ABSTRACT

Title of Document: CALCIUM BINDING PROTEINS AND GAD IMMUNOREACTIVITY IN THE AUDITORY SYSTEM OF GEKKO GECKO

KAI YAN, Master of Science, 2008

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Geckos use vocalizations for intraspecific communication, but little is known about the organization of their central auditory system. We therefore used immunohistochemical techniques to delineate the auditory nuclei of the hindbrain, midbrain and thalamus. We used antibodies against the calcium binding proteins calretinin (CR), parvalbumin (PV), calbindin-D28k (CB) as well as antibodies against Glutamic Acid Decarboxylase (GAD) and synaptic vesicle protein 2 (SV2). Western blots showed that all five antibodies were specific within gecko brain. The fluorescence double labeling of calcium binding proteins yielded partly overlapping but mostly complementary distributions in the gecko auditory pathway. The differential expression of all calcium binding proteins and GAD, as well as SV2, characterized the structures of the gecko auditory system. The results indicate that the auditory structures and organization in Gekko gecko bear many similarities to the other land vertebrates.

CALCIUM BINDING PRTOTEINS AND GAD IMMUNOREACTIVITY IN THE AUDITORY SYSTEM OF GEKKO GECKO

By

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List of abbreviations

AVCN: Anteroventral cochlear nucleus

CaBP: Calcium binding protein

CB: Calbindin

CR: Calretinin

DVR: Dorsal ventricular ridge

MM: Nucleus medialis

NM: Nucleus magnocellularis

NMM: Nucleus magnocellularis, medial

NML: Nucleus magnocellularis, lateral

NA: Nucleus angularis

NAM: Nucleus angularis, medial

NAL: Nucleus angularis, lateral

NL: Nucleus laminaris

NLL: Nucleus of the lateral lemniscus

dNLL: Nucleus of the lateral lemniscus, dorsal

vNLL: Nucleus of the lateral lemniscus, ventral

PV: Parvalbumin

SO: Superior olivary nuclei

dSO: Superior olivary nuclei, dorsal

vSO: Superior olivary nuclei, ventral

TS: Torus semicircularis

TSc: Central nucleus of Torus semicircularis

TSl: Laminar nucleus of Torus semicircularis

VIII: Eighth nerve (auditory nerve)

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Chapter 1: Introduction

The organization and connections of the central auditory system are well known in mammals and birds (Carr, 1992; Carr and Boudreau, 1991; Carr and Boudreau, 1993; Carr and Code, 2000; Manley et al., 2003; Parks, 1981; Parks and Rubel, 1975; Parks and Rubel, 1978; Rubel and Parks, 1975; Rubel and Parks, 1988; Webster et al., 1992). They share similar projections and structures, although issues of homology remain unclear. Both avian and mammalian ascending auditory pathways are characterized by monaural and binaural projections from the first order nuclei to the superior olivary nuclei (SON) and the nuclei of the lateral lemniscus (NLL) (Grothe et al., 2005). These pathways converge at the auditory midbrain, termed the torus semicircularis in most vertebrates and the inferior colliculus in birds and mammals (Grothe et al., 2005). The auditory midbrain projects to thalamic and then telencephalic targets (Grothe et al., 2005).

Much less is known about the projections of the auditory system in lizards than in birds. In many lizards, four nuclear populations that receive primary auditory inputs have been recognized: a medial and lateral nucleus magnocellularis and a medial and lateral angularis (Miller, 1975; Szpir et al., 1990; Szpir et al., 1995). The ascending auditory pathways of the *Iguana iguana* pathways were described by Foster and Hall (1978). Although some basic anatomical information about *Gekko gecko's* auditory brainstem is already known (Miller, 1975), the auditory structures and their connections in the gecko remain relatively unknown.

Immunohistochemical techniques were used to delineate the auditory nuclei of the hindbrain, midbrain and thalamus in *Gekko gecko*. Antibodies against the calcium

binding proteins calretinin (CR), parvalbumin (PV), calbindin-D28k (CB) and antibodies against Glutamic Acid Decarboxylase (GAD) and synaptic vesicle protein 2 (SV2) were used. Calcium binding proteins have been proposed to be useful markers for specific functional auditory pathways since they labeled neurons in the central auditory system of mammals such as rat (Lohmann and Friauf, 1996) and guinea pig (Caicedo et al., 1996); in birds such as the chicken (Parks et al., 1997) and barn owl (Kubke et al., 1999; Takahashi et al., 1987), and in lizard (Dávila et al., 2000). We also used an antibody against GAD (glutamic acid decarboxylase) to determine the distribution of GABAergic neurons and terminals in the auditory nuclei. GAD was previously used in auditory system of barn owl and chick (Carr et al., 1989; Lachica et al., 1994; von Bartheld et al., 1989). An antibody against synaptic vesicle protein 2 was used to identify synapses.

The goal of the present work was to provide a comprehensive study of the distribution pattern of the above antibodies in the auditory nuclei of *Gekko gecko* to identify morphological and organizational features of the gecko auditory system. The second goal of this study was to compare the distribution of these markers with other vertebrates and to show that the gecko shared many features of its auditory system.

