB+ Cells	CB+ Cells/100μm^2
MGBv	PAL64 2-4	57.1	201	103	51.2	25.8
	PAL65 2-3	57.1	161	79	49.1	19.8
	PAL65 2-4	42.9	161	68	42.2	17.0
	PAL67 2-6	71.4	176	80	45.5	20.0
	PAL67 2-7	57.1	166	88	53.0	22.0
	PAL67 2-8	42.9	161	94	58.4	23.5
	PAL67 2-9	28.6	210	66	31.4	16.5
	PAL68 2-5	57.1	235	44	18.7	11.0
	PAL68 2-6	42.9	214	69	32.2	17.3
	PAL68 2-7	28.6	198	82	41.4	20.5
	PAL69 2-2	57.1	147	74	50.3	18.5
	PAL69 2-3	42.9	149	85	57.0	21.3
MGBd	PAL64 2-4	57.1	190	97	51.1	24.3
	PAL65 2-3	57.1	155	79	51.1	19.8
	PAL65 2-4	42.9	253	78	30.8	19.5
	PAL67 2-6	71.4	191	62	32.5	15.5
	PAL67 2-7	57.1	175	96	54.9	24.0
	PAL67 2-8	42.9	163	79	48.5	19.8
	PAL67 2-9	28.6	204	79	38.7	19.8
	PAL68 2-5	57.1	208	72	34.6	18.0
	PAL68 2-6	42.9	196	88	44.9	22.0
	PAL68 2-7	28.6	146	86	58.9	21.5
	PAL69 2-2	57.1	162	51	31.5	12.8
	PAL69 2-3	42.9	169	68	40.2	17.0
SG	PAL64 2-4	57.1	187	23	12.3	5.8
	PAL65 2-3	57.1	179	42	28.2	10.5
	PAL65 2-4	42.9	237	29	12.2	7.3
	PAL67 2-6	71.4	195	42	21.5	10.5
	PAL67 2-7	57.1	175	44	25.1	11.0
	PAL67 2-8	42.9	180	40	22.2	10.0
	PAL67 2-9	28.6	205	59	28.8	14.8
	PAL68 2-5	57.1	173	29	16.8	7.3
	PAL68 2-6	42.9	177	31	17.5	7.8
	PAL68 2-7	28.6	183	62	33.9	15.5
	PAL69 2-2	57.1	175	49	28.0	12.3
	PAL69 2-3	42.9	159	45	28.3	11.3

[1] % Location based on Nissl-stained sections. The caudal to rostral extent of the MGB was assessed by assigning the first caudal section where the MGB was visible 0% and the last rostral section in which the MGB is visible 100%.

Table 3.2: Quantification of CB+ Cells in the Medial Geniculate Body

3.4 Discussion

The main goal of this study was to quantify PV and CB staining in parallel thalamocortical pathways: (1) the thalamic SG nucleus and its cortical projection zone, the FM-selective region (putative echolocation pathway) and (2) the thalamic MGBv division and its cortical projection zone, the noise-selective region (putative prey-localization pathway). There were two major findings. First, the calcium binding proteins CB and PV show differential staining patterns across the various regions of the MGB. CB+ cells were present across the MGB but was differentially distributed. Specifically for the functional pathways, a greater percentage of CB+ cells were found in the MGBv compared to the SG. There was no difference between the MGBd and the MGBv. PV staining was, however, constrained to the SG and absent in the MGBv and parts of the MGBd outside the SG. Most PV expression was limited to diffuse staining of the neuropil in the SG. Second, in the auditory cortex there was a higher percentage of PV+ than CB+ cells in layers V-VI, but not in layers I-IV. However, this was seen in both the FM- and the noise-selective areas, with no area-specific difference. Thus, the MGB, but not cortical, expression pattern of CB/PV appears functional pathway-specific.

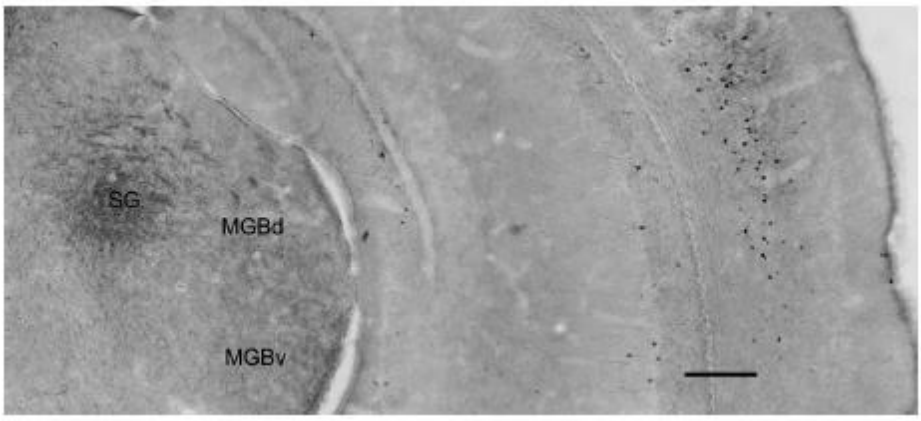

Figure 3.8: Photomicrograph of PV staining (10x) showing intense cell body staining in the cortex (note this is not auditory cortex) and neuropil staining in the SG, but no staining in the MGBv or the MGBd.

This image illustrates the low level of PV expression in the MGB compared to the cortex in the same section. Scale bar is 250 µm.

The FM-selective region contains neurons tuned between 30-60 kHz. The majority (~70-75%) of neurons in this region are selective for the 60→30 kHz downward FM sweeps used by the pallid bat to echolocate obstacles. This form of response selectivity suggests this region's involvement in echolocation. The noise-selective neurons are tuned between 6-35 kHz. Most neurons respond robustly to 5-40 kHz noise and this region contains a systematic map of binaural and azimuth selectivity that may be involved in localization of prey-generated noise (Razak, 2011). Taken together with electrophysiological (Razak and Fuzessery, 2002) and tract-tracing studies (Razak et al.,

2007), the immunostaining data indicate different patterns of calcium binding protein expression in parallel thalamocortical pathways involved in different behaviors.

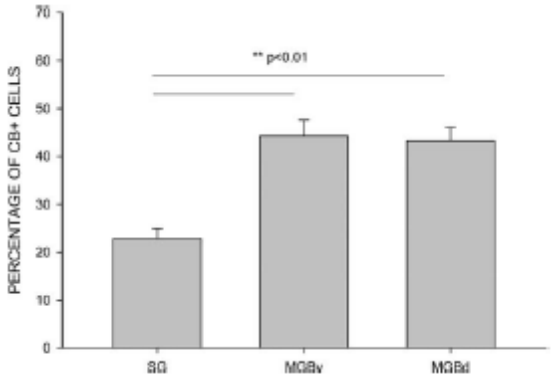

Figure 3.9: Quantification of percentage of CB+ cells relative to Nissl-stained cells in the different regions of the MGB.

The percentage of CB+ cells was significantly lower in the SG compared to the MGBd and MGBv. There was no difference between the MGBd and MGBv. The data for this figure are shown in Table 3.

3.4.1 Comparison across species

MGB: Considerable species-specific differences exist in the expression patterns of PV and CB in the MGB (Table 4). The general trend in rodents and the rabbit is a complementary expression pattern of PV and CB in a lemniscal/non-lemniscal or core/shell organization (de Venecia et al., 1995, Cruikshank et al., 2001, Ouda et al., 2008). PV expression is strongest in the MGBv and weak/absent in the MGBd while CB expression is weak/absent in the MGBv and strong in the MGBd. The SG in the rabbit is devoid of PV expression. The SG was not explicitly identified in the rodent studies. In

rodents, unlike the rabbit, strong PV expression in the MGBv is limited to the neuropil with sparse labeling of the somata. Although less specific in terms of identified MGB divisions, studies of gerbil and guinea pig MGB also support the trends observed in rabbits and mice (Bruckner et al., 1994, De Biasi et al., 1994, Budinger et al., 2000). The macaque monkey is similar to rodents and rabbit in that PV expression is strong and CB expression is weak in the MGBv (Hashikawa et al., 1991, Molinari et al., 1995). However, the monkey differs from rodents/rabbit in that PV+ cells are also common in the MGBd. More PV+ cells than CB+ cells are found in the anterodorsal MGB of the macaque. Similar number of PV+ and CB+ cells are found in the posterodorsal MGB.

