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Convergence of Excitatory and Inhibitory Projections in the Mouse Medial Geniculate Body

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CONVERGENCE OF EXCITATORY AND INHIBITORY PROJECTIONS IN THE MOUSE
MEDIAL GENICULATE BODY

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ABSTRACT

The medial geniculate body (MGB) is the target of excitatory and inhibitory inputs from several neural sources. Among these, the inferior colliculus (IC) is an important nucleus in the midbrain that acts as a nexus for many auditory pathways and projections, ascending and descending, throughout the rest of the central auditory system and provides both excitatory and inhibitory projections to the MGB. In addition, the thalamic reticular nucleus (TRN) is a major source of inhibition to the MGB, particularly in rodents. Finally, the auditory cortex (AC) is a major source of descending input to the MGB, providing direct excitation and indirect inhibition via the TRN.

In our study, we assessed the relative contribution from these excitatory and inhibitory projection sources to the MGB of the auditory system in mice. Using retrograde tract tracing with CT_β -Alexa Fluor 594 injected into the MGB of the mouse, we quantitatively mapped the projections from both the ipsilateral and contralateral IC, the TRN, and the AC to the ipsilateral MGB. Our results indicate significant GABAergic projections from the IC and TRN to the MGB and excitation from the AC that play an overlooked role in shaping auditory processing. These results complement prior studies in other species, which suggests that these pathways are important factors in the regulation of neuronal activity in the auditory forebrain.

CHAPTER 1
INTRODUCTION

The primary function of the auditory system is to develop the sense of hearing. To do this, the system is divided into two subsystems – the peripheral auditory system and central auditory system. Being a very intricate process, involving many regions and mechanisms, audition has a lengthy process in the goal to translate sound waves into neural electrical signals. This process begins from sound waves entering the ear, and eventually culminates in the cochlea, where the neural signals are created (Hudspeth, 1997). These signals then proceed as cranial nerve VIII to the cochlear nuclei, where one will also see nerve afferents crossing to the superior olivary complex. To help prevent loss of hearing due to unilateral damage, most inputs are distributed bilaterally throughout the brain. From the lower brainstem nuclei, all ascending inputs make their way to the inferior colliculi (IC) and medial geniculate body (MGB) in the thalamus, reaching the cortex as the last stop. The MGB is the target of many excitatory and inhibitory projections, has a wide integration of auditory connections throughout the central auditory system, and receives inputs from all lower brainstem and midbrain nuclei (Buentello et al., 2015; Ito and Oliver, 2010).

As an overview, sound processing in the mammalian auditory pathway proceeds as follows: sound enters the outer ear as time-varying air-pressure waves, which is then eventually transduced into neural signals via the cochlea (Hudspeth, 1997). The frequency of those sounds are analyzed, along with the location and timing of the sounds, by the lower brainstem nuclei, specifically the cochlear nuclei (CN), lateral leminscus nuclei (NLL), and the superior olivary complex (SOC) (Blosa et al., 2013; Cant, 1992; Hudspeth, 1997; Oliver, 2000; Schwartz, 1992).

These nuclei are also involved in intensity and interval analysis for localization of sound. The dorsal region of the NLL is a major source of GABAergic input to the inferior colliculus, as well as all other lower brainstem nuclei, all projecting excitatory and inhibitory inputs (Buentello

et al., 2015; Ito and Oliver, 2010). IC nuclei, like those in the brainstem, are located in both hemispheres of the brain, and along with the superior olivary complex (SOC), they are the first region where sound location is fully integrated, fusing vertically and horizontally oriented data (Oliver, 1984; Reetz and Ehret, 1999; Winer and Schreiner, 2005). Furthermore, like most brain areas, excitatory and inhibitory inputs from other auditory regions is integrated in the IC; excitatory input is integrated from the brainstem and auditory cortex, and inhibitory input is integrated from nearly every peripheral brainstem nucleus found in the auditory system (Winer and Schreiner, 2005). Once this integration is completed, it is then sent to an ascending auditory center, namely the MGB, which then projects to the primary auditory cortex (Hackett, 2011; Kaas and Hackett, 2000; Lee and Winer, 2011). Once here, the primary AC integrates with the secondary AC, and interconnects with the frontal lobes for further processing, such as speech perception (Hackett, 2011; Kaas and Hackett, 2000; Lee and Winer, 2011).

The higher auditory regions in the brain include the inferior colliculus (IC), medial geniculate body (MGB), thalamic reticular nucleus (TRN), and the auditory cortex (AC), structures that all play an important role in higher-order auditory processing (Wenstrup, 2005; Winer, 1992). Among these, the auditory thalamic nucleus, i.e. the MGB, receives convergent information from several structures, including the IC, the TRN, and AC (Lee, 2013; Lee, 2015; Winer, 1984). All auditory information processed through lower brainstem and midbrain structures eventually reaches the MGB, where that information is then sent to the auditory cortex (Lee, 2013). Information from each of these sources to the MGB may have an excitatory, inhibitory, or mixed influence of the activity of neurons in the MGB (Winer, 1992). Consequently, understanding the neural interactions between these structures and the MGB is crucial to understanding how auditory information is processed in the brain and how it is altered during diseases of the auditory system.

Of the upstream inputs to the MGB, the inferior colliculus (IC) is the main auditory structure found in the midbrain, and is a major hub for ascending and descending pathways in the central auditory system (Buentello et al., 2015; Ito and Oliver, 2010; Oliver, 1984; Wenstrup, 2005). The IC is mostly involved in auditory orientation and perception, and acts as a nexus for many auditory pathways, receiving and sending projections from other nuclei and regions (Winer and Schreiner, 2005). The inferior colliculus is composed of three main subregions - the central nucleus (CN), dorsal cortices (DC), and lateral cortices (LC). These three subdivisions are generally similar in cellular makeup, but have stark differences in their projections (Saldaña and Marchán, 2005; Wenstrup, 2005). The central nucleus of the IC (ICC) projects its axons mainly to the ventral division of the medial geniculate body (MGBv). The dorsal and lateral cortices project their axons to the dorsal and medial MGB subdivisions (Hackett, 2011; Wenstrup, 2005). All components of the IC mainly send their projections to the ipsilateral MGB, with very few going to the contralateral MGB (Mellott et al., 2014a).

Glutamate is the main excitatory neurotransmitter of the central auditory system, with gamma-aminobutyric acid (GABA) and glycine being the main inhibitory neurotransmitters. The IC contains both types of neurotransmitters, with varying degrees of concentration; approximately 21% of IC neurons are GABAergic, with the remaining 75% of neurons being almost solely glutamatergic. The glutamatergic neuron percentage is based on the findings of those neurons expressing vesicle glutamate transporters (VGLUT) (Altschuler et al., 2008; Caspary et al., 2008). As the IC has both GABAergic and glutamatergic neurotransmitters, the IC would also have both excitatory and inhibitory effects in the auditory system (Mellott et al., 2014a; Peruzzi et al., 1997; Winer et al., 1996). The ascending inhibitory component of the IC projection to the MGB is a novel discovery within the IC (Mellott et al., 2014a; Peruzzi et al., 1997; Winer et al., 1996). The

IC integrates nearly all lower brainstem auditory information before sending its output to the medial geniculate body (MGB), but said output is particularly intriguing, as it contains both GABAergic and glutamatergic projection neurons, unlike many other areas of the brain. These cells are involved in what is known as the tectothalamic pathway, the largest output pathway of the IC, mainly sending inhibitory projections to the MGB (Wenstrup, 2005). The rest of the tectothalamic pathway is involved in excitation, sending ascending glutamatergic projections to the MGB. Interestingly, it has been shown that the inferior colliculus displays signs of reduced GABA release as it ages (Caspary et al., 2008).

Decreasing GABA levels imply a decreased level of neural inhibition, thus understanding the cellular makeup and layout of the IC and its projections to other structures is invaluable information in truly appreciating and comprehending its role in auditory processing, particularly as it pertains to age-related hearing loss. Furthermore, the studies described in this thesis are concentrated on the distribution of the IC's tectothalamic pathway as it projects to the MGB, but, as noted, there are many more structures involved in the MGB's auditory processing function.

The medial geniculate body (MGB) is the main auditory center of the thalamus. It is the target of excitatory and inhibitory inputs from many structures of the central auditory system (Hackett, 2011; Llano and Sherman, 2008; Villa et al., 1991; Wenstrup, 2005). A knee-shaped structure, the MGB receives inhibitory input from the TRN, excitation and inhibition from the IC, and direct excitation from the AC (Winer, 1984). It acts as a thalamic way station, receiving input from the IC, and sending to, as well as receiving projections from the auditory cortex (Lee and Winer, 2008; Lee, 2013). Most of its output is directed towards to the auditory cortex, but projections are sent to other regions as well, such as the TRN, amygdala, and striatum (Lee, 2015).

The medial geniculate body can be separated into three subdivisions – ventral (MGBv), dorsal (MGBd), and medial (MGBm) (Figure 1) (Winer, 1992). The ventral subdivision (MGBv) is the main nucleus of the MGB, projecting axons via the thalamocortical pathway to layers III and IV of the auditory cortex (Huang and Winer, 2000; Smith, 2012). The MGBv also receives input mainly from the ICC. The dorsal MGB (MGBd) is very similar to the MGBv, projecting to the same layers of the AC. The difference is that MGBd's axons terminate in what is known as the extralemniscal auditory cortex (Hackett, 2011; Kaas and Hackett, 2000). The medial MGB (MGBm) is unique, having an axonal output terminating in many cortical layers, particularly layers I and VI of the auditory cortex. MGBv and MGBd's projections to the AC are excitatory, being shown to be non-GABAergic (Lee, 2015).