Chapter 2: Materials and Methods

2.1 Tissue preparation

This study was based on 20 adult *Gekko gecko* of both sexes. All animal care and anesthesia procedures followed the procedures approved by the University of Maryland College Park Animal Care And Use Committee. Geckos were anesthetized by isofluorane in a small chamber, followed by i.p. euthasol injection of euthasol at a dose of 7 mg/kg BW. Once the geckos were deeply anesthetized (no response to toe pinch, depressed respiration), they were perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1M PB for one hour. The brains were postfixed in the fixative overnight at 4°C and cryoprotected in 30% sucrose in 0.1M PB at 4°C until it sank. The brains were sectioned on a freezing microtome at 40µm thickness and all the sections were collected in order in PB. Most brains were cut in the coronal plane, while three brains were cut in the horizontal plane.

2.2 HRP immunohistochemistry

Standard immunohistochemical procedures were applied using the avidin-biotin-peroxidase complex (ABC, Vector Laboratories) method in conjunction with horseradish peroxidase substrate reagent SG kits (Vector Laboratories). Free-floating sections were pre-incubated for one hour in a blocking solution of 10% normal goat serum diluted in 0.1M PBS (PH 7.4) containing 0.3% Triton X-100. Subsequently, sections were incubated with antisera against (1) CR (Swant 7699/66, Switzerland) diluted in 1:5000; (2) PV (Sigma, PA-235) diluted 1:2000; (3) CB (Swant 300, Switzerland) diluted in 1:3000; (4) GAD (Sigma G5163) diluted 1:1000; (5) SV2

(Hybridoma Bank, University of Iowa) diluted in 1:1000 for two days at 4°C. Following multiple washes, sections were incubated for one hour in biotinylated goat anti-rabbit (for GAD, CR) or goat anti-mouse (for PV, CB, SV2) secondary antisera, diluted in 1:500. Sections were incubated in ABC followed by a horseradish peroxidase reaction. Sections were mounted on gelatin coated glass slides, air dried overnight, dehydrated through an ascending series of ethanol, cleared in Xylene and coverslipped with permount. Some sections were additionally counter-stained with neutral red.

Negative controls were obtained by incubating cerebellar tissue in the absence of primary antibodies. In these cases, the immunostaining of Purkinje cells and interneurons was eliminated. By contrast, the Purkinje cells heavily expressed CB and PV but not CR, while CR labeled interneurons of the granule cell layer in positive controls (Figure 1).

Figure 1. Calcium binding protein and GAD specific immunoreactivity in gecko cerebellum. CB, PV and GAD labeled Purkinje cells and CR only labeled interneurons in the granule cell layer. In the absence of primary antibody, A and B, with goat anti rabbit and goat anti mouse respectively, the cerebellum was unlabeled. All scale bars = 50µm.