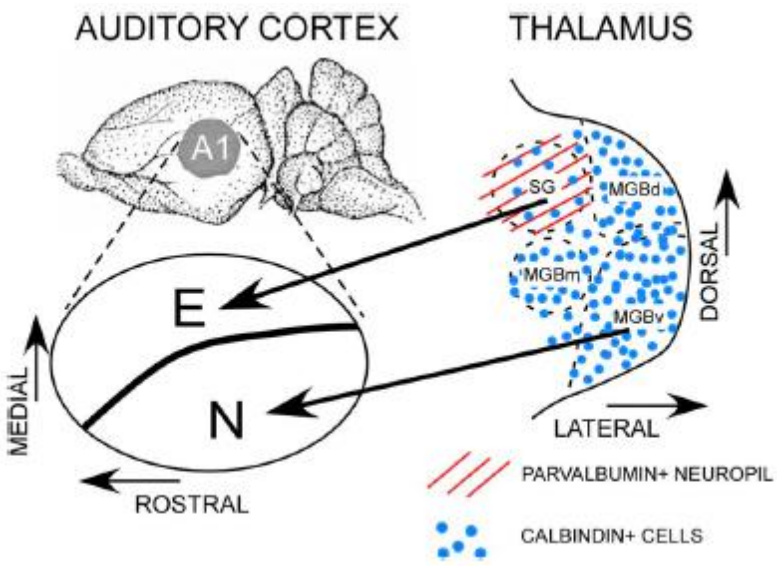

Figure 3.10: Parallel pathways used in different behaviors show distinct staining patterns for calbindin and parvalbumin.

The pallid bat thalamocortical connections contain two parallel pathways: 1) from the suprageniculate (SG) nucleus in the MGB to the echolocation call-selective (E) auditory cortical region and 2) from the ventral MGB (MGBv) to the noise-selective (N) cortical region. These pathways likely serve echolocation for obstacle avoidance and passive localization of ground-dwelling prey (e.g., crickets), respectively. At the level of the MGB, these two functional pathways show differential patterns of staining for the calcium binding proteins parvalbumin (PV) and calbindin (CB). The most surprising finding is the virtual lack of PV+ neurons in any division of the MGB. Only in the SG was PV staining seen, and that only in the neuropil. CB+ cells were presented throughout the MGB, but the density was significantly lower in the SG compared with the other MGB regions.

Horseshoe bat and mustached bat are two species of bats in which PV/CB expression in the MGB has been studied (Zettel et al., 1991, Vater and Braun, 1994). Both species are called 'constant frequency-frequency modulation (CF-FM)' bats that are obligate echolocators (use echolocation for obstacle avoidance and prey-tracking). In general, the PV expression patterns in the MGB of these bats are similar to each other and to the macaque monkey, but differ from rodents/rabbit. PV+ cells and neuropil staining are found in both MGBv and MGBd (including the SG). CB+ cells are also found in both MGBv and MGBd in these bats. CB+ cells are absent in the SG of the mustached bat. The pallid bat differs from the other species examined (including bats) in terms of PV expression in the MGB. PV staining is absent in the MGBv, and is constrained to only the SG of the MGBd. In fact, PV staining appears to be a suitable method to demarcate the SG from other areas of the MGB. As in the CF-FM bats, but different from other species, expression of CB in the pallid bat is similar in MGBd and MGBv. However, similar to the mustached bat, there appears to be reduced expression of CB in the SG in the pallid bat.

Species	MGBv		MGBd		SG	
	PV	CB	PV	CB	PV	CB
Rabbit					none	
Rat					?	?
Mouse					?	?
Macaque monkey				* 	?	?
Mustached bat						none
Horseshoe bat						
Pallid bat	none		none			

Table 3.3: Comparison of PV/CB expression patterns in the MGB across species.

The size of the circle indicates qualitative strength of staining (number of cells or strength of neuropil staining). The solid circumference indicates presence of cell body staining. Absence of a solid circumference line indicates mostly neuropil/fiber staining. Question mark indicates unclear. 'None' indicates absence of staining. * - in the monkey, there are differences between the antero-dorsal and postero-dorsal areas of the MGB. It is important to note that the size of the circles does not indicate similarity of absolute numbers/percentage of stained cells across species. It is only intended to show the relative levels of CB *versus* PV staining within each species.

Taken together, comparative data do not support a general mammalian plan for expression of PV and CB in the MGB. The only trend is that there is stronger PV than CB expression in the MGBv of non-chiropterans, but not in chiropterans. The functional significance of the species-specific CB/PV expression pattern is unclear. One possibility is that PV expression is higher in thalamic targets of central inferior colliculus (ICc) projection (de Venecia et al., 1995). In support of this suggestion, MGBd in bats show ICc inputs and high PV expression (Wenstrup et al., 1994). Likewise, the SG in the pallid bat also receives input from the ICc (Razak and Fuzessery, unpublished observations). However, this does not explain the high number of PV+ cells in the MGBd of the monkey, or the lack of PV expression in the MGBv of the pallid bat. A second possibility is that PV/CB expression correlates with cellular metabolic demands (Braun et al., 1985, Celio et al., 1986) or temporal processing requirements. In echolocating bats, both the MGBd and MGBv exhibit adaptations for echolocation signal processing (Wenstrup and Grose, 1995, Wenstrup, 1999). During the approach phase of hunting (terminal buzz), many bat specie echolocate at a high rate. The homogenous and relatively high expression levels of PV in both MGBv and MGBd in the mustached and horseshoe bat may therefore be a specialization for the metabolic and/or neural temporal fidelity demands of rapid echolocation call processing. The pallid bat data support this suggestion in that the SG, but not the MGBv, appears to be used in echolocation call processing and shows PV expression.

Auditory cortex: Data from rodents, monkey and rabbit show cortical areas with MGBv input strongly express PV. This suggests that MGBv input is correlated with PV

expression. Alternately, cortical PV expression may be an intrinsic property of the primary auditory cortex based on processing requirements such as selectivity for rapid temporal modulations (Atencio and Schreiner, 2008) and/or metabolic demands. The pallid bat data does not support the hypothesis that MGBv input is necessary for high cortical PV expression. The echolocation call- and noise-selective cortical regions are overlain on a tonotopic map of the pallid bat's audible range (6-70 kHz). Together with tonotopy, the short latency and narrowly frequency tuned responses of both areas indicate they constitute the primary auditory cortex in this species. But only the noise-selective region (5-35 kHz) receives input from MGBv. Thus, if MGBv input was necessary for PV expression, only the noise-selective region part of the primary auditory cortex will show such expression. However, both FM- and noise-selective cortical regions contain similar number of PV+ cells. The present study also showed that both PV+ and CB+ cells were seen in similar number of cells in layers I-IV but PV expression was stronger than CB expression in the deeper layers. In the mouse cortex, more intense CB labeling is seen in the superficial layers compared to the deeper layers (Hof et al., 1999, Cruikshank et al., 2001). A PV+ circuit linking the MGBv and the primary auditory cortex seen in the rabbit (de Venecia et al., 1998) is absent in the noise-selective pathway but may be present in the FM selective pathway in the pallid bat. Future studies will examine this possibility. Together, these data indicate species-specific patterns in the expression of calcium binding proteins in the auditory thalamocortical pathways.

3.4.2 Conclusions:

Bat species use diverse foraging strategies. Broad classification schemes of bats have focused on the type of echolocation calls used (CF-FM *versus* FM bats) or the relative importance of passive *versus* active hearing in prey-localization. The neuroanatomical and neurophysiological adaptations for various foraging strategies are only beginning to be understood. The two CF-FM bats (mustached and horseshoe bat) studied show more similarities to each other than to the pallid bat in terms of calcium binding protein expression in the MGB. All three species belong to different families (mustached bat: Mormoopidae, horseshoe bat: Rhinolophidae, pallid bat: Vespertilionidae). It remains unclear if the differences in calcium binding protein expression are related to foraging strategy (obligate echolocators versus passive gleaner) and/or other factors such as the different types of echolocation calls used. Comparative studies of bats, including bats that depend on gleaning to different extents across families, will shed light on the functional basis for differential patterns of calcium binding protein expression and on the convergent neural adaptations for gleaning.