Hearing loss is one of the most prevalent conditions in the world today (Dalton et al., 2003; Salomon, 1986; Yamasoba et al., 2013; Yueh et al., 2013). It affects many members of both older and younger generations, being the third most seen chronic condition in the elderly (Salomon, 1986; Yamasoba et al., 2013; Yueh et al., 2013). It affects approximately one in three people over the age of 65, and one in two people over 85 (Yamasoba et al., 2013; Yueh et al., 2013). Modern technology has equipped us with the means to treat some of these hearing-related problems through the use of hearing aids (Dalton et al., 2003; Yueh et al., 2013). However, these devices primarily manage hearing loss and are rarely fully restorative (Bielefeld et al., 2010). It thus remains an arduous issue for society to tackle. Even with hearing-aids, many elderly people continue to struggle with engaging in daily conversation, and the essential skill of blocking out unwanted auditory information while focusing on a particular auditory source (Bielefeld et al., 2010; Dalton et al., 2003; Humes et al., 2006). The world is full of a wide array of unique, special sounds, which vary in terms of their location, intensity, frequency, or pitch (Hudspeth, 1997). The brain, over

time, loses efficiency in processing these sounds, and because of this, people eventually exhibit difficulty comprehending the incredible amount of auditory stimulation experienced on a daily basis (Dalton et al., 2003; Yamasoba et al., 2013).

On a physiological level, one of the current theories to explaining age-related hearing loss (presbycusis) is the gradual inability of the auditory system's inhibitory circuitry to "filter out" unwanted information (Allen and Eddins, 2010; Caspary et al., 2008; Llano et al., 2012; Stebbings et al., 2016). This filtering ability becomes less potent as our brains age (Caspary et al., 2008; Llano et al., 2012; Wehr and Zador, 2003), and can occur centrally or peripherally. Peripheral issues stem from the destruction of inner ear hair cells, usually due to physical trauma or loud noises, and are generally well researched. Central issues, on the other hand, can range from several physiological changes in the central auditory system, and is far less understood. These issues are shown by molecular changes in inhibitory processes in many auditory regions at differing levels of the auditory system. Lower levels of the auditory system are specialized for the initial detection and localization of sounds, while higher levels of the auditory system specialize in the integration of these sounds and stimuli with other sensory modalities to form holistic auditory percepts (Geissler and Ehret, 2004; Harkrider et al., 2005; Hipp et al., 2011; Hudspeth, 1997).

Sounds don't exist in isolation - there is a massive amount of stimuli in our environment surrounding a wanted auditory target, and our brain faces the daunting task of integrating and pruning those stimuli into behaviorally relevant meaning. The difficulty of this task is only increased when introducing localization of sound, temporal scales, and filtering out unwanted information.

One way that the auditory brain accomplishes this task is through the descending projections of the auditory cortex in modulating lower auditory structures, such as the IC, TRN, and MGB (Lee, 2015; Llano and Sherman, 2008).
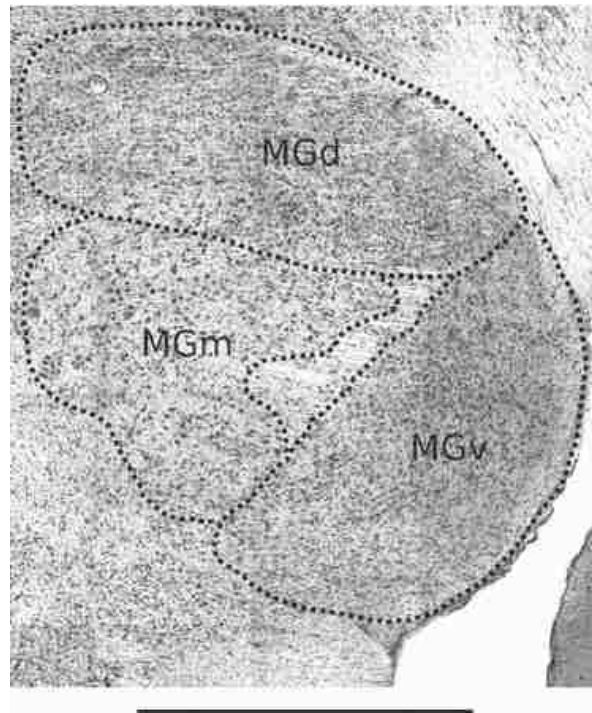
Figure 1. Coronal section of the medial geniculate body depicting the distinct cytoarchitectonic subdivisions; MGd, the dorsal nucleus; MGv, the ventral nucleus; MGm, the medial nucleus. The MGv is characterized by neurons that are oriented in sheets parallel to the tonotopic axis. The MGd has larger unoriented cells, while the MGm contains many large neurons that project broadly across the auditory cortex. Coronal section from a fox. Scale bar: 2 mm. (Nadjzion et al., 2011). Used under the terms of John Wiley and Sons, License #4081481200894.

The auditory cortex (AC) sends descending projections from its fifth and sixth layers to the MGB via the corticothalamic projection system. The corticothalamic projection of the AC is one of the greatest found throughout the entire brain, in terms of relative contribution to several systems. Other corticothalamic projections, such as the ones found in the visual and somatosensory systems, seem to perform similarly (Briggs and Usrey, 2008), allowing the cortex to communicate

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with the thalamus continuously. Most of those projections are glutamatergic, resulting in an excitatory response, mainly ending in the ventral region of the MGB (MGBv), for the layer VI projection (Llano and Sherman, 2008; Winer et al., 2001). One function of the corticothalamic system may be rooted in modulation, involving different terminals depending on cortical layer of origin (Llano and Sherman, 2008).

The thalamic reticular nucleus is a structure in the ventral thalamus, and is one of the only thalamic nuclei not to project axons to the cerebral cortex (Figure 2) (Crabtree, 1998). However, as stated, the AC provides indirect inhibition via the TRN; it sends projections to the auditory region of the TRN, which then provides inhibitory output to the MGB.
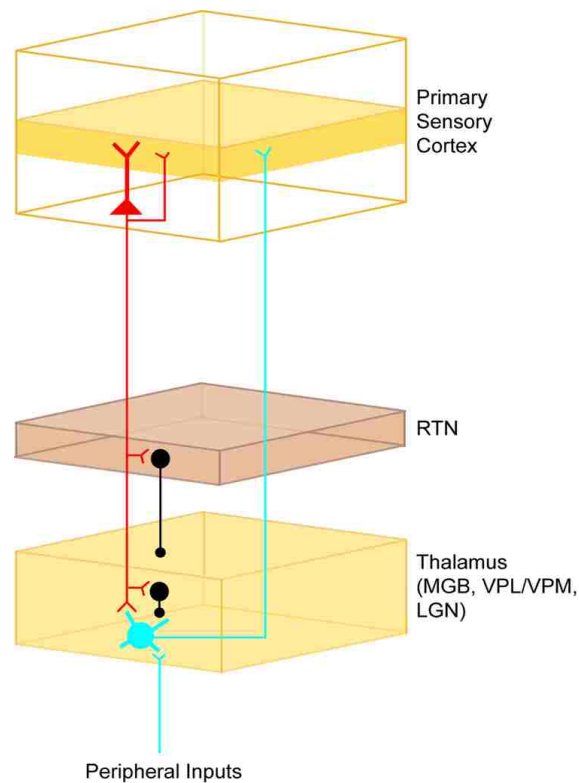
Figure 2. Schematic diagram of circuitry between the thalamus and cortex. Thalamocortical neurons (blue) receive peripheral inputs and project axons to layer 4 of primary sensory cortex. Corticothalamic neurons (red), local thalamic interneurons (black). receive local input from thalamocortical recipient layer 4 and provide output to layer 4 and to the thalamus (Briggs and Usrey, 2008). Used under the terms of the Creative Commons Attribution License (CC BY).

The TRN is the main source of inhibition to many thalamic structures, including the MGB (Lam and Sherman, 2005; Sherman and Guillery, 2006). The corticocollicular system projections arise from the AC ending in the IC, originating mainly from layers V and VI (Figure 3) (Llano and Sherman, 2009; Llano et al., 2014).
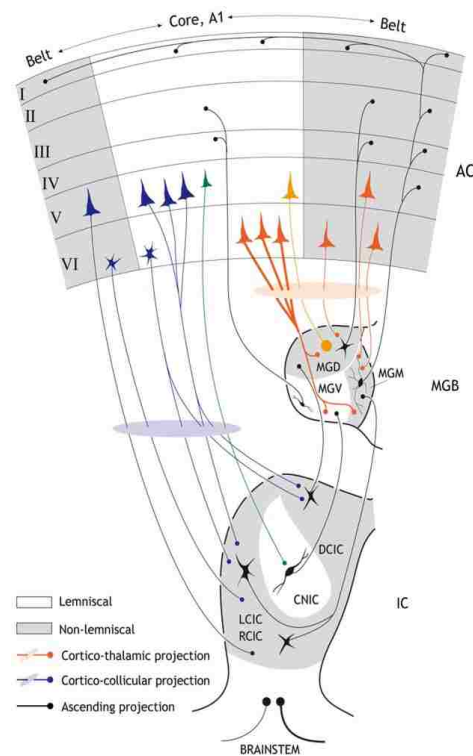
Figure 3. Schematic diagram showing the major anatomical subdivisions of the IC, MGB and AC that illustrates the pathways from the midbrain up to the cortex and back. A1, primary auditory cortex; AC, auditory cortex; CNIC, central nucleus of the inferior colliculus; DCIC, dorsal cortex of the inferior colliculus; LCIC, RCIC; lateral and rostral cortex of the inferior colliculus; MGD; dorsal division of the medial geniculate body; MGM; medial division of the medial geniculate body; MGV; ventral division of the medial geniculate body. (Malmierca et al., 2015). Used under the terms of the Creative Commons Attribution License (CC BY).