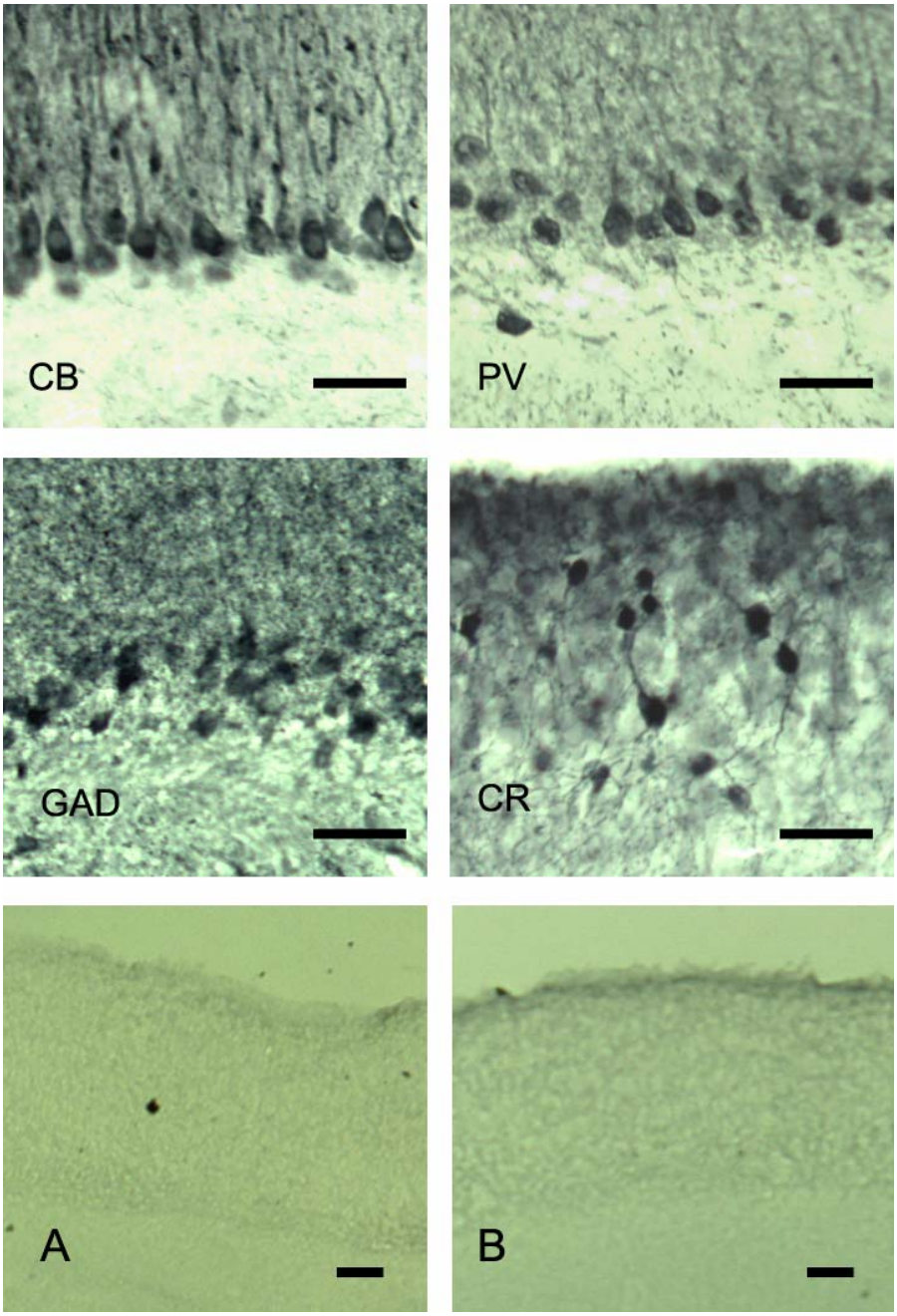
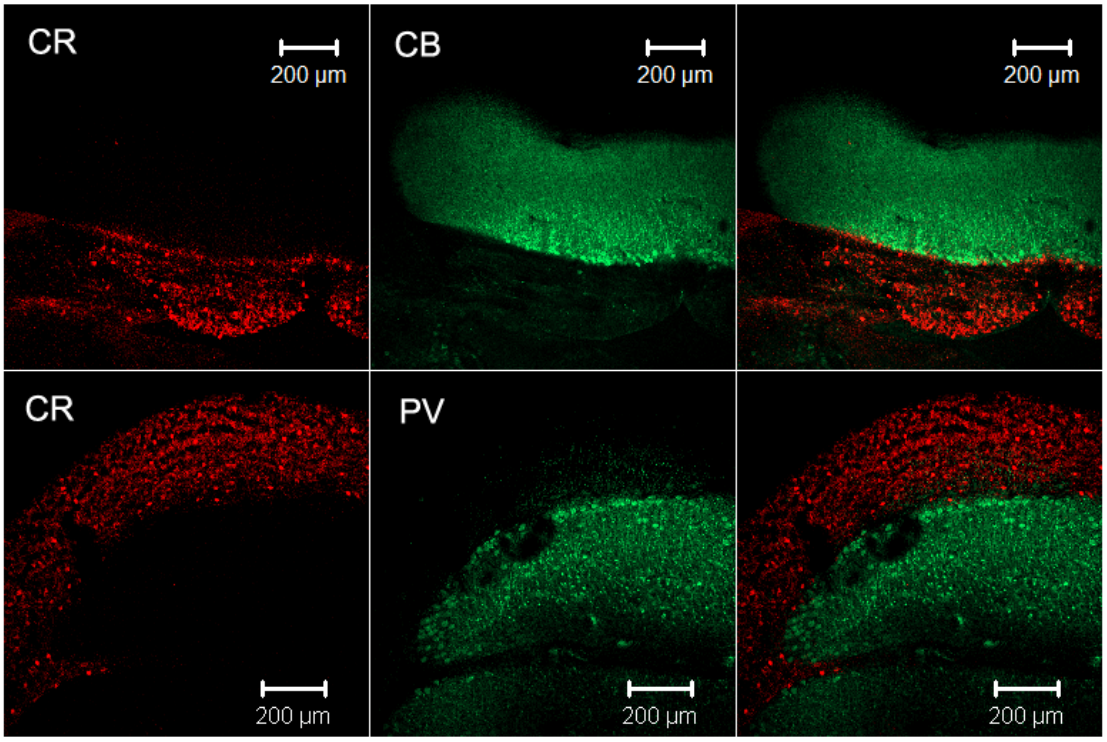
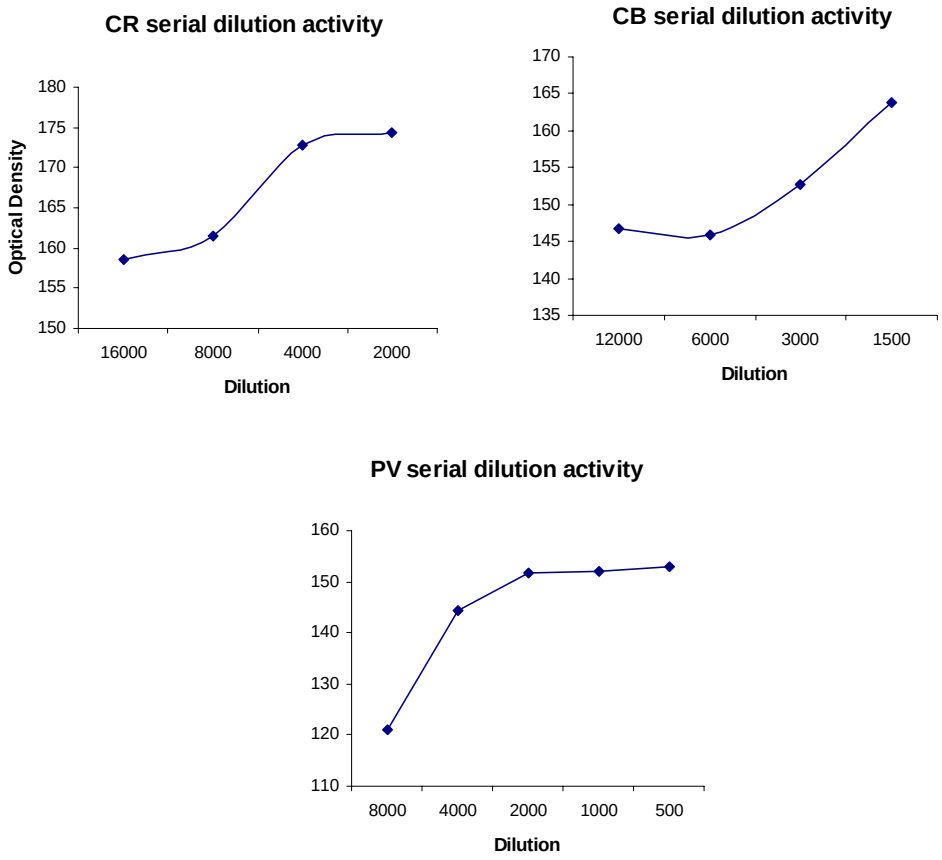