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Chapter 4: Thalamocortical Projections to Intensity Selective Neurons in the Pallid Bat Auditory Cortex

Abstract

Neuronal response selectivity for low intensities may play an important role in processing the returning echoes in a bat's echolocation behavior. The pallid bat, *Antrozous pallidus*, is a gleaning bat that uses echolocation for obstacle avoidance. Response selectivity for intensities is found in a vast majority of cortical neurons in the high-frequency region (HFR) of the pallid bat auditory cortex and is topographically organized. In this study the thalamocortical projections to the HFR of the pallid bat cortex were examined under the hypothesis that there would be an organization in the projection pattern from the medial geniculate body (MGB) of the thalamus related to the intensity selectivity of cortical neurons. Retrograde tracing was utilized to label MGB cells that projected to intensity selective and/or non-selective areas of the HFR. Data supported previously reported findings that the HFR does not receive projections from the ventral division of the MGB (vMGB) and that its inputs are from non-lemniscal nuclei. Data also showed an increase in labeling in the medial division of the MGB (mMGB) than was previously reported in the pallid bat, suggesting that this nuclei may play an important role in processing the echolocation call. The hypothesis of a topographically organized projection pattern to intensity selective regions of the HFR was not supported by the results of this study.

4.1 Introduction

The connections in neural circuits play a fundamental role in determining response properties of neurons in the sensory systems. Circuits between different nuclei in the auditory system have been responsible for either passing response properties on

from one nuclei to the next or for providing the inputs that shape the properties, *de Novo,* in the subsequent nuclei. For the auditory cortex much of its sound related inputs come directly from the thalamus (see review in (Winer et al., 2005). Cortical organization of simple properties can often be inherited from lower levels of the sensory system as seen with the thalamocortical projections that lead to a tonotopic organization in the cortex (Andersen et al., 1980, Imig and Morel, 1984, Redies et al., 1989) see also review in (Imig and Morel, 1983). Inherited organization can also be in the form of functional response properties as the topography of sound localization parameters in the cortex that has been form by a systematic representation of inputs from the thalamus (Middlebrooks and Zook, 1983, Razak et al., 2007). Intensity selectivity has been implicated in echolocation behavior (see chapter 2) and may represent another functional response property in which the thalamocortical projections are organized.

In this study we are using the pallid bat, *Antrozous pallidus*, as a model to study the thalamocortical projections involved in shaping intensity selectivity in the cortex. The pallid bat is a gleaning bat that hunts for food by passively listening to prey generated noise. The pallid bat also uses an echolocation call comprised of a downward frequency modulated (DFM) sweep of a 60-30kHz bandwidth (Brown, 1976, Brown, 1978, Bell, 1982, Fuzessery et al., 1993). The auditory cortex of the pallid bat forms one continuous tonotopic region that is split into two areas that are specially adapted for processing these two different auditory behaviors (Razak and Fuzessery, 2002). The cortex has a low frequency region (LFR) that is selective for low frequencies (5-35kHz) and broadband noise that would be generated by prey and a high frequency region (HFR)

that is selective for frequencies between 30 and 60 kHz and for DFM sweeps that approximate the natural echolocation call (Razak and Fuzessery, 2002). The thalamic connections to these areas have been studied previously and have been shown to be segregated and form parallel pathways (Razak et al., 2007, Razak et al., 2009, Razak and Fuzessery, 2010). The low frequency region receives strong inputs from the ventral subdivision of the medial geniculate body (vMGB) as an adult and connections from the suprageniculate nucleus (SG) of the dorsal MGB (dMGB) that seem to disappear after development. The high frequency region receives strong inputs from the SG with weaker connections from the dMGB and the medial MGB (mMGB), but does not at any time during development and adulthood receive inputs from the vMGB (Razak et al., 2007, Razak et al., 2009, Razak and Fuzessery, 2010). These connections have only been studied in terms of functional organization in relationship to the thalamic connections to the cortical IID map present in the pallid bat. In that case there was a systematic organization in the rostral/caudal orientation of vMGB with respect to the values of IIDs seen in the connection to the low frequency region (Razak et al., 2007). This study attempts to discover if there is a similar organization of thalamocortical projections to the intensity selective cortical neurons.

Intensity selectivity is often shaped by inhibition arising throughout the ascending auditory system, this is because at the level of the auditory nerve where there is no inhibition present the response to increasing intensity is monotonic in nature (Winter and Palmer, 1990, Zhou et al., 2012). It is not clear as to how much each level of the system relies on an ascending input to create or sharpen the intensity selectivity seen at that level.

It has been suggested that monotonic responses to increasing intensity in the cortex are inherited directly from thalamic inputs (Wu et al., 2006). That same study proposed that non-monotonic responses may be created or enhanced *de Novo* in the cortex with a mixture of inhibitory inputs that are recipients of monotonic thalamic inputs and excitatory inputs that inherit their non-monotonic response from thalamic inputs(Wu et al., 2006). There is evidence to support the notion that intensity selectivity can be enhanced in the cortex because as bicuculline, a competitive antagonist of GABA-A receptors, is applied to cortical neurons, non-monotonic responses can become monotonic (Chen and Jen, 2000) (also supported by unpublished data from our lab using another GABA-A antagonist, gabazine), although this does not explain why there is not residual intensity selectivity seen by the non-monotonic thalamic excitatory inputs that are suggested by (Wu et al., 2006). Both of the previous studies also used simple CF tone bursts to classify selectivity. In this study we are determining the intensity selectivity as classified using the more complex stimuli of frequency modulated (FM) sweeps.

In this study we attempt to trace the origins of thalamic inputs to cortical neurons that are intensity selective in the HFR of the pallid bat auditory cortex. We hypothesize that the clustered organization of intensity selective neurons found in the cortex will inherit much its organization from the MGB. We used retrograde tracers to identify neuronal cell bodies in the MGB that projected to regions of the cortex that had been probed using single-unit electrophysoiology for intensity selectivity. Results from this study will be used to expand the knowledge of thalamocortical projections and their role in organizing the cortex around functional properties. To our knowledge this is the first

time that the thalamocortical connections have been studied in their relationship to shaping the topography of intensity selectivity in the cortex.

4.2 Methods

Pallid were netted in Arizona, New Mexico, Texas, and California and housed in a 11 x 14 ft room. The bats were able to fly in this room and were provided with crickets/mealworms and water *ad libitum*. The room was maintained on a reversed 12:12 light cycle. All procedures followed the animal welfare guidelines required by the National Institutes of Health and the Institutional Animal Care and Use Committee at the University of California, Riverside

4.2.1 Surgical Procedures

Recordings were obtained from the right auditory cortex of bats (both males and females, n =20 bats) anesthetized with isoflurane inhalation, followed by an i.p. injection of pentobarbital sodium (Nembutal, 30 μg/g). To expose the auditory cortex, the head was held in a bite bar, a midline incision was made in the scalp, and the muscles over the dorsal surface of the skull were reflected to the sides. The front of the skull was scraped clean and a layer of glass microbeads applied, followed by a layer of dental cement. The bat was then placed in a custom-made Plexiglass holder. A cylindrical aluminum head pin was inserted through a cross-bar over the bat's head and cemented to the previously prepared region of the skull. This pin served to hold the head secure during the recording session. The crossbar holding the head pin was secured behind the bat, leaving no interference between the speaker and the ear. The location of the auditory cortex was

determined relative to the rostrocaudal extent of the midsagittal sinus, the distance laterally from the midsagittal sinus, and the location of a prominent lateral blood vessel that lies parallel to the midsagittal sinus (Razak and Fuzessery, 2002). The size of the exposure was usually ~2mm^2. Exposed muscle was covered with petroleum jelly, and exposed brain surface was covered with silicone oil to prevent desiccation. At the end of the recording session the incision was sutured and lidocaine and a topical antibiotic were applied to the wound. The animal was allowed to recover in isolation before the next surgery Following 1-3 recording sessions (depending on the timing of tracer placement) the bat was euthanized with an overdose of pentobarbital solution and trans-cardially perfused with 4% paraformaldehyde with subsequent removal of the brain for histological processing.