Almost all regions of the AC project their axons to the ipsilateral IC, mainly the dorsal and lateral nuclei (Winer et al., 1998). Layer V and VI neurons have been described as mostly pyramidal (Llano and Sherman, 2009). The majority of layer V neurons are large pyramidal neurons that project to the ipsilateral IC, while layer VI neurons are smaller and tend to terminate

their projections in the central nucleus of the IC (Winer et al., 1998). These projections are glutamatergic as well, implying a role of direct excitatory modulation to the IC (Llano et al., 2014).

Corticocollicular projections can induce depression of sounds and activity, suggesting some type of an inhibitory relationship in this circuitry, which would conflict with the direct glutamatergic projections from the AC to the IC, but may be a function of indirect inhibition via local IC interneurons (Saldaña and Marchán, 2005). These findings have been shown in a variety of species, from cat (Winer and Larue, 1996) to gerbil (Cant and Benson, 2006) to bat (Winer et al., 1992). One theory claimed that the target cells of the AC axons may be inhibitory, but a previous study ruled that just 4% of these target neurons were GABAergic.

To truly understand the general integration of these auditory inputs and outputs, characterizations of each of these projection systems are required. Previous studies have attempted to characterize the inhibitory and excitatory projections from the IC to the MGB in a fairly wide range of species (Mellott et al., 2014a; Peruzzi et al., 1997; Winer et al., 1996). GABAergic tectothalamic neurons can be detected throughout the IC, and it was shown that IC GABAergic cells contribute approximately 40% of the tectothalamic pathway in the rat, but just 20% of the tectothalamic pathway in cats (Peruzzi et al., 1997; Winer et al., 1996). The stark difference is particularly unusual, given that 20-25% of both species' IC cells are GABAergic. This could be due to the differences in frequency ranges between the cat and rat (Cat: 45 Hz to 64 kHz; Rat: 200 Hz to 76 kHz), but nonetheless stimulates the idea that rats have a disproportionately high amount of GABAergic cells involved in the tectothalamic pathway compared to cats (Winer and Larue, 1996). Rats may have a higher concentration of GABAergic cells compared to cats due to their almost complete lack of MGB interneurons (<1%) (Winer, 1992). Cats have been shown to display

25% of total MGB neurons as interneurons, which may explain the lower percentage of tectothalamic GABAergic neurons (22%) (Winer and Larue, 1996).

There may also be a difference in how the excitatory and inhibitory inputs are integrated within the MGB, depending on MGB subdivision. Focusing on the IC's output to the MGB, in guinea pigs, non-GABAergic cells from the IC were the most numerous cells involved in the pathway; GABAergic cells supplied various levels of intensity, specifically 22% of the projections, ending in distinct subdivisions of the MGB (Mellott et al., 2014b). These findings also hamper previous studies' hypotheses about the basis behind interneuron count. The guinea pig has 22% of its tectothalamic cells as GABAergic, similar to the cat's, yet a very low interneuron count. (Mellott et al., 2014b). This substantiates the idea that a high GABAergic cell count is not simply making up for a low interneuron count. If this were not so, then the guinea pig should have a far higher GABAergic tectothalamic count than shown. The variability of the amount of GABAergic cells in these three species makes it difficult to translate and generalize to overall auditory processing (Mellott et al., 2014b; Peruzzi et al., 1997; Winer et al., 1996).

Studies of the contralateral IC projections to the MGB also receive far less attention than ipsilateral studies (Mellott et al., 2014a; Wenstrup, 2005). Much work has been covered on the ipsilateral IC and MGB's projections, while the contralateral structures only contribute a small amount in comparison (Mellott et al., 2014a). A thorough characterization of excitatory and inhibitory projections to MGB from bilateral structures would be informative and beneficial. As stated earlier, there has been a large number of studies completed in other species, focusing on the corticothalamic and corticocollicular pathways of the auditory system (Llano and Sherman, 2008; Winer et al., 1998; Winer, 2006). Differences have been shown in these studies, sometimes to a great degree, and because of this, an inclusion of other species is worthy of study. There also exists

a substantial lack of characterization of the TRN's involvement in auditory thalamic inhibition, particularly as it pertains to excitation and inhibition via the AC (Crabtree, 1998; Crabtree et al., 1998). These projections have been examined individually in various species, but there has not been an overarching amount of research done to quantify these convergent projections to the MGB.

In this thesis, I used quantitative analyses of convergent projections using retrograde tracing and transgenic immunofluorescence of inhibitory neurons. I examined the relative contribution of excitatory and inhibitory projections from the IC, TRN and AC to the MGB via mapping of their respective convergent projections. These analyses, in addition to past studies of these regions, proposes the idea that there lies a meaningful and compelling role for these structures in the regulation and modulation of auditory processing.

Audition is an extremely complicated and intricate process, involving many brain structures, regions, mechanisms, and pathways, ultimately resulting in what is perceived as a simple and quick deciphering of the auditory environment. The medial geniculate body in particular is a substantial contributor to this process - a thalamic station that acts as a key mediator in the integration of these auditory signals. Many axonal projections converge in the MGB, which integrates these excitatory and inhibitory inputs. The neuroanatomical mapping of the GABAergic and glutamatergic projections throughout this area will only serve to advance an understanding of these pathways as it relates to each structure. This would prove invaluable in the eventual goal to reduce and perhaps ultimately reverse age-related hearing loss in the future.

CHAPTER 2
LITERATURE REVIEW

The layout, divisions, and connections between the medial geniculate body (MGB) and other thalamic structures have been investigated for decades, if not centuries (Winer, 1992). Focus on the MGB alone began in simpler terms - as far back as the early 1900s, where confirmed sections or subdivisions of the structure were denoted. The number of defined subdivisions in the MGB has evolved over time, with the earliest noting between two (Rioch 1929), a lateral pars principalis and medial pars magnocellularis, and three (Cajal 1911), with many nuclei to be described later (Morest 1964). D. Kent Morest was and is still considered the "father of modern neuroanatomy of the auditory system" (Winer, 1992). His work focused on the neuroanatomy of the thalamic areas and the then novel usage of the Golgi histological stain, to identify the morphology of the cells in the MGB (Figure 4).
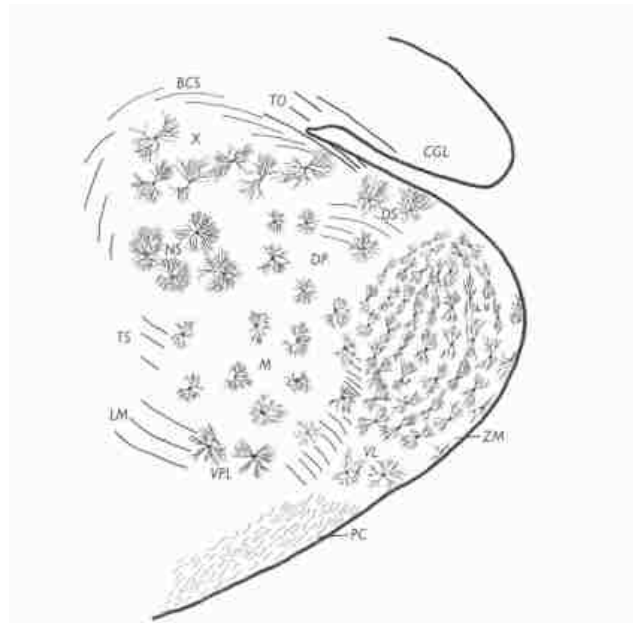

Figure 4. Typical distribution of principal neuronal morphologies at the junction of the anterior and middle thirds of the medial geniculate body. Transverse section, Golgi–Cox. 15-day-old cat. (Morest, 1964). Used under the terms of John Wiley and Sons, License # 4081711367257.

The subdivisions identified by these morphological criteria were furthered by studies of the connections of subthalamic nuclei to the MGB ending in different regions (Morest 1964), each correlating with various functions in the auditory pathway (Morest 1965). These early studies were the first to suggest that auditory, somatosensory, and visual systems all had distinct processing roles within different subdivision of the MGB, further detailing its uniqueness and internal variability (Morest, 1965). Due to these studies, the MGB had been well described in terms of cell morphology and basic connectivity with subcortical structures, but the complexity of the MGB, particularly its diverse functions and connections with other auditory structures of the brain, were yet to be unveiled.

Broadly, one can consider the MGB as being composed of three main subdivisions, the ventral (MGBv), dorsal (MGBd), and the medial subdivision (MGBm), although further parcellation of these regions exist in some species (Winer, 1992). The dorsal subdivision of the MGB (MGBd) is mostly auditory with higher order auditory duties, while the ventral subdivision of the MGB (MGBv) is entirely an auditory nucleus. The medial subdivision (MGBm) organization is multimodal (Cajal 1911). Within the MGBv, neurons are oriented to form a laminar structure (Morest 1965) - that is, neurons form different layers or sheets within the structure, much like the layering of an onion – which was first hinted by Cajal (1955). Lamination had been shown in other thalamic structures involved in audition before; the inferior colliculus (IC) (Katsuki et al. 1958, Morest 1964b) displayed signs of lamination, and in other auditory structures as well (Wenstrup, 2005), strengthening the claim that laminated regions were a staple of the auditory system. The 1965 study by Morest went further, implying that the laminar structure of the MGBv was almost certainly due to a topographic auditory organization, perhaps "frequency discrimination." Among the nuclei, only the MGBv exhibits such a laminated organization, while

the MGBm and MGBd are nonlaminated (Winer 1984). Jeffrey Winer, who described much of the architectonic organization of the MGB, was one of the leading figures in the development of auditory neuroscience and one of the protégés of Kent Morrest, and his research provided a "major influence on our conceptions of the neuroanatomical organization of the auditory forebrain and its sources of input" (Schreiner and Cant, 2011).