Figure 2. Fluorescence double labeling of calcium binding proteins in cerebellum. CR and CB or PV had complementary distributions in cerebellum. CR labeled interneurons in the granule cell layer while CB and PV labeled Purkinjie cells.



Serial dilution controls were used to optimize the working concentration of each antibody. Primary antibodies were diluted into four serial dilutions ranging from 1:500 to 1:16,000. Sections were processed using the same immunohistochemical methods above. Except for the different primary antiserum dilutions, all sections shared common reaction baths and were incubated simultaneously. The optical density of the target neurons versus antibody dilution were plotted (Figure 3). All the antibody concentrations used were in the linear stages of the optical density vs. concentration plot. Note that the dilution used for PV (1:2000) was at the end of the linear range. This concentration was used due to the fairly light staining for PV (Figure 3).

Figure 3. Calcium binding protein antibody serial dilution activity plots. Optical density was measured in NM for CR, and in Purkinje cells for CB and PV. The optical density increased with the antibody concentrations. Linear stages were obtained among the tested concentration range. All the antibody concentrations used in this study were within the linear range, except for PV, where a higher concentration of 1:1000 was used.



2.3 Fluorescence immunocytochemistry

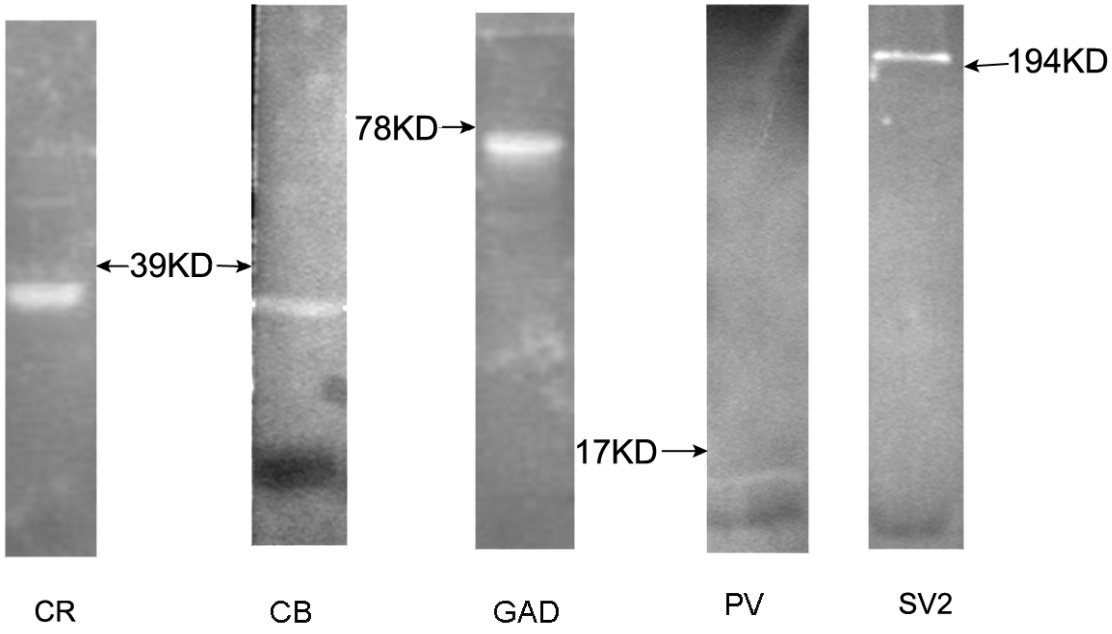
To double-label CR and PV or CB, sections were incubated in two primary antiserums simultaneously at the same dose as applied in regular HRP staining after one hour blocking. After washing, sections were incubated in a mixture of two secondary antibodies, alexa 594 donkey anti- rabbit and alexa 488 donkey anti-mouse (Invitrogen, Carlsbad, CA) in the dark at a dilution of 1:500. The labeled sections were coverslipped with anti-fade kit (Molecular probes). The images were obtained by Zeiss LSM 510 confocal microscope. Corresponding to the HRP immunohistochemistry results, CR was present in non-overlapping cerebellar cell types when compared with the distribution of CB and PV labeled cell types (Figure 2)