4.2.2 Recording Procedures

Experiments were conducted in a warm (~80°F), sound-proof chamber lined with anechoic foam (Gretch-Ken Industries, Oregon). Bats were kept anesthetized throughout the course of the experiments with additional pentobarbital sodium (one-third of pre-surgical dose) injections. Acoustic stimulation and data acquisition were driven by custom software (Batlab, Dr. Don Gans, Kent State University) and Microstar DSP board based hardware. Programmable attenuators (PA5, Tucker-Davis Technologies, Florida) allowed control of sound intensities before amplification by an integrated amplifier (Yamaha AX430). Stimuli were delivered using an LCY- K100 ribbon tweeter (Madisound, Wisconsin) placed 38 cm directly in front of the bat at an elevation aligned

with the snout. Because a major goal of this study was to examine the thalamocortical projections to intensity selective neurons responding to the FM sweeps used in echolocation, the 0^o elevation/azimuth speaker location was chosen as it approximates echoes coming back along the flight path. In addition, most FM sweep selective neurons tuned between 30–60 kHz in the pallid bat auditory cortex are binaurally insensitive (EO/O type neurons) when tested with interaural intensity differences (Razak and Fuzessery, 2002). Therefore, except for changes in absolute thresholds, the results presented below are likely to be similar at least for a narrow range of azimuths around 0^o from which echoes return.

The frequency response curve of the sound delivery system, measured with a 1/4-in microphone (Bruel and Kjaer, Denmark) had a roll-off from 30 to 80 kHz that was gradual at a rate ~20 dB/octave. Sound intensity levels for FM sweeps were calculated as the average intensity across the range of frequencies in the sweep. Recordings were obtained using glass electrodes (1M NaCl, 2 – 10 MΩ impedance) at depths between 200 and 600μm. Penetrations were made orthogonal to the surface of the cortex. Action potentials were amplified by a Dagan extracellular preamplifier (2400A) and a spike signal enhancer (FHC, Maine) and band-pass filtered (0.3–3 kHz, Krohn-Hite, MA). Extracellular single-unit recordings were identified based on window discriminator threshold-crossing and consistency of action potential amplitude and waveform displayed on an oscilloscope. Waveforms and peri-stimulus time histograms were stored. Responses were quantified as the total number (20 stimulus repetitions, 1 Hz repetition rate) of action potentials occurring within 200 ms of stimulus onset. Adjustments for

spontaneous activity were not necessary because there was no spontaneous activity in these recordings.

4.4.3 Data Acquisition

The focus of this study was on the high-frequency, FM sweep-selective region of the pallid bat A1 (Razak and Fuzessery, 2002). This region is likely to be involved in echolocation behavior. The FM sweep-selective region contains neurons tuned between 25 – 70 kHz and is located rostral and medial to the lower frequency neurons (tuning 5 – 35 kHz) that are noise-selective (Razak and Fuzessery, 2002). The FM sweep-selective neurons respond better to downward sweeps than to noise or upward sweeps with energy in the same spectral band. Using tones, noise, and downward sweeps as search stimuli, neurons with characteristic frequency (CF) >30 kHz, and with stronger response to downward FM sweeps than noise and upward FM sweeps were isolated.

Once single units were isolated various response properties were obtained. Pure tones (25–70kHz, 5 ms duration, 1 ms rise/fall times,1 Hz repetition rate) were used to determine the CF and minimum threshold (MT) for tones. CF was defined as the frequency that elicited action potentials to at least four of five successive stimulus repetitions at the lowest intensity, this intensity being the MT.

4.2.4 Dye injection

Once an area of intensity selectivity was chosen fluorescent dyes, either Fluoro-ruby (FR) dextran tetramethyl rhodamine (Invitrogen, Carlsbad, CA), and/or Fluoro-gold

(FG) (Fluorochrome, Denver, CO) were injected in that location. Tips of glass electrodes (~10 µm diameter) were capillary-filled with 20mg/mL of the dye and back filled with 1 M NaCl. The dye was injected using a Midgard precision current source (Stoelting, Wood Dale, IL) with 7 seconds on-7 seconds off current stimulus of +4 µA. The duration of injection was 5 minutes at a depth of ~300 -600 µm. Following histological processing, dye injection sites were viewed with a Nikon Eclipse 80i Microscope with epiflourescent light filters. Images were taken with a Nikon Digital Sight Camera.

4.2.5 Intensity selectivity

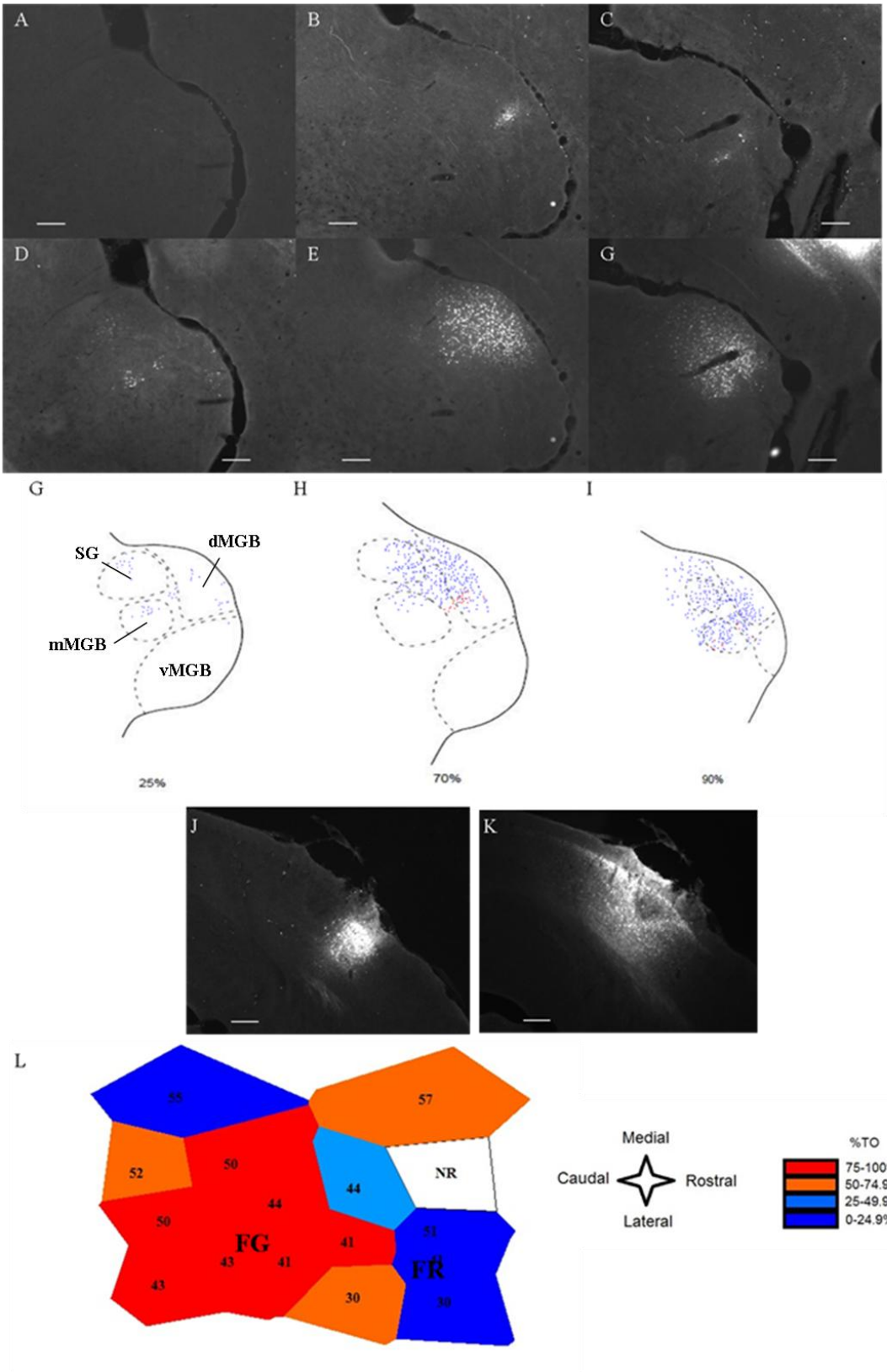
To determine de placement the intensity selectivity was assessed. Downward FM sweeps (5 ms duration, 1 ms rise/fall times, 1 Hz repetition rate) were presented at different intensities (in 5 dB steps increasing from MT). Responses to 20 stimulus presentations were recorded and an intensity selectivity function was plotted. The percent turnover (%TO) indicates the monotonicity of the intensity selectivity function. %TO was calculated as:

$$\%TO = \frac{\text{Max. Response} - \text{Response at highest intensity}}{\text{Max. Response}} \times 100\%$$

(Phillips and Kelly, 1989). Because of the varying values of MT not all neurons were tested with the same number of intensities, so the %TO uses the highest intensity tested or 40 dB above threshold, whichever value is lower. %TO ranges between 0 and 100, with a higher number indicating stronger non-monotonicity.