Regarding the topography of the MGB, there are many findings that supported the claim that the MGB contained an orderly representation of frequencies, i.e. a tonotopic organization. Lesioning different areas of the MGB resulted in specific losses to frequency discrimination abilities in cats (Ades et al. 1939), and a topographic degeneration of MGB connections to regions of the auditory cortex (Woollard and Harpman, 1939). More work would be done focusing on the MGBd and MGBm, two subdivisions that were mostly ignored or forgotten in comparison to the MGBv (Lee, 2015). The MGBm contains different types of neurons, varying in size and shape and has different sensory functions, with connections to multiple areas of the brain (Erulkar 1975). The MGBm was eventually further characterized, revealing a striking difference in neuronal types, compared to the MGBv and MGBd. The MGBm was found to receive connections from the extraleminscal parts of the IC and auditory cortex (Winer and Morest 1983) and primarily sends divergent axonal projections to several areas of the auditory cortex, terminating in upper cortical layers (Winer and Morest 1983). The MGBd sends connections to non-primary auditory cortical areas, while the MGBv almost solely sends output to the primary auditory cortical regions (Winer et al. 1977, Morest 1965). Interestingly, no interconnections exist between the MGB subdivisions themselves, with some interactions possible through the thalamic reticular nucleus, but limited overall (Morest 1985). These findings began the idea of a parallel pathway system, with each subdivision organized to send projections to distinct targets, not between themselves.

Much of the work until the late 1980s was performed using the cat as a model system, although comparative efforts started examining the neuronal structure of these regions using other model systems, such as the opossum (Morest and Winer 1986), and demonstrated that homologous structures were relatively unchanged between them. Some early work had been completed on the anatomy of the opossum MGB, resulting in a cursory layout of the neuronal architecture with respect to subdivisions (Chu 1932), but very little had been done in revealing the homology between the models. Focusing on the cat and opossum models, early signs showed that there were similarities in the organization of the MGB among these structures, but Morest and Winer (1986) confirmed many of the homologies that had been suggested. All of these subregions are directly relatable to the organization of the human MGB (Winer 1984; Van Buren and Borke, 1972). Neurons of the human MGB also function in both hearing and other sensory modalities depending on their separate subdivisions, with the MGBv almost entirely auditory. One interesting finding in humans is a direct pathway between the cochlear nucleus and the MGB, a pathway not realized in cats. Winer (1984) proposed that this was a potential route for quick networking between subthalamic nuclei and the MGB, a trait that cats may not necessarily require. These discoveries strengthened the idea that there was no general, common pattern of thalamic organization for all mammals, and that characterization of different species was needed and valuable.

Clearer roles for the MGB's subdivisions began to be observed. Using a rat model, an all-inclusive architecture of the MGB was done, and with it, subdivisions and the types of neurons within them were marked (Winer et al., 1999). The starkest difference between the MGB's subdivisions lies in the ventral and medial regions, the MGBv being almost entirely auditory (Aitkin and Webster, 1973), while the MGBm is polysensory (Wepsic, 1966). In addition to having dissimilar sensory functions, there is also a difference in GABAegic neuronal proportions between

different species' MGBs (Winer and Larue, 1996). If different species' MGBs exhibited a varied balance of GABAergic neurons, the proportion with respect to subdivision needed closer examination as well.

The majority of studies of this time further characterized the tonotopic and functional organization of the MGB, examining at length the connections of the MGB, particularly with the auditory cortex. The laminar origins of auditory cortical projections were extensively characterized during this time; layer IV of the auditory cortex sending projections via the ipsilateral corticocortical pathways (Winguth and Winer, 1986), layer III neurons being found to also receive thalamic inputs (Gilbert and Kelly, 1975), layer V neurons having axonal connections to the inferior colliculus was discovered using retrograde transport (Beyerl, 1978). In addition to the projection neurons, which are glutamatergic, increasing attention was paid to gamma-aminobutyric acid (GABA)-ergic neurons. By this point, GABA was known to be one of the primary neurotransmitters in the brain, found in a number of auditory regions (Winer and Larue, 1988), ranging from subthalamic nuclei such as the cochlear nucleus, all the way to the auditory cortical areas (Winer and Larue, 1989). However, the role of GABAergic neurons' within the MGB was not entirely known.

Using a mustached bat model, GABAergic neurons were found in the dorsal and ventral subdivisions of the MGB, with few in the medial subdivision (Winer et al., 1992). In addition, GABAergic neurons were only a small part of all of the subdivisions; just 1-2% of the cells in the MGBd and MGBv were GABAergic, with the MGBm having an extremely small amount. But why did the MGBm of the mustached bat have so few GABAergic neurons compared to the other two regions? Why did each subdivision have such a small proportion of GABAergic neurons? Winer et al. (1992) looked in the direction of the thalamic reticular nucleus (TRN) as a potential

source of much of the GABAergic input to the MGB, similar to the study of Rouiller (1985); but took it one step further and made the claim that multiple regions were provided input as well.

The conventional view is that most mammals have a highly conserved auditory system, with the type of neurons involved and their circuitry essentially unchanged throughout many brains regions, regardless of physical differences between the neurons. This brings about the assumption that the true species differences between neurological systems have more to do with neuronal size and shape, than their actual features. The MGB counters this manner of thinking, having a "species-specific arrangement," (Winer and Larue, 1996), meaning that there lies a system of physiological and functional differences of the MGB, depending on species. The MGB exhibits a wide range of variation in the MGB with respect to its GABAergic neuronal composition - bats and rats displaying <1% of GABAergic neurons, while cats and monkeys exhibiting 25% or more. Interestingly, the MGB and somatosensory thalamus (VB) exhibit this trait, while the visual thalamus (LGN) does not (Sherman and Guillery, 2006). Moreover, the IC and other structures retained similar GABAergic neuronal counts across species (Winer and Larue, 1996). This being the case, it raises questions about the MGB's role in inhibition depending on species, particularly the GABAergic neurons. Could TRN neurons play a major role in species with a low GABAergic neuronal count?

The neuronal topography of the inferior colliculus (IC) was also increasingly investigated during this time. GABAergic neurons had been described in the IC (Roberts and Ribak, 1987), with different GABAergic cell types present in the IC as well. Yet, there had been no true quantification of the amount of the different types of neurons. Oliver et al. (1994) found that 20% of the central complex of the IC (ICC) cells were GABAergic, and furthermore, like the MGB, displayed different types of neurons with respect to morphology (Oliver et al., 1994). On a quest

to discover the physiological function behind nearly every IC neuron producing local axon collaterals, Oliver et al. (1994) hypothesized that IC neurons could be heavily dependent on local circuitry. Could a "feedforward excitation/inhibition" mechanism arise out of this local circuitry of neurons? Their study implied that GABAergic neurons might potentially play two roles in the IC - projecting axons to other regions of the brain, and functioning as local circuit neurons, connecting to other neurons within the IC. But where were these synapses originating? It had been known that the dorsal nucleus of the lateral lemniscus (DNLL) projected to the IC (Roberts and Ribak, 1987), but notably, Oliver et al. (1994) expanded that finding to multiple areas outside of the IC.

More studies emerged examining the IC's output to the MGB and other areas of the central auditory system using anterograde tracers in the mustached bat. As stated earlier, the concept of hearing and perception involves a wide array of mechanisms, ranging from localization, pitch, frequency, intensity, and familiarity, among many others. For the auditory system to accomplish this, the IC is needed to act as a nexus for this input, and for its eventual distribution to other thalamic regions. The IC integrates input from lower brainstem nuclei, but what of its output? For the first time, an inhibitory tectothalamic pathway of GABAergic cells from the IC to the MGB was described; the pathway represents 10-30% of the neurons involved in the central auditory system (Peruzzi et al., 1997; Winer et al., 1996). Before this, it was thought that input projections to the MGB were completely and solely excitatory. Showing a tectothalamic pathway, arising from the IC to the MGB, using both excitatory and inhibitory projections raises many questions about the role of GABAergic input within the auditory system. These findings suggest a "convergence" of excitatory and inhibitory neurons to the MGB, further underscoring its complexity. This changed the idea of how information is refined in the thalamus. Ideas of converging pathways of

feed-forward inhibition began to be considered. The inhibitory GABAergic tectothalamic pathway provided an additional avenue for inhibition of the MGB, besides local interneurons and projections from the TRN. With at least three sources of possible inhibition in the MGB, the consideration of the relative influence of each was considered. Moreover, how these multiple inhibitory influences interact with the convergent ascending and descending excitatory inputs has not yet been fully investigated.

Learning the proportions and types of GABAergic neurons within the MGB was important for many reasons. Most notably, the subdivisions provide different sets of output to cortical areas (Calford and Aitkin, 1983) and inputs as well (Diamond et al., 1969). Possible differences between subdivisions' GABAergic makeup could divulge differences in how information is processed as it ascends to the auditory cortex. In the rat, it was found that ~26% of neurons found in the MGB were GABAergic (Huang et al., 1999), giving the MGB a potential title as possessing the highest count of GABAergic neuronal count in the auditory system (IC: 20% (Oliver et al., 1994), AC: 25% (Prieto et al., 1994)). The MGBv was found to contain an average of 33% of those cells, the MGBd and MGBm containing 26% and 18%, respectively (Huang et al., 1999). As the variation within the MGB becomes wider, and GABAergic contribution deviates from significant to almost nonexistent, the potential for morphological differences in subdivision function increases, and the larger role of interneurons becomes apparent.

Subdivisions of thalamic structures create pathways that are specific to one another, projecting from the IC to the MGB, and ultimately from the MGB to the auditory cortex. These pathways are involved in an assortment of functions, ranging from frequency organization to integration. The tectothalamic pathway from the IC to the MGB varies among species as well. In rats, 40% of the tectothalamic pathway's cells are GABAergic, but just 20% of the cells in cats are

(Peruzzi et al., 1997). The deviation between these two species becomes more compelling, given that both species' IC total GABAergic count is roughly the same, 20-25%.