2.4 Western blotting

Western blotting procedures were used to confirm antibody specificity in gecko. A gecko brain was removed after the animal was deeply anesthetized by isoflurane and euthasol as described above. Pre-chilled RIPA buffer containing a protease inhibitor mixture was added by a volume of 5ml per gram tissue, and the tissue was broken down by homogenizer and vortex for 60 seconds. The suspension was stored on ice for 45 minutes followed by centrifuging at 14000G for 10 minutes at 4°C. The cytosolic protein was collected from the supernatant, whose concentration was measured by Bradford protein assay (Bio-Rad). 10ug protein and 5ul standard (161-0324, Bio-Rad) were loaded to 12% SDS/PAGE gel. The protein was separated by SDS/PAGE and transferred to PVDF membrane. The membrane, blocked with TBST containing 3% BSA for 1 hour at room temperature, was incubated in primary antisera that was diluted to 1:2000 in 1% BSA in TBST overnight at 4°C. Afterward,

it was probed with alkaline phosphatase conjugated Goat anti-Rabbit or Goat anti-Mouse (1:5000, Pierce 31340 and 31320) for 1 hour at room temperature. The blots were visualized by exposing the membrane to ECF substrate (Amersham Bioscience) at dark for 5 to 10 minutes, scanned on Storm™ (Molecular Dynamics). Blots with Calretinin showed a major band at estimated molecular weight of 29 KD, identical to the measurement in chick (Hack et al., 2000). CB, PV and GAD blots also yielded single bands at estimated molecular weights of 28kD (Ellis et al., 1991), 15kD (Celio et al., 1988; Lohmann and Friauf, 1996) and 65-67kD (Sloviter et al., 1996) respectively (Figure 4). Western blot analysis of the anti-SV2 antibody was previously tested against zebra finch brain tissue and produced a broad band over a range of protein sizes of 66-200KD (Nealen, 2005). This may be due to the difficulty in electrophoretically resolving glycoproteins. Our western blot of the SV2 against gecko brain tissue obtained a clear single band at protein size around 200 KD that was within the range of previous results in zebra finch (Figure 4).

Figure 4. Western blots for calcium binding proteins, GAD and SV2 in gecko brain. All blots yield a single band at estimated molecular weights of 29kD, 28kD, 65-67kD, 15kD and 200kD for CR, CB, GAD, PV and SV2 respectively.



Chapter 3: CaBP and GAD distribution in the auditory system of Gekko gecko

3.1 Auditory hindbrain overview

The auditory nerve terminates in the first order nuclei. Our studies of calcium binding protein and SV2 expression in the lower auditory brainstem suggested that two groups of auditory nerve fibers projected to the first order nuclei. The first was a dorsal-caudal fiber bundle that bifurcated upon entering the brainstem. One branch ran caudally to NMM and NML, while the other branch terminated more rostrally in NAL. The second group was a more ventral-rostral fiber bundle that appeared to project only to medial NA without bifurcating.

NM, NL, NA were recognized in Gecko auditory brainstem. NM was divisible into two subregions, NMM and NML. NMM was caudally located along the medial edge of the acoustic tubercle. NML was located lateral and rostral to NMM and caudal to NA. The NML was localized at the region between NMM and NA. NL was located ventral to NM and consisted of strands of cell bodies with bitufted dendrites. NA was the largest hindbrain auditory nucleus and was located rostrally to NM. NA contained a more heterogeneous grouping of cells than NM and NL. The superior olivary nuclei (SO) were located in the ventral hindbrain and consisted of two nuclei, the dorsal SO and ventral SO. The nuclei of the lateral lemniscus (NLL) were made up of dorsal and ventral nuclei.

3.1.1 Auditory ganglion, first order nuclei and nucleus laminaris

Calretinin-immunoreactive (CR-ir) auditory nerve fibers projected to the first order nuclei, NM and NA via CR-ir auditory ganglion neurons. Most of the auditory ganglions co-expressed CR, CB and PV (Figure 5). In horizontal sections, CR-ir auditory nerve branches bifurcated and projected into NA and NM. The auditory nerve terminals and NM somata were densely CR-ir (Figure 6). NMM and NML could be identified by CR expression (Figure 13). The NAM contained large round CR-ir cell bodies (diameter =12.84 ± 0.92 μm, n=23; Figure 5). There were also CR-ir neurons below NM (Figure 8). We hypothesize that these ventrally located bitufted neurons correspond to the second order nucleus laminaris (NL). The dorsal CR-ir dendrites of NL extended into the overlying NM. The lateral region of the putative NL merged with NM, with no clear border detected between the two nuclei.