4.3 Results

In this study we used retrograde tracers injected into different areas of the auditory cortex to trace the projections from the MGB. We recorded our findings from 9 pallid bats (more were attempted, however 4 did not survive the recovery and 2 provided results that were indiscernible.) Figure 1 shows an example subject in which both FR and FG tracer were injected into two different areas, both representing neurons with a 40kHz characteristic frequency (CF). FG was injected into an area with intensity selective response (%TO = 50%+), whereas FR was injected into a intensity non-selective region (%TO < 25%). This is depicted in the Voronoi projection map (Figure 1 L.) in which the color represents the %TO range and the number represents the CF of the penetration. The FR injection (Figure 1 A-C.) showed sparser labeling than the FG injection (Figure 1 D-G.). The position of the labeled cells were digitized for each of the MGB sections: 25% (G), 70% (H), and 90% (I) (percentages are distance from the caudal edge of the MGB) and show the location of the cells relative to approximate positions of the supragenicular (SG), and then ventral (vMGB), medial (mMGB), and dorsal (dMGB) subdivisions of the MGB.


A
B
C
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SG
dMGB
mMGB
vMGB
25%
70%
90%
J
K
L
55
57
52
50
44
NR
50
44
FG
41
51
43
41
FR
43
30
30
Medial
Caudal
Rostral
Lateral
%TO
75-100%
50-74.9%
25-49.9%
0-24.9%

Figure 4.1. Example of Retrograde Tracing Study

This is an example of MGB tracing from cortical neurons using Fluororuby (FR) and Fluorogold (FG). (A-C) 4X fluorescent micrographs of FR labeled cells at (A) 25%, (B) 70%, and (C) 90% caudal-rostral locations in the MGB. Percent values are the approximate location in the MGB from the caudal end. (D-F) 4X fluorescent micrographs of FG labeled cells at (D) 25%, (E) 70%, and (F) 90% caudal-rostral locations in the MGB. (G-I) Digitized cell labeling of FR (red) and FG (blue) labeled cells at (G) 25%, (H) 70%, and (I) 90% caudal-rostral with subdivisions of the MGB labeled. (J-K) 4X fluorescent micrographs of FR (J) and FG (K) cortical injections site. (L) Voronoi projection map of single-unit recording sites. The center of the polygon represents the recording placement. The number in the center of the polygon is the characteristic frequency. Colors represent different %TO ranges: red = 75-100%, red-orange = 50-74.9%, light blue = 25-49.9%, and dark blue = 0-24.9%. (all scale bars = 250μm).

Two bats were successfully doubled label using both FR and FG. Figure 2 shows bats in which FG and FR was injected into a cortical area of intensity selective (non-monotonic) and non-selective (monotonic) neurons. In PAL44 the overall labeling of cells varied in position as the sections went from rostral (90%) to caudal (25%). In the more rostral slices (70% and 90%) most of the labeled cells were found in the mMGB and dMGB (in 90% there were some cells labeled cells in the SG) and as the position moves more caudal (50% and 25%) more cells appeared in the SG and became less apparent in the vMGB and dMBG. In the most caudal slice, only cells labeled with FR (intensity selective) were seen with these cells only being located in the SG. In PAL64 the overall labeling of cells was less varied in position as the sections went from rostral to caudal, showing labeling in all three non-lemniscal nuclei (mMGB, dMGB, and SG) regardless of rostral/caudal position (data was missing from the 50% section that was damaged during processing). Just like PAL44, labeled cells from intensity selective neurons were seen in the most caudal section (25%) while labeling from non-selective neurons were absent. Unlike PAL 44 the intensity selective labeling in the most caudal section was distributed between the three nuclei.

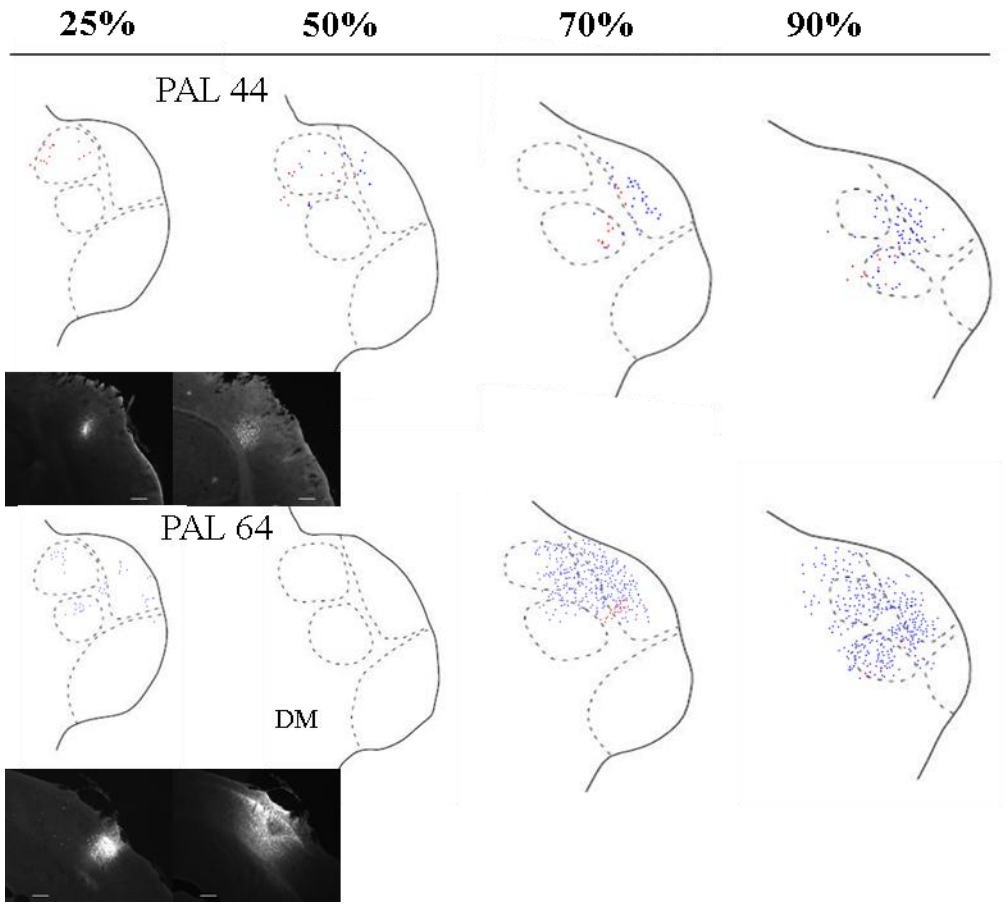

Figure 4.2. MGB cells from both intensity selective and intensity non-selective cortical neurons

Two examples of MGB FR and FG labeled cells with origin at 34-35kHz (PAL44) and 40kHz (PAL64). PAL44 was injected in both intensity selective (red labeled cells) (%TO ~50%+) area and intensity non-selective (blue labeled cells) (%TO <25%) area of the cortex. PAL64 was injected in both intensity selective (blue labeled cells) (%TO ~50%+) area and intensity non-selective (red labeled cells) (%TO <25%) area of the cortex. Percent values are the approximate location in the MGB from the caudal end. Inset picture is of 4X fluorescence micrograph of the injection site (scale bar = 250μm). NT = no tracer seen in the section.