Why does the rat's tectothalamic pathway involve far more GABAergic neurons in proportion to the IC's total count, than the cat's? Interneurons may be the key; rats feature a higher percentage of its GABAergic neurons due to their lack of interneurons in the IC, at <1% (Winer and Larue, 1988). Cats, on the other hand, have a far higher interneuron count at 25% (Huang et al., 1999). One theory that could account for this is that interneurons were an integral component of the tectothalamic pathway, and species lacking interneurons compensate by simply increasing the involvement of GABAergic neurons form other sources. To strengthen this theory, other species have been examined (Mellott et al., 2014). Characterizing the guinea pig, it was discovered that 22% of the ipsilateral IC's cells involved in the tectothalamic pathway were GABAergic (Mellott et al., 2014). Contrary to the interneuron theory, guinea pigs were more similar to cats than rats; the guinea pig MGB contains very few GABAergic cells, but does not have an inflated amount of GABAergic cells involved in that pathway. High GABAergic count was found to not be merely compensating for low interneuron count.

The concept of these thalamic structures participating in an array of grouped connections began to surface. Perceiving, detecting, and ultimately integrating sound requires a joint effort from multiple regions and structures. The thalamic reticular nucleus (TRN) is one of those structures, designed to act as a relay station between the auditory cortex (AC) and other thalamic regions, such as the MGB (Lam and Sherman, 2005). It is a relatively small region of the thalamus, and is involved in a number of different sensory characteristics, not committed solely to hearing. (Sherman and Guillery, 2006). The AC, MGB and TRN are all intertwined, as thalamocortical and corticothalamic neurons branch to innervate TRN targets. The inferior colliculus' (IC) connection

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with the MGB is an evident component of this converging and diverging projection system; the IC's inputs to the MGB are organized by subdivision and terminate in different areas of the MGB. The MGB also sends projections to the AC, terminating in a number of different layers of the cortex. The first projection mostly terminates in layer IV of the AC, while the second does so in layers II and III (Huang and Winer, 2000). In terms of output, the MGB receives axonal projections from layers V and VI of the AC (Winer et al., 1999). All of these pathways result in a notable contribution to the auditory system's overall function of integrating and extracting meaning out of sound. The challenge remaining is to integrate all of these projection systems into a coherent framework that describes auditory neural operations. This thesis extends these past studies by examining the convergence of excitatory and inhibitory inputs to the MGB from three sources in the mouse: the inferior colliculus, the thalamic reticular nucleus and the auditory cortex.

CHAPTER 3
INVESTIGATING CONVERGENT PROJECTIONS TO THE MGB

3.1 Introduction

The medial geniculate body (MGB) is a prominent region of the central auditory system, acting as a critical neural hub for ascending and descending axonal projections (Winer and Larue, 1996; Winer et al., 1996). It is part of the thalamus, and as such, is a gateway for flowing most sensory input on its way to the cerebral cortex (Winer et al., 1996). The MGB contains three subdivisions (dorsal, ventral, and medial), composed of different neuronal cell types, including excitatory and inhibitory neurons in most species (Winer and Wenstrup, 1994). The MGB has been studied extensively over a century (Cajal 1911) in terms of its anatomy (Morest 1964), tonotopic organization (Winer 1984), neuronal makeup, and connections to other auditory regions of the midbrain (Winer and Larue, 1996). However, a unified framework of the converging projections to the MGB from midbrain, thalamic and cortical regions, particularly in the mouse model, have not been extensively examined (Winer 2005, Smith et al. 2012).

The goal of this project is to examine the contribution of excitatory and inhibitory projections to the MGB, with a goal of establishing a comprehensive neuroanatomical map marking these projections from its main input sources, i.e. the ipsilateral/contralateral inferior colliculus (IC), thalamic reticular nucleus (TRN), and the auditory cortex (AC). Here we used a VGAT-Venus transgenic mouse model which enabled the rapid and unambiguous identification of inhibitory neurons in these structures (Wang et al., 2009). We employed retrograde tract-tracing to identify the various connections from the aforementioned input regions that ultimately terminate in the MGB. An additional objective for this project is to assess each projection's proportional input to the MGB, quantitatively marking the intensity of each projection based on retrograde-

labeled neuronal count, and to also confirm the type of projection, excitatory and inhibitory, involved in each pathway.

3.2 Injection of Cholera Toxin Beta subunit (CT$_\beta$) Conjugate

The following procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Louisiana State University School of Veterinary Medicine. Vesicular GABA transporter (VGAT)–Venus transgenic mice were anesthetized with a ketamine/xylazine mixture (0.1 ml / 20 g). The reasons for choosing this strain of mouse are strong; the VGAT-Venus mouse natively contains the vesicular GABA transporter (VGAT), which expresses the Venus protein, an autofluorescent protein. Being naturally fluorescent, it eliminates the need for less efficient or effective antibody staining and allows for high detection of inhibitory neuronal populations. The head was shaved and cleaned with hydrogen peroxide prior to injection, then placed in a stereotaxic head-holder frame. Body temperature was maintained with a heating pad. Sterile instruments and aseptic techniques were used throughout the surgery.

An incision in the scalp and a drilled opening in the skull were made above the stereotactic target coordinates dorsal to the medial geniculate body (MGB). Once the opening to the brain was made, a Hamilton microsyringe with a sterile needle was lowered to the medial geniculate body (MGB) and used to pressure inject Cholera Toxin Subunit B (recombinant) Alexa Fluor® 594 Conjugate (Thermofisher Scientific, Waltham, MA), a fluorescent tracer dye, according to the manufacturer's recommendations for retrograde tracing volume. After the tracer dye was deposited and equilibrated, the animal's scalp was then sutured, and the head removed from the stereotaxic framework. The animal was observed and monitored post-surgery until fully recovered.

3.3 Post-Surgical Processing of VGAT-Venus Mouse Brain

The animals were then housed for three to five days for the tracer to transport throughout the brain optimally, and were monitored for health during this period. Following the transport time, the animal was then deeply anesthetized with isoflurane and sacrificed via transcardial perfusion, using 5 mL of 10 mM phosphate-buffered saline (PBS) solution, followed by 6 ml of 4% paraformaldehyde (PFA) in 10 mM PBS fixative. The brain was then removed and postfixed in 4% PFA / in 10 mM PBS at 4° C for 24 hours, after which it was stored in a 4% PFA with 30% sucrose solution in 10 mM PBS for 24 hours for cryoprotection at 4° C. Subsequently, the brain was then prepared for cryosectioning; the brain was blocked with a 35-degree cut on the dorsal surface and sliced to preserve the IC, MGB and AC (Lee and Sherman, 2009). Using a cryostat, each brain was frozen and sectioned at 50 μM, then sections were collected in 48-well plates containing 10 mM PBS.

3.4 Fluorescence Imaging of Brain Tissue Samples

A series of brain tissue sections were mounted and coverslipped on slides using Vectashield anti-fade mounting medium with DAPI (Vector Labs, Burlingame, CA). Once the slides had dried, they were then scanned using a Nanozoomer digital slide scanner (Hamamatsu, Naka-ku, Hamamatsu) and a FluoView microscope for confocal microscopy (Olympus, Center Valley, PA), in preparation for the counting of retrogradely-labeled and Venus-expressing cells. Tissue sections that exhibited labeled tracer were counted for quantitative analysis. Double-labeled (Venus+tracer) and single-labeled (tracer alone) cells were counted and plotted using a marker, from the ipsilateral auditory cortex (AC) and thalamic reticular nuclei (TRN), and both ipsilateral and contralateral inferior colliculi (IC). The cells were identified based on labeled structures having the general size

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and characteristics of cells in those areas. The cells were counted manually from processed images produced by the FluoView confocal microscope and a Nanozoomer digital slide scanner. An automatic counter analysis was also done via ImageJ for further confirmation of the results. Completing this process, the results of this analysis was used for an overall summary of the tracer's distribution, labeling, and plotting of cells. Figures showing the distribution of the tracer throughout the aforementioned auditory regions were taken using NDP.view2 (Hamamatsu).

CHAPTER 4
ANALYSIS OF CONVERGENT PROJECTIONS TO THE MGB

Retrograde tract tracing of convergent inputs to the MGB with CTβ Alexa Fluor 594 showed a significant number of single and double-labeled retrograde cells within the regions being observed. Single-labeled cells were identified as VGAT (vesicular GABA transporter)-negative retrograde cells (i.e. red only), ones that did not express the Venus protein. Double-labeled cells were identified as VGAT-positive, while also being identified as retrograde cells via the tracer dye (i.e. red and green). The VGAT-Venus transgenic mice are very beneficial for identifying and tracing GABAergic circuitry; cells expressing the Venus protein with the vesicular GABA transporter, and also labeled with the tracer dye, are confirmed to be GABAergic (Lee at al., 2015). The single-labeled cells are VGAT-negative, and as such, are not GABAergic, but presumed excitatory. The labeling process does not provide absolute proof of glutamatergic populations, and can only contrast GABAergic from non-GABAergic populations.

4.1 Convergent Excitatory and Inhibitory Projections to the MGB

The injection of the tracer dye was made in the medial geniculate body, to ensure correct and accurate retrograde tracing (Figure 5). The mouse received an injection at a volume of 0.2-0.5μl in the area. The injection site displayed deep diffusion of the tracer dye as expected, across much of the lateral sector of the MGB, and labeled many cells locally.