CB labeling differentiated the branches of the eighth nerve. Dense CB-ir characterized auditory nerve terminals in NM and NA (Figures 6 and 7). Additionally, the NM cytoplasm was lightly stained (Figure 6). A few CB-ir cell bodies and many CB-ir axon terminals were detected in NAM (Figure 7). The NL was slightly CB-ir.

PV-ir labeled auditory ganglion, auditory nerve and auditory nerve terminals in the first order nuclei. In NM, PV-ir perisomatic puncta surrounded unlabeled somata (Figure 6). PV-ir labeled all nerve terminals and occasional cell bodies in NA (Figure 7). There was no PV-ir in NL.

Figure 5. Calcium binding proteins in auditory ganglion. CR, PV and CB labeled the majority of ganglion cell bodies. Most of them were co-labeled by all three calcium binding proteins (arrows).

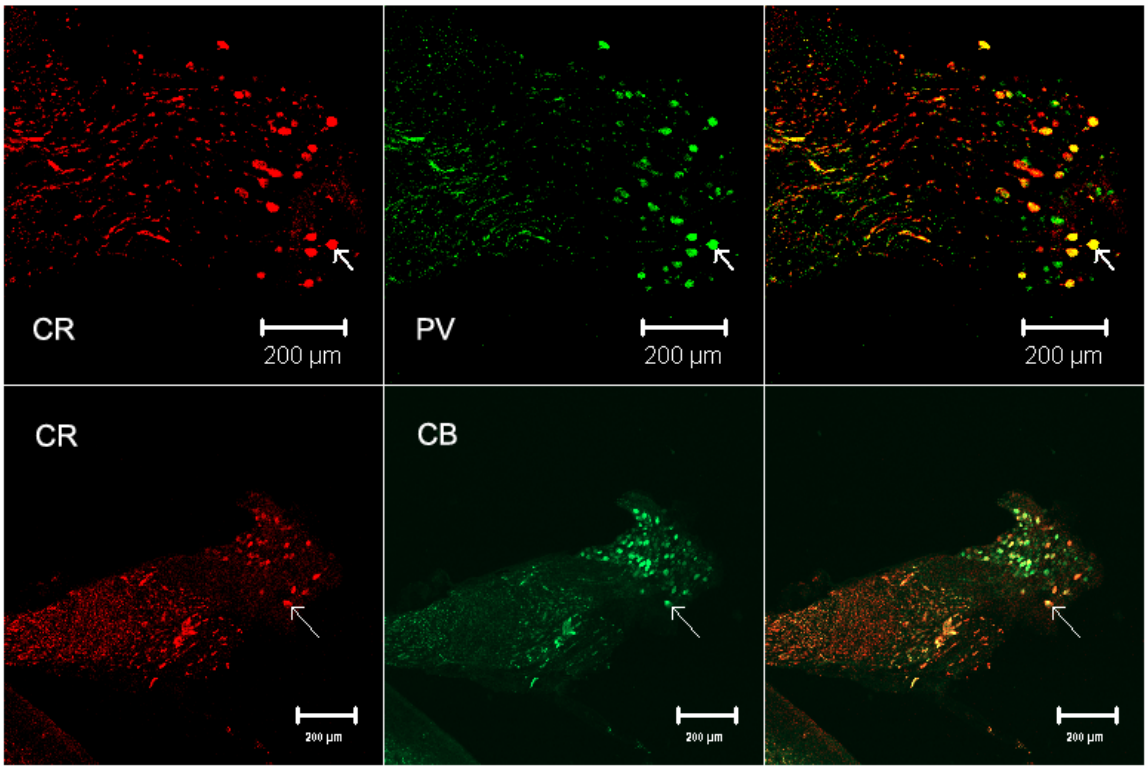


Figure 6. Calcium binding proteins and GAD immunoreactivity in Nucleus Magnocellularis. CR-ir heavily labeled both cell bodies and neuropil in NM. PV labeled auditory nerve terminals around NM neurons. CB was located in the auditory nerve terminals and lightly stained NM cell bodies. GAD-ir terminals surrounded unlabeled somata in NM. All scale bars = 20μm.