Four bats were successfully labeled in intensity selective areas that had a high characteristic frequency (CF) of 50+ kHz (Figure 3). All four bats were labeled with FR which produced sparser labeling than intensity selective neurons that were labeled with FG (Figure 2). In PAL53 the labeling of cells was only seen in the mMGB and was absent in the most rostral section. PAL65 had labeled cells that were in all three nuclei, with labeling in the mMGB in the most rostral sections (90% and 70%) and labeling in the SG and dMGB in the 70% and 50% sections. No labeling was seen in the most caudal section. PAL69 had labeled cells only in the most rostral sections (90% and 70%). The labeling was centered in the SG in the 90% section and in the dMGB in the 70% section. PAL71 had a similar pattern to PAL53 in that labeling was limited to mostly the mMGB (a small amount of cells were seen in the margins of the SG ad the dMGB in the most caudal section). Based on these results there was no discernible pattern in the labeled cells that projected to high CF intensity selective cortical neurons.

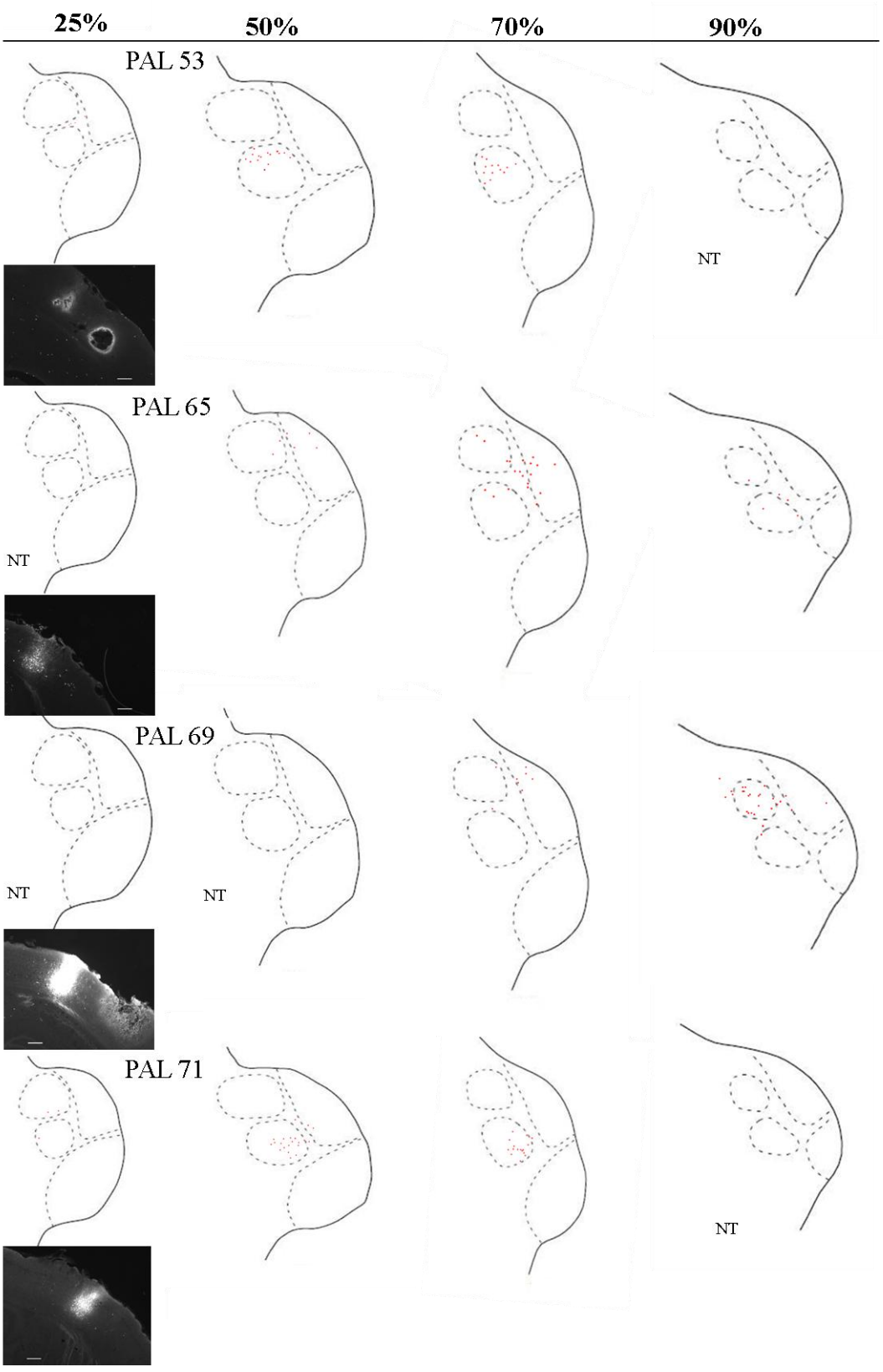
25%
50%
70%
90%
PAL 53
NT
PAL 65
NT
PAL 69
NT
NT
PAL 71
NT

Figure 4.3. MGB cells from high frequency intensity selective cortical neurons

Four examples of MGB Fluororuby labeled cells with origin at high frequency (50kHz+) intensity selective (%TO ~50%+) areas of the cortex. Percent values are the approximate location in the MGB from the caudal end. Inset picture is of 4X fluorescence micrograph of the injection site (scale bar = 250μm). NT = no tracer seen in the section.

Two bats were successfully labeled in intensity selective areas that had a lower characteristic frequency (CF) of 40-49 kHz (Figure 4). MGB cells in the bats were differentially labeled with either FG (PAL78) or FR (PAL68). FG produced a greater number of cells labeled in the MGB than did FR. This differential level of staining is consistent with what was seen in the doubled labeled experiments (Figure 2). In PAL68 the labeling of cells was seen in the both mMGB and dMGB. Labeling was sparse in the most rostral section (90%) and was completely absent in the most caudal section (25%) and was absent in the most rostral slice. PAL78 had labeled cells that were limited to the SG and the margins of dMGB. Labeled cells were absent in the two most caudal sections (25% and 50%) despite the large amount of cells that were labeled with FG. This suggests that although FG led to a larger number of cells being labeled (as compared to FR) in all the bats where it was utilized, the spread of the injection site does not seem to affect the distribution of labeled cells in the rostral/caudal direction. As with intensity selective cortical neurons with high CF values, there seems to be no discernible organization of the projections to intensity selective neurons with lower CF values.