Retrograde labeling in both IC, the AC, and TRN were found as well; to compound on the evidence of single and double-labeled retrograde cells in these areas, high-magnification images of the regions were also produced, the IC being one example (Figure 6).
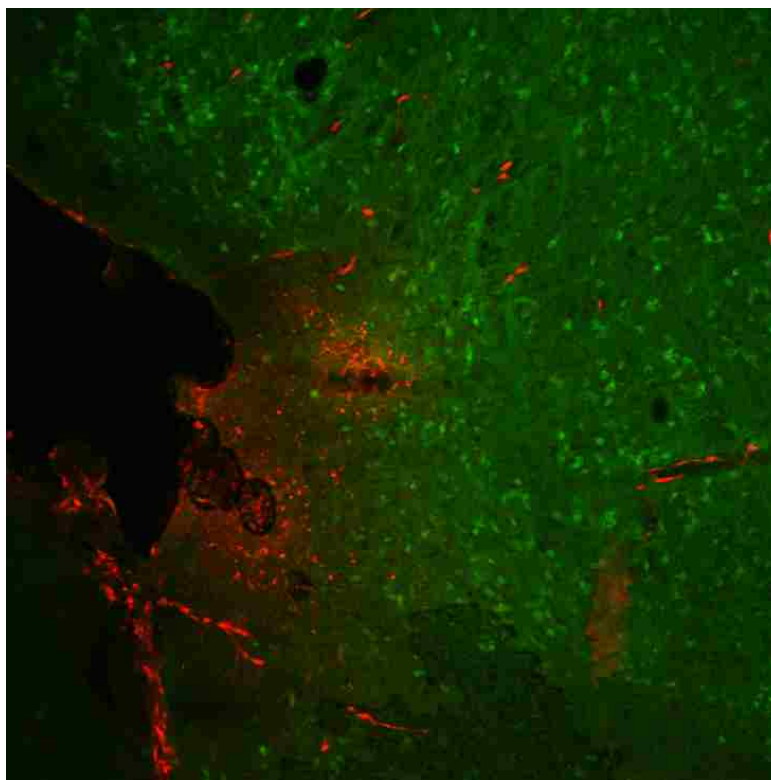
Figure 5. Confocal microcopy imagery of the injection site for the CTβ-Alexa 594 tracer dye (red), located in the medial geniculate body of the VGAT-Venus transgenic mouse.

The ipsilateral inferior colliculus (IC) contained a large amount of single- and double-labeled retrograde cells projecting to the medial geniculate body (MGB); 30.4% of the IC's retrograde cells were double-labeled, proving them to be GABAergic, while the rest were VGAT-negative, resulting in them being defined as excitatory. Being a nexus for ascending and descending projections in the central auditory system, these results confirmed previous studies (Saldaña and Marchán, 2005; Wenstrup, 2005; Buentello et al., 2015; Ito and Oliver, 2010; Oliver, 1984). The ipsilateral IC (Figure 6) displayed less GABAergic (14, 30.4%) and non-GABAergic (32, 69.6%) neurons labeled by the tracer dye, in comparison to the contralateral IC (12, 36.3%; 21, 63.6% respectively). While these results are close enough to make it likely of an inconsequential difference, they further confirm past studies in other species, such as the guinea

pig and the mustached bat (Mellott et al., 1994; Winer, 1992). The distribution of single- and double-labeled retrograde cells in both IC were similar; they displayed a larger count of retrograde cells near the dorsal edges of the region. A large majority of the two types of retrograde cells were also overlapping, as many single-labeled cells were clustered adjacent to the double-labeled cells.
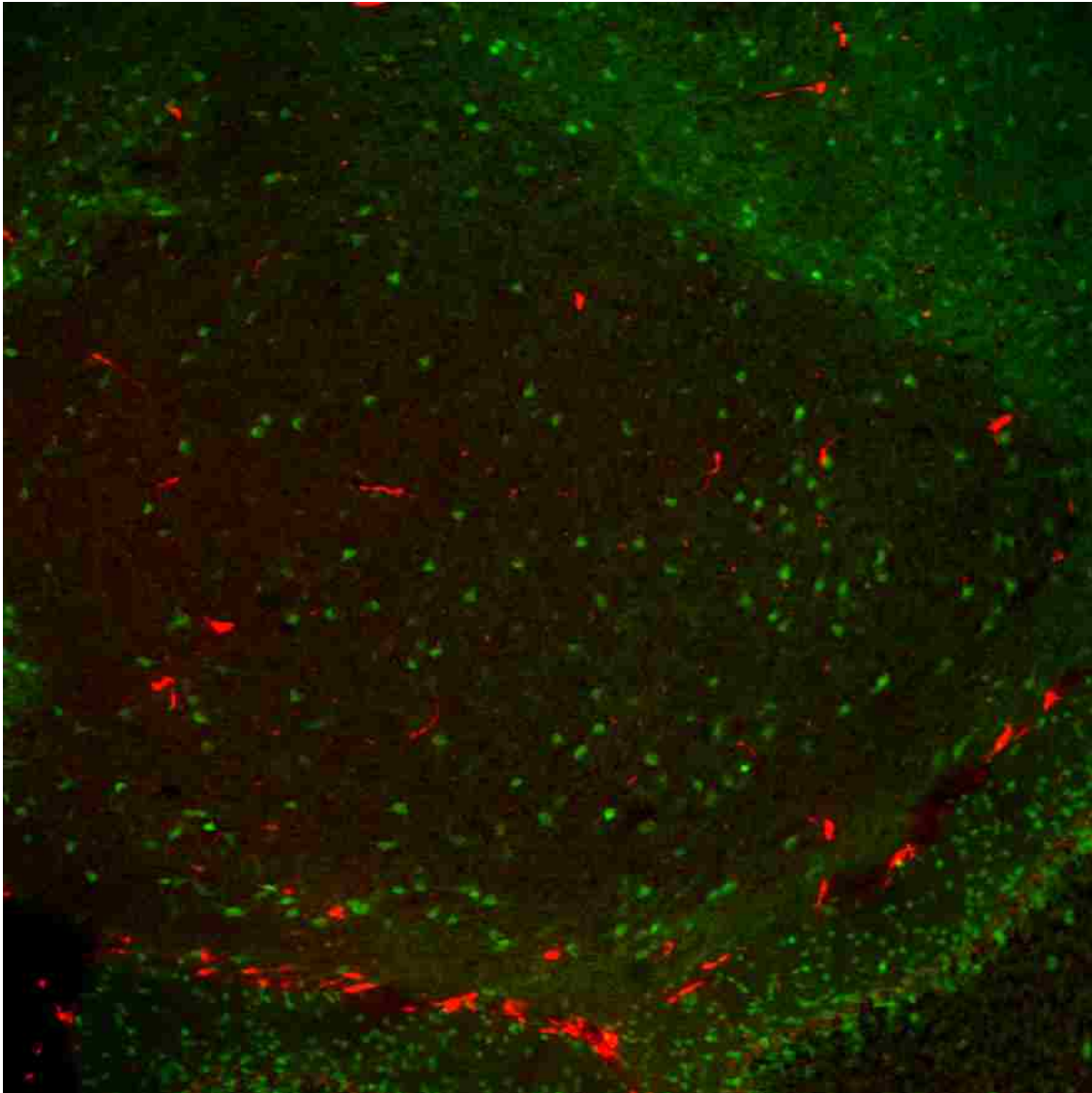

Figure 6. Confocal microscopy imagery of ipsilateral IC. VGAT-negative retrograde cells are labeled red, VGAT-positive retrograde cells are labeled red and green.

The thalamic reticular nucleus (TRN) also exhibited GABAergic neuronal projections to the MGB, as seen by double-labeled retrograde cells; 77.8% of retrograded cells found in the TRN were GABAergic. (Figure 7). As the TRN provides a major source of inhibition to the MGB, these findings demonstrate a noteworthy source of inhibition to the MGB from the TRN. The distribution of the TRN's retrograded cells, single- and double-labeled, were found in the region of the TRN closest to the hippocampus.

Figure 7. Confocal microscopy imagery of the ipsilateral thalamic reticular nucleus. VGAT-negative retrograde cells are labeled red, VGAT-positive retrograde cells are labeled red and green.

The ipsilateral auditory cortex was also scanned and shown to display projections to the