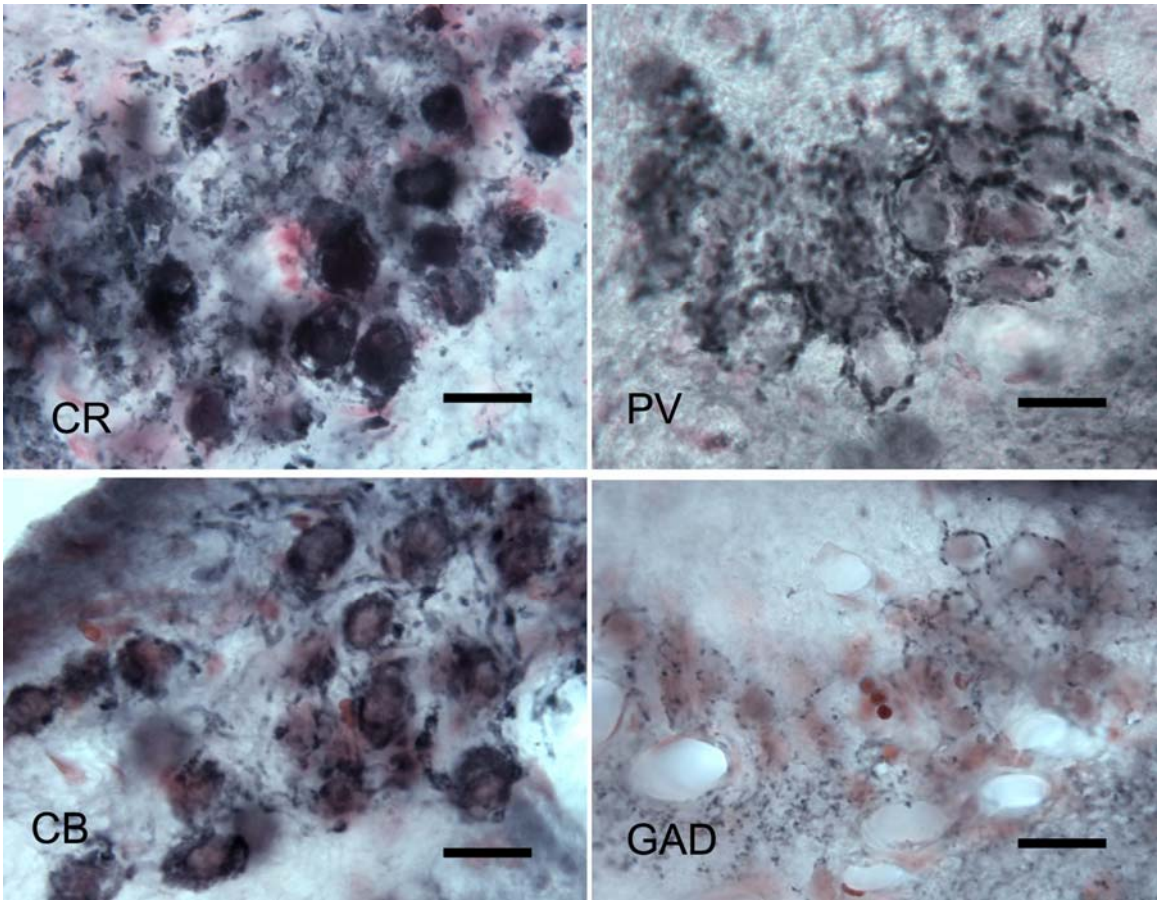


Figure 7. CaBP immunoreactivity in Nucleus Angularis. CaBP-ir auditory nerve fibers projected to the medial nucleus of NA. CR heavily labeled cell bodies in the medial area of NA; very few NA neurons were CB-ir or PV-ir; CB and PV labeled fine terminal fields at NA. Scale bar for the top left = 500µm, all others =100µm.