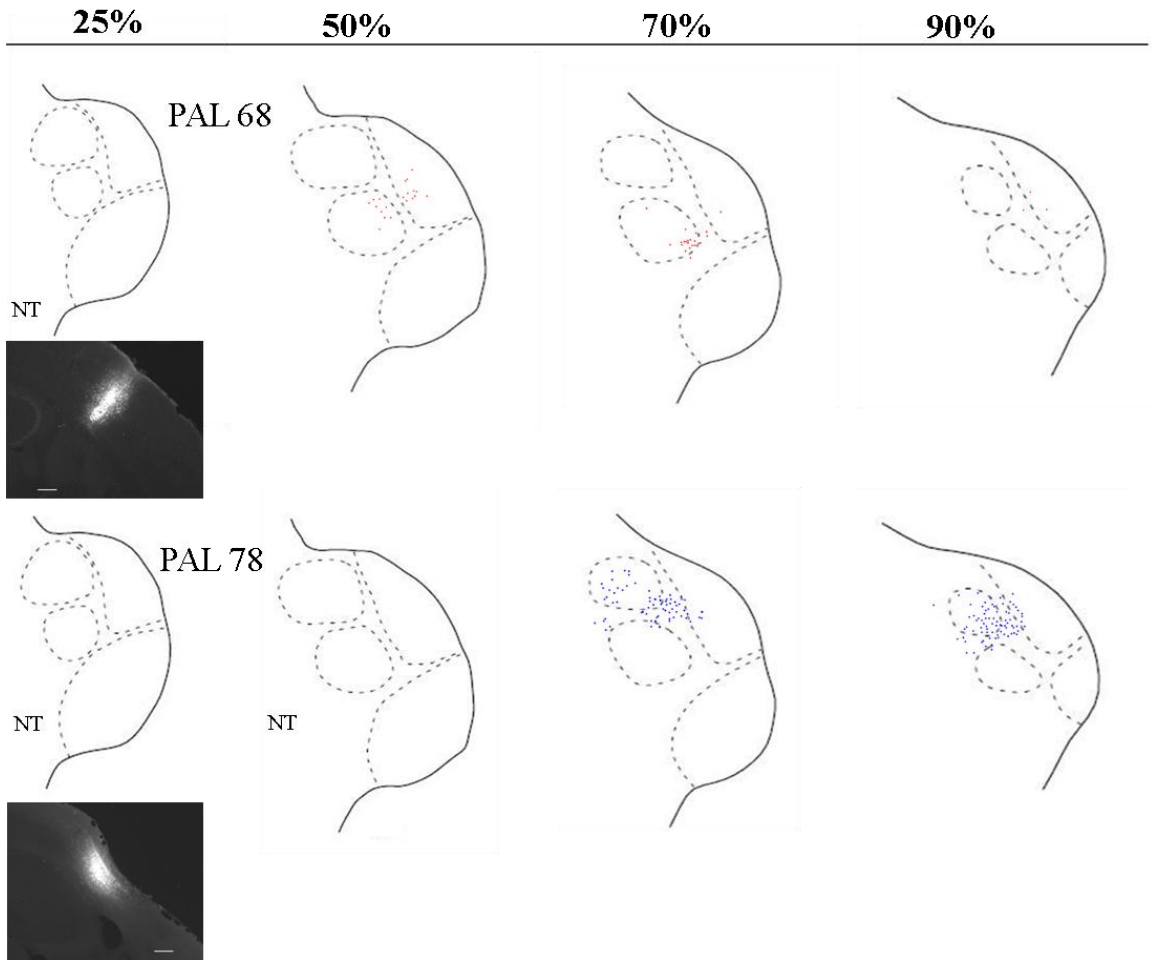

Figure 4.4. MGB cells from middle frequency intensity selective cortical neurons

Two examples of MGB Fluororuby labeled cells with origin at middle frequency (40kHz-49kHz) intensity selective (%TO ~50%+) areas of the cortex. Percent values are the approximate location in the MGB from the caudal end. Inset picture is of 4X fluorescence micrograph of the injection site (scale bar = 250μm). NT = no tracer seen in the section.

Only one bat was singly labeled from a cortical area that was non-selective for intensity (Figure 5). The low numbers of non-selective subjects was primarily due to the difficulty in consistently finding neurons that had a %TO value of less than 25% (see Chapter 2). PAL67 had labeled cells that were mostly in the dMGB and were located in the most rostral sections (70% and 90%). This data along with non-selective labeling in the double labeled subjects (Figure 2) produced no discernible pattern of projections to cortical neurons that are non-selective to intensity.

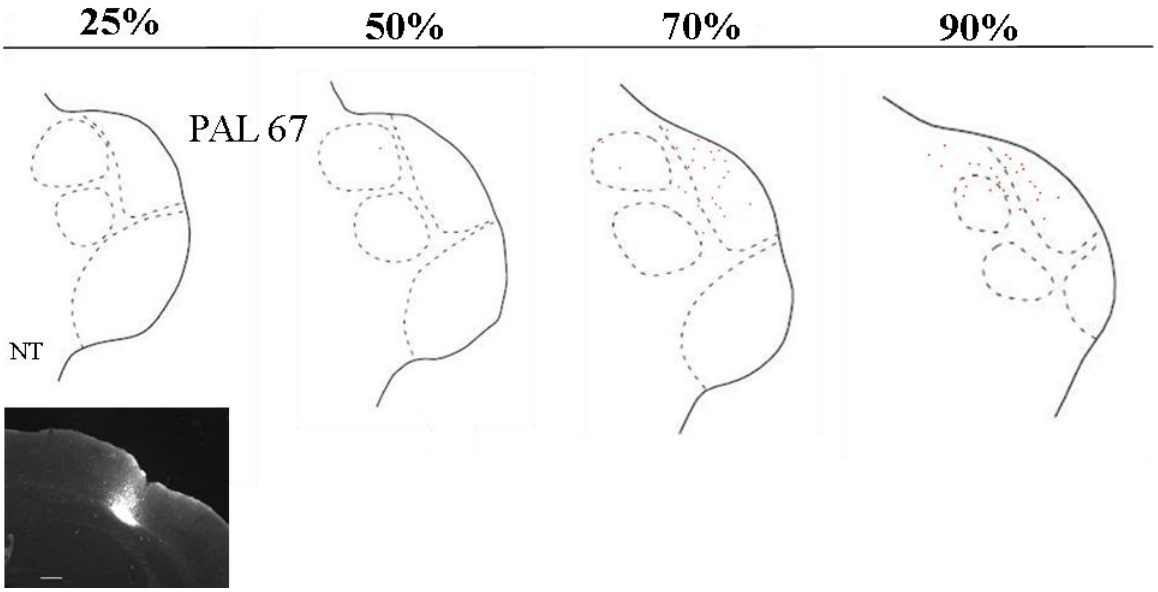

Figure 4.5. MGB cells from intensity non-selective cortical neurons

One example of MGB Fluororuby labeled cells with origin at 51-54kHz intensity selective non-selective (%TO <25%) areas of the cortex. Percent values are the approximate location in the MGB from the caudal end. Inset picture is of 4X fluorescence micrograph of the injection site (scale bar = 250μm). NT = no tracer seen in the section.

When looking at the population of cells as a whole (Table 1.) a two-way ANOVA shows that there is a significant difference in the percentage of cells labeled in the different rostral/caudal locations ($p<0.001$). However, there was not a significant difference in the nuclei that the labeled cells were located in. A pair wise comparison using the Holm-Sidak method shows that within the rostral/caudal locations there is a significant difference ($p<0.001$) for 90% vs. 25%, 90%, vs. 50%, 75% vs. 25%, and 75% vs. 50%, where no significant difference was seen in 90% vs. 70% and 50% vs. 25%. This means that labeled cells mostly populated the most rostral part of the MGB. This pattern was similar when you examined intensity selective (Table 2) and non-selective (Table 3) labeled cells separately.

		25%	**%**	**50%**	**%**	**70%**	**%**	**90%**	**%**
Total	1243	65	5.2	101	8.1	531	42.7	546	43.9
SG	269	25	9.3	18	6.7	109	40.5	117	43.5
dMGB	439	18	4.1	30	6.8	236	53.8	155	35.3
mMGB	243	12	4.9	44	18.1	83	34.2	104	42.8
NA	292	10	3.4	9	3.1	103	35.3	170	58.2

		SG	**%**	**dMGB**	**%**	**mMGB**	**%**	**NA**	**%**
Total	1243	269	21.6	439	35.3	243	19.5	292	23.5

Table 4.1. Location of labeled cells. SG = suprageniculate nucleus, dMGB = dorsal division of the MGB, mMGB = medial division of the MGB, NA = Areas outside of or between discernible nuclei.

		25%	%	50%	%	70%	%	90%	%
Total	1040	65	6.3	82	7.9	447	43.0	446	42.9
SG	246	25	10.2	12	4.9	105	42.7	104	42.3
dMGB	323	18	5.6	19	5.9	168	52.0	118	36.5
mMGB	219	12	5.5	44	20.1	79	36.1	84	38.4
NA	252	10	4.0	7	2.8	95	37.7	140	55.6

		SG	%	dMGB	%	mMGB	%	NA	%
Total	1040	246	23.7	323	31.1	219	21.1	252	24.2

Table 4.2. Location of labeled cells: Non-monotonic origin. SG = supragenicular nucleus, dMGB = dorsal division of the MGB, mMGB = medial division of the MGB, NA = Areas outside of or between discernible nuclei.

		25%	%	50%	%	70%	%	90%	%
Total	203	0	0	19	9.4	84	41.4	100	49.3
SG	23	0	0	6	26.1	4	17.4	13	56.5
dMGB	116	0	0	11	9.5	68	58.6	37	31.9
mMGB	24	0	0	0	0.0	4	16.7	20	83.3
NA	40	0	0	2	5.0	8	20.0	30	75.0

		SG	%	dMGB	%	mMGB	%	NA	%
Total	203	23	11.3	116	57.1	24	11.8	40	19.7

Table 4.3. Location of labeled cells: Monotonic origin. SG = supragenicular nucleus, dMGB = dorsal division of the MGB, mMGB = medial division of the MGB, NA = Areas outside of or between discernible nuclei.