MGB via retrograde cell tracing (Figure 8).
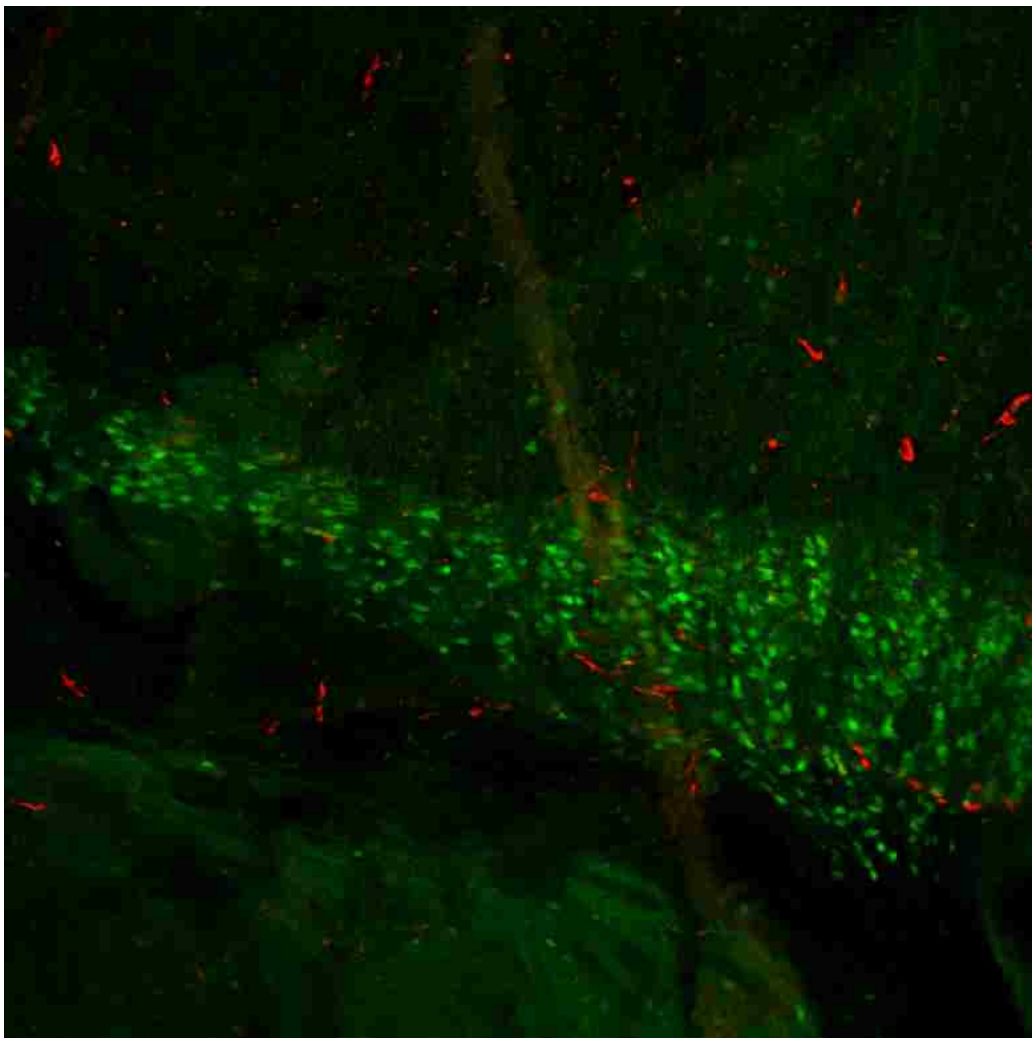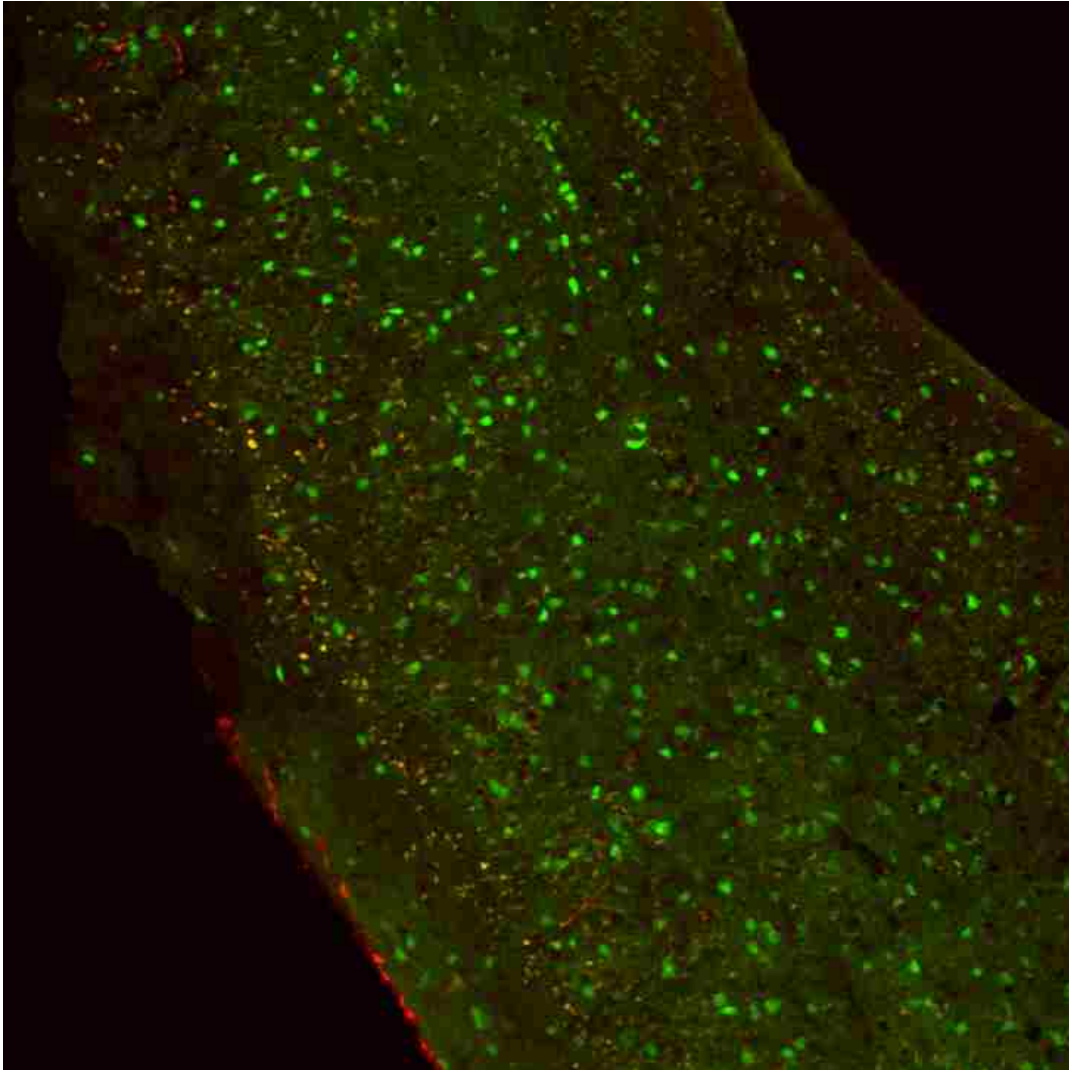

Figure 8. Confocal microscopy imagery of the ipsilateral auditory cortex. VGAT-negative retrograde cells are labeled red, VGAT-positive retrograde cells are labeled red and green.

All of the retrograded cells found in the AC were single-labeled; most of the distribution

of these cells was found in the lower layers of the AC, which could be described as layers V and

VI. These findings strengthen the established idea of the AC providing a substantial excitatory contribution to the MGB.

4.2 Quantitative Analysis of GABAergic and Excitatory Projections

The distribution of single- and double-labeled retrograde cells were counted, from the confocal and Nanozoomer digital microscopy images (Figure 9).
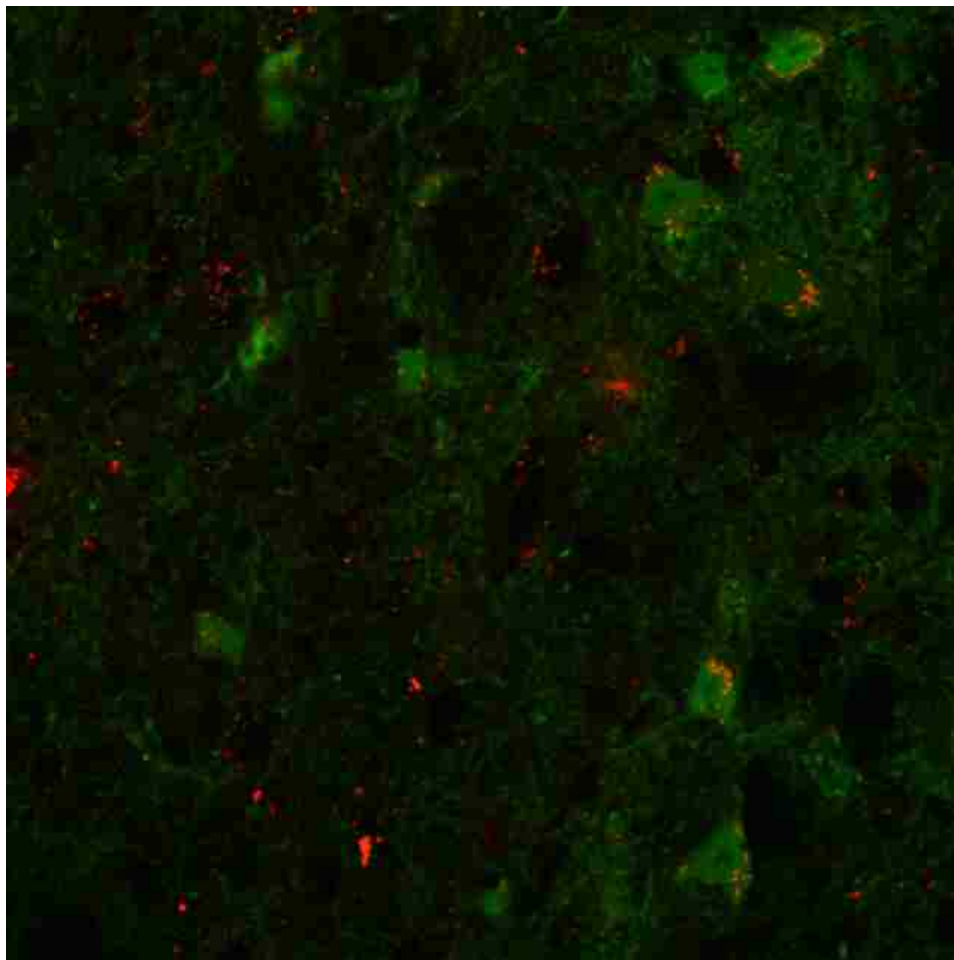

Figure 9. High-magnification confocal microscopy imagery of an example of single- and double-labeled cells in the medial geniculate body. VGAT-negative retrograde cells are labeled red, VGAT-positive retrograde cells are labeled red and green.

Cells were counted several times to ensure precise findings; the cell counts further displayed and characterized what was found via imaging, in that a strong GABAergic projection from the three of the neuronal regions observed (IC, contralateral IC, TRN) to the MGB was found. Manual cell counting was completed using plotter and marker. The TRN was shown to provide just 25% of total inhibitory neuronal count (Table 1).