4.4 Discussion

This study examined the thalamocortical projections in the auditory pathway of the pallid bat, *Antrozous pallidus*, and the role they play in organizing intensity selective neurons in the auditory cortex. Retrograde tracers were placed in either intensity

selective or non-selective regions in the high-frequency region (HFR) of the cortex , which is selective for echolocation calls (Razak and Fuzessery, 2002). The purpose of the study was to determine if the topographic organization of intensity selectivity that has been seen in the HFR (chapter 2) had its origin in an organization in the thalamocortical projections to this area. Overall, the results of this study did not provide evidence for an organization of these projections, although due to the low number of animals surveyed and the sparseness of non-selective neurons (Chapter 2) available in the cortex, this study does not rule out a possibility for some form of organization.

4.4.1 Methodological considerations

A possible confound to this study may be the ability to precisely deliver either the fluroruby or fluorogold to a small enough region of the cortex so that it only represents an area with similar intensity selectivity. The injection sites averaged approximately 350 microns in diameter in both the rostral/caudal and medial/lateral directions. Attempts were made to place the dye in the middle of regions that had similar intensity selectivity so that this would minimize spread to other areas but it is possible that the spread of the dye could have invaded regions that had an intensity selectivity other than the one intended. This could occur especially in areas that are close to the border of two adjacent clusters of neurons with different selectivity. Another possible confound in the study is that the spread of the dye may also invade the white matters tracks just below layer six cortical neurons. Dye injections were made at 200-450 microns so that the dye would be

present at thalamocortical receipts layers, however it is possible that tracer may have spread to the white matter as well.

4.4.2 Organization of thalamic projections to intensity selective cortical neurons

The data from this study does not support the hypothesis for an organization of thalamocortical projections to intensity selective neurons in the cortex. There are models to suggest that monotonic responses could be inherited from thalamic inputs while non-monotonic responses may be created *de Novo* in the cortex (Wu et al., 2006). This could mean that retrograde tracing from cortical neurons will not label MGB neurons that are directly responsible for creating cortical selectivity but one that are actually just providing a simple monotonic excitatory response. Methods that could highlight local cortical circuits that feed into intensity selective neurons might provide more insight into the connections responsible for creating this selective response and the topographic organization around these responses. Studies show that in mouse auditory cortex, that PV+, presumptive inhibitory neurons have a monotonic rate-level function in response to increasing intensity (Moore and Wehr, 2013). This may also be the case for PV+ neurons in the MGB. In a recent study, the pallid bat MGB was nearly absent of PV+ cells, although PV+ neuropil was seen in the SG (Campo et al., 2014). In the pallid bat, the SG is a major input to the HFR region of the cortex (Razak et al., 2009). Monotonic inhibitory responses have been shown to be important for shaping intensity selective responses (Wu et al., 2006). It is possible that PV+ input from the SG to the HFR is providing this inhibition in a monotonic fashion.

4.4.3 Thalamic inputs to the high frequency region of the pallid bat auditory cortex

This study confirmed previous studies that showed that the HFR of the pallid bat auditory cortex does not receive inputs from the ventral division of the MGB (vMGB) (Razak et al., 2007, Razak et al., 2009). Tracing from both intensity selective neurons and non-selective neurons labeled cells in the SG, mMGB, and the dMGB. The data does suggest that the mMGB may have more inputs into the HFR than has been previously reported (Razak et al., 2007), as many of the subjects of this study had retrogradely labeled neurons in the mMGB. This could suggest a role for the mMGB in processing echolocation calls. The mMGB has been implicated in associative learning (Edeline and Weinberger, 1992, Bordi and LeDoux, 1994). In rats selectivity for intensity ranges can change by associative learning processes (Polley et al., 2004). Perhaps thalamocortical connections from the mMGB to the HFR reflect the need for cortical plasticity in echolocation behaviors. It is also possible that spread of the injected dye was taken up by projections to layer I and VI of the cortex which have been shown, in limbic associated areas of the cat auditory cortex, to receive projections principally from the mMGB (see review in (Winer et al., 2005)).

4.4.4 Conclusions

Although the data in this study did not support the hypothesis of a topographic organization of thalamocortical inputs to intensity selective cortical neurons, it could be explained by the reality that the nature of these connections are more complex than what could have been elucidated using simple retrograde labeling techniques. MGB neurons

in the cat have been shown to have a large percentage of intensity selective neurons (Rouiller et al., 1983). Future studies may include recording the response-intensity functions in pallid bat MGB neurons and using anterograde labeling to determine the projections patterns of thalamic intensity selective neurons. It is possible that the MGB has a topographic organization involving intensity selectivity as does the HFR of the pallid bat cortex (see Chapter 2), however that organization, if present, may not be responsible for the responses or the organization that is seen in the HFR.

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Chapter 5: Conclusion

Bat echolocation calls are specific sounds produced by the bat to locate prey and avoid obstacles. Often these calls are made "on the wing" and provide information that guides the bat's flight. Having neural structures that are selective for the properties specific to the call are crucial for the detection and fast processing of the returning echoes that is needed for the bat to make precise movements. The pallid bat, *Antrozous pallidus*, was used in these studies as a model for studying intensity selectivity. These studies show the importance that intensity selectivity may play in processing of echolocation calls.

Electrophysiological studies (Chapter 2) showed that the high-frequency region (HFR) of the pallid bat auditory cortex, which had already been shown to be selective for the rate and direction of the FM sweep echolocation call, is also well adapted for processing the low intensities present in the returning echoes. The data from the electrophysiology study supports previous studies in which FM sweeps, that mimic the echolocation call, contain the inhibition that is necessary for shaping selectivity for direction, rate, and now intensity. The spectrotemporal components of the pallid bat echolocation call provide a mechanism for shaping this cortical selectivity. Immunohistochemical studies (Chapter 3) showed a differential staining pattern of calcium binding proteins in the cortex and the auditory thalamus that differed from some other mammals studied. These differential staining patterns may be a biochemical marker that can be used to differentiate echolocation selective and non-echolocation selective regions. Tracing studies (Chapter 4) failed to elucidate any thalamocortical projections that are specialized to the intensity selective neurons in the cortex. Data from

this study did support previous studies that showed projections from non-lemniscal thalamic nuclei to the tonotopically organized, "primary-like" echolocation region of the cortex.

There is evidence provided in these studies to suggest that the pallid bat deviates from some general mammalian plans that have been studied. This study showed a higher percentage of intensity selective neurons than seen in other mammals. The distribution of calcium binding proteins also revealed a deviation from what has been seen in other mammals. Lastly, the thalamocortical projections of the pallid bat may represent another deviation from a general mammalian plan. Perhaps these differences highlight the unique requirements for processing echolocation calls that are not utilized by other non-chiropterans. Differences seen between species of bats may reflect the unique requirements of different habitats and ecological niches. In the recent study of another gleaning bat, *M. waterhousii*, there seems to be a similarity to the pallid bat auditory cortex in that the cortex contains one large continuously tonotopic gradient (Macias et al., 2014). However, *M. waterhousii* does not seem to have an over representation of echolocation frequencies that are seen in the pallid bat. This is another adaption that may be present in the pallid bat and other bat species but is not necessarily a feature common to all bats.

This dissertation research, as a whole, supports the concept of studying sensory processing using behaviorally relevant stimuli. Much like the information that codes for life is not processed with the single monomer of a nucleotide, but must extracted from the

DNA or RNA polymer, so does the processing of sensory information need to reflect the complexities of the natural and meaningful stimuli and not just its constituent features. Auditory neuroscientists must explore how sound processing makes use of spectrotemporal and intensity components of complex sounds. We also must study the dynamic nature of these sounds in both their production and processing by the brain. By studying how the bat processes its echolocation call we can achieve this level of analysis because of: the spectrotemporal features of the call; the dynamic nature of call production and the ability of some bats to modulate the call for optimum processing of echoes; the variability that is seen in the properties (i.e. spectrotemporal interactions, timing, intensity, etc.) of the returning echoes; and the richness of species specific calls and strategies of processing that can be found in the order Chiroptera. Future studies can expand on this work by: examining the behavioral ability of the pallid bat to modulate the intensity of its call; continuing to look at the differential expression of biochemical markers that may play a cellular role in neuronal responses to intensity; examining the intensity selective nature of other areas of the pallid bat cortex to determine if this is truly a feature adapted for echolocation; and trying to unravel the complex thalamocortical projections that may play a role in shaping intensity selectivity in the cortex.

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