Table 1. Quantitative analysis of proportional convergence of retrogradely labeled GABAergic and non-GABAergic projections to the MGB in the VGAT-Venus mouse. VGAT+ and VGAT-retrograde cells values are described as percentages of total excitatory and/or inhibitory neurons relative to overall labeling.

	Inferior colliculus (ipsi)	Inferior colliculus (con)	Thalamic reticular nucleus	Auditory cortex
Mean VGAT+ retrograde cells (%)	41.8 ± 0.9	32.9 ± 24.1	25.3 ± 33.6	0 ± 0
Mean VGAT-retrograde cells (%)	32.2 ± 0.1	21.2 ± 0.1	2.3 ± 0.2	44.3 ± 1.0
Mean total retrograde cells (%)	34.5 ± 0.01	24 ± 1.33	7.7 ± 1.7	33.8 ± 0
Type of projection	Excitatory/Inhibitory	Excitatory/Inhibitory	Inhibitory	Excitatory

We found that most of the presumed excitatory inputs arose from the auditory cortex (Table 2, 44% of total excitatory retrograde cells), with the ipsilateral and contralateral IC showing lesser of an excitatory count (32% and 21%, respectively). The number of VGAT-negative retrograde cells exceeded the VGAT-positive retrograde cells in every region besides the TRN, indicating a larger non-GABAergic projection from the majority of the structures than GABAergic. Focusing on GABAergic projections, the region shown to provide the most GABAergic output to the MGB

was the ipsilateral IC, with the contralateral IC as the next highest. The AC, being involved in direct excitation of the MGB, had a large amount of VGAT-negative retrograde cells, indicating a more robust non-GABAergic, excitatory projection to the MGB. Most of the inhibitory inputs labeled were sourced from the inferior colliculi (Table 2, 42% and 33%, respectively).

Table 2. Quantitative analysis of retrograded GABAergic and non-GABAergic projections to the MGB in the VGAT-Venus mouse. VGAT+ and VGAT- retrograde cells values are described as percentages of total excitatory and/or inhibitory neurons in that region.

	Inferior colliculus (ipsi)	Inferior colliculus (con)	Thalamic reticular nucleus	Auditory cortex
Mean VGAT+ retrograde cells (%)	30.4 ± 2.0	36.4 ± 5.0	77.8 ± 1.0	0 ± 0
Mean VGAT- retrograde cells (%)	69.6 ± 2.0	63.6 ± 5.0	22.2 ± 1.0	100.0 ± 0
Type of projection	Excitatory/Inhibitory	Excitatory/Inhibitory	Inhibitory	Excitatory

Table 2 also shows once more, the type of output that the structures project to the MGB. Table 1 compounds on Table 2's finding, showing each region's percentage of labeling in comparison to overall labeling done in all structures. What was counted in the structures matched with the type of projection the regions were previously known to have.

CHAPTER 5
DISCUSSION

In this study, we used retrograde tract tracing with the CTβ Alexa Fluor 594 tracer dye to quantitatively characterize the neuronal pathways arising from the thalamic reticular nucleus (TRN), auditory cortex (AC), and both inferior colliculi (IC), that project to the medial geniculate body (MGB). The AC exhibited a substantial presumed glutamatergic projection to the MGB, while both IC and TRN provided inhibitory GABAergic and/or excitatory glutamatergic projections. Our data, using the VGAT-Venus mouse, reveals a clear convergence of excitatory and inhibitory projections from multiple IC, TRN and cortical regions to the MGB. This tract-tracing strengthens past findings of homologous regions in various species, such as the cat, rat, and gerbil (Winer and Larue, 1996; Mellott et al., 2014b; Cant and Benson, 2006). Our use of the VGAT-Venus transgenic mouse strain is advantageous compared to prior studies, since the native expression of the Venus-fluorescent protein enabled the ready and relatively unambiguous identification of inhibitory neuronal cells, where prior studies were reliant on immunohistochemical detection of these cells.

Prior studies have typically focused on examining projections from one particular region to the MGB, but assessing the convergence of multiple projections quantitatively to the MGB has not been accomplished in the mouse. In addition to the topographical location of these projections, the relative contribution of presumed excitatory and inhibitory inputs was also assessed, based on retrograde cell counting from each region. The intensity of these projections varied from structure to structure. The auditory cortex displayed a high count of VGAT-negative retrograde cells, indicating a powerful and robust glutamatergic projection to the MGB. Next in intensity was the ipsilateral IC, then the contralateral IC, and ending with the TRN. These results underscore the

complex interactions between these structures, and further refine the neuroanatomical organization established by previous studies. (Figure 10)
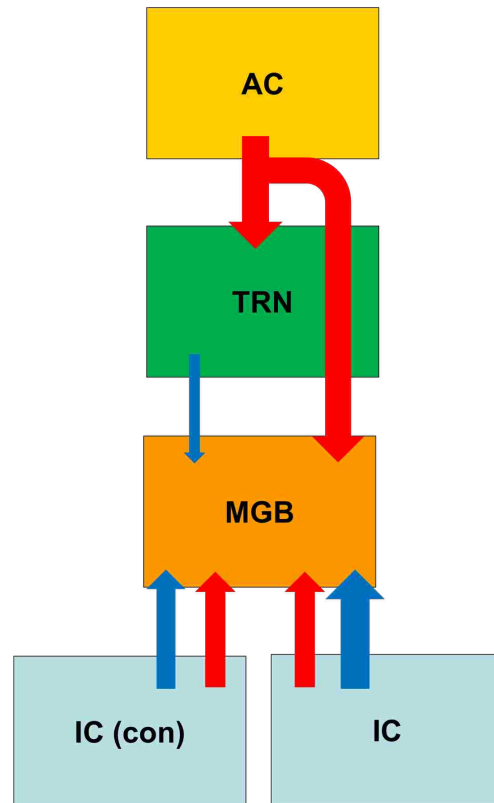

Figure 10. Schematic diagram of excitatory (red) and inhibitory (blue) projections of the auditory cortex (AC), thalamic reticular nucleus (TRN) , medial geniculate body (MGB), and inferior colliculus (IC and IC (con)). Thickness of arrows represents the intensity of the projection.

The analysis of the MGB's importance dates back nearly 60 years, continuing with further discoveries of its anatomical subdivisions (Morest 1964, Aitkin and Webster 1972, Winer and Morest 1983), tonotopic organization (Aitkin 1973, Winer 1984, Winer 1999), function (Morest and Winer 1986), and connections with other thalamic regions (Rouiller et al. 1985, Winer and Larue 1996). Further dissection has been carried out on the auditory cortex's important role in integrating and refining ascending input from the MGB (Winer 2005, Smith et al. 2012) via corticothalamic projections, sending excitatory input to the TRN and MGB (Wenstrup, 2005). The

AC is also charged with the task of indirectly inhibiting the MGB via the TRN. The IC is particularly important in this framework, acting as a nexus for all lower brainstem nuclei, such as the superior olivary complex and cochlear nuclei, and is also the first region where inputs begin to integrate in the central auditory system (Winer and Schreiner, 2005). The connectivity between the IC and MGB cannot be understated; the tectothalamic pathway is the largest output pathway of the IC, and is responsible for sending a substantial amount of GABAergic and glutamatergic inputs to the MGB. Our results augment these prior findings by grouping and quantifying all of these connections in one overall schema, in a species not extensively characterized.

The results found and described in this thesis correlate previous studies in this area of study. As mentioned before, retrograde tracing of central auditory regions has been accomplished in many other models, such as the mustached bat (Winer et al., 1992), gerbil (Cant and Benson, 2006), guinea pig (Mellott et al., 2014), and mouse. These studies have characterized thalamic and cortical regions extensively, and provided a substantial foundation for quantitative analysis of the neuronal architecture within these areas.

A major finding of this thesis consists of a framework of excitatory and inhibitory projections from multiple auditory regions, converging in the MGB, providing a singular look at a unified neuroanatomical map. Of note, the projections from the contralateral IC have been especially interesting, as there has not been a substantial amount of bilateral IC studies involving those structures' connections to the MGB (Mellott et al., 2014a.). Our study shows that the contralateral IC provides a less intense, but apparently inhibitory projection to the contralateral MGB, which may enable further ascending inhibitory control of auditory processing. Overall, extending the mapping of the many pathways and projections of the auditory system should provide a broader framework for interpreting and stimulating further research on this topic.

CHAPTER 6
CONCLUSION

Real-world application of the findings from this thesis may emerge and have the potential to affect the treatment of disorders of the auditory system. The neural processing of sound is an extremely complicated task, and the labyrinthian aspects of that task have taken many years to uncover, all concluding in what is perceived as a quite simple and speedy process (Lee and Winer, 2008). Our auditory system uses excitation and inhibition to stimulate, prune, filter, decipher, and integrate the massive amount of stimuli obtained almost constantly throughout the usual day (Lee and Winer, 2011). This system is further complicated by the daunting task of understanding this integration; in order to effectively interpret the integration of ascending and descending inputs, and excitatory and inhibitory projections to the MGB, a thorough characterization of these projection systems is needed (Mellott et al., 2014a; Peruzzi et al., 1997; Winer et al., 1996).

More than that, an overall characterization of every main structure involved in the contribution of those projections to the MGB is necessary for an increased comprehension of these pathways. Obtaining this knowledge only furthers our insight into the excitation/inhibition balance of hearing; one of many theories states that loss of GABAergic activity may play a role in the onset and persistence of presbycusis (Tang et al., 2014; Gao et al., 2015; Winer, 1992). Further studies of how these neuroanatomical connections change temporally would be very beneficial in testing its role in this disorder and will almost certainly prove to be a welcome addition to the future goal of reducing and alleviating age-related hearing loss.

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