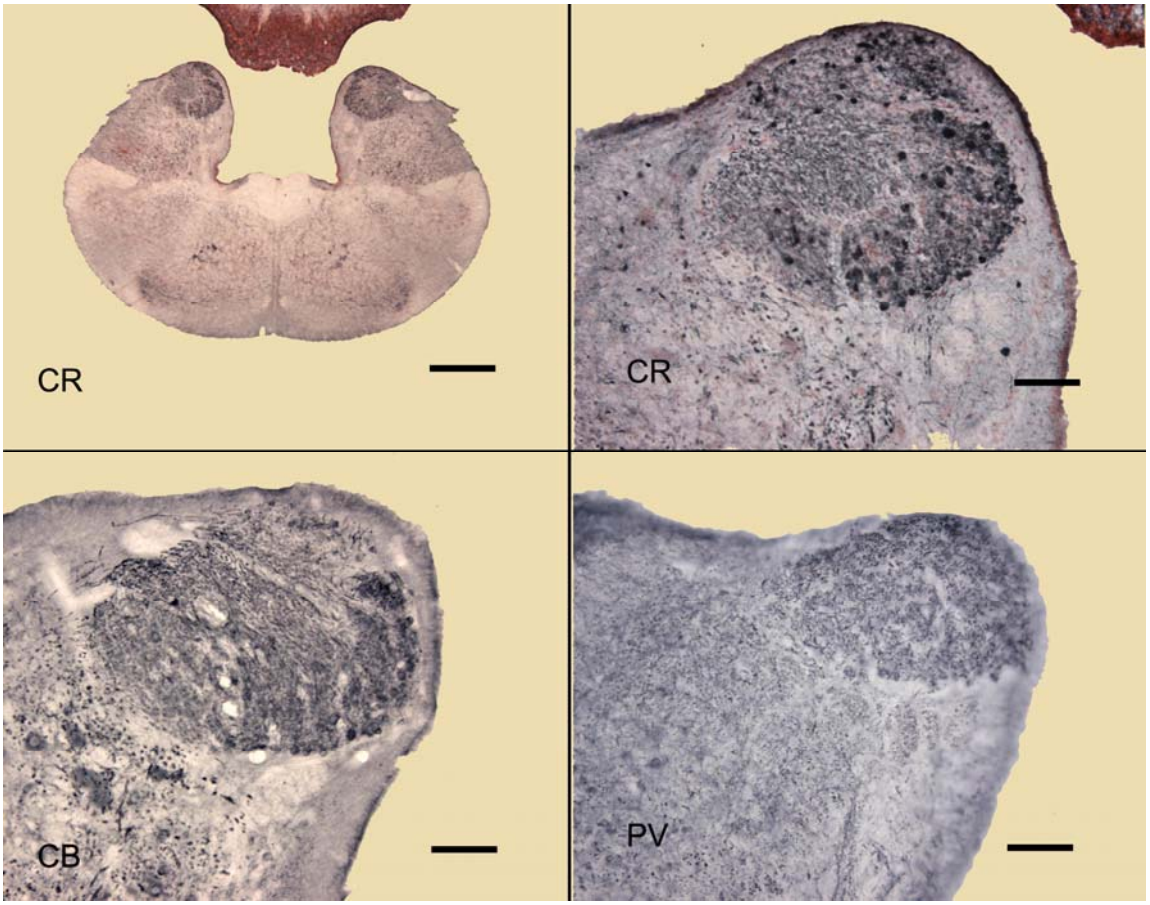
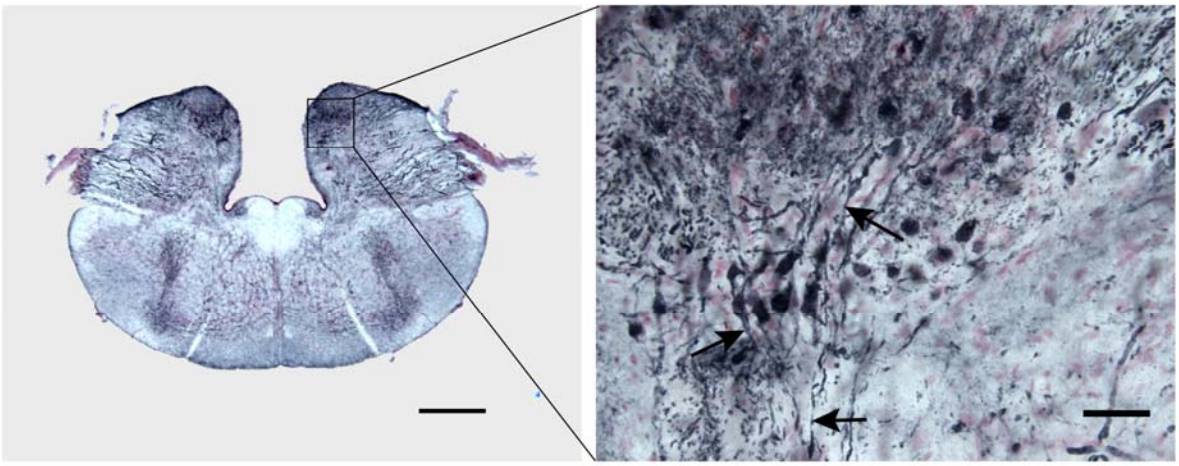


Figure 8. Nucleus Laminaris. Calretinin-ir strand of bitufted cells below NM. Left: low-power view of cochlear nuclei and SO. Right: cochlear nuclei area showing two strands of NL bipolar cells ventral to NM. Arrows showed the CR-ir neuropil connected to ventral side of NM and dorsal sides of SO. Left scale bar 500 µm, right 50 µm.



GAD-ir presynaptic terminals surrounded unlabeled somata in NM (Figure 6). GAD-ir terminals could be distinguished from the auditory nerve terminals by their distribution and small size, and were observed throughout NM and NA. There was a small population of GAD-ir neurons in NA. Synaptic vesicle protein 2 (SV2) labeled all synaptic terminals (Buckley and Kelly, 1985). The auditory nerve input patterns were well identified by SV2 (Figure 13).

3.1.2 Co-expression of Calcium binding proteins in first order nuclei and nucleus laminaris

Double label immunofluorescence was used to determine whether different calcium binding proteins were localized within the same neurons. The majority of auditory nerve ganglion cell bodies were immunoreactive for all three calcium binding proteins (Figure 5). Most cochlear nerve fibers co-expressed two or three calcium binding proteins. CR was not only present in NM somas but also localized to presynaptic nerve terminals, which also co-localized with PV-ir and hence appeared yellow (Figure 9). CB was expressed in cytoplasm of NM neurons near the membrane and in nerve terminals but was not found throughout the soma (Figure 9). In double labeled material, NL neurons and neuropil were CR-ir, while PV was completely absent. CB lightly labeled cell bodies in NL (Figure 11). In NA, the nerve terminals co-expressed CR, PV and CB. CR-ir was localized throughout the somata of NAM, while perisomatic region in a small population of NA neurons co-expressed CR and CB (Figure 10). The ascending efferent fibers from the first order nuclei were all CR-ir (Figure 10).

Figure 9. Fluorescence double labeling of calcium binding proteins in Nucleus Magnocellularis. A. CR and PV in NM. CR and PV were co-localized to many nerve fibers and puncta around NM neurons (arrow heads). The somata of NM expressed CR but not PV (arrow). B: CR and CB in NM. CB was located in the auditory nerve terminals and cytoplasm of NM neurons. All the nerve fibers lateral to NM were double labeled by both CR and CB. Co-expression of CR and CB in the perisomatic cytoplasm of a NM neuron (arrow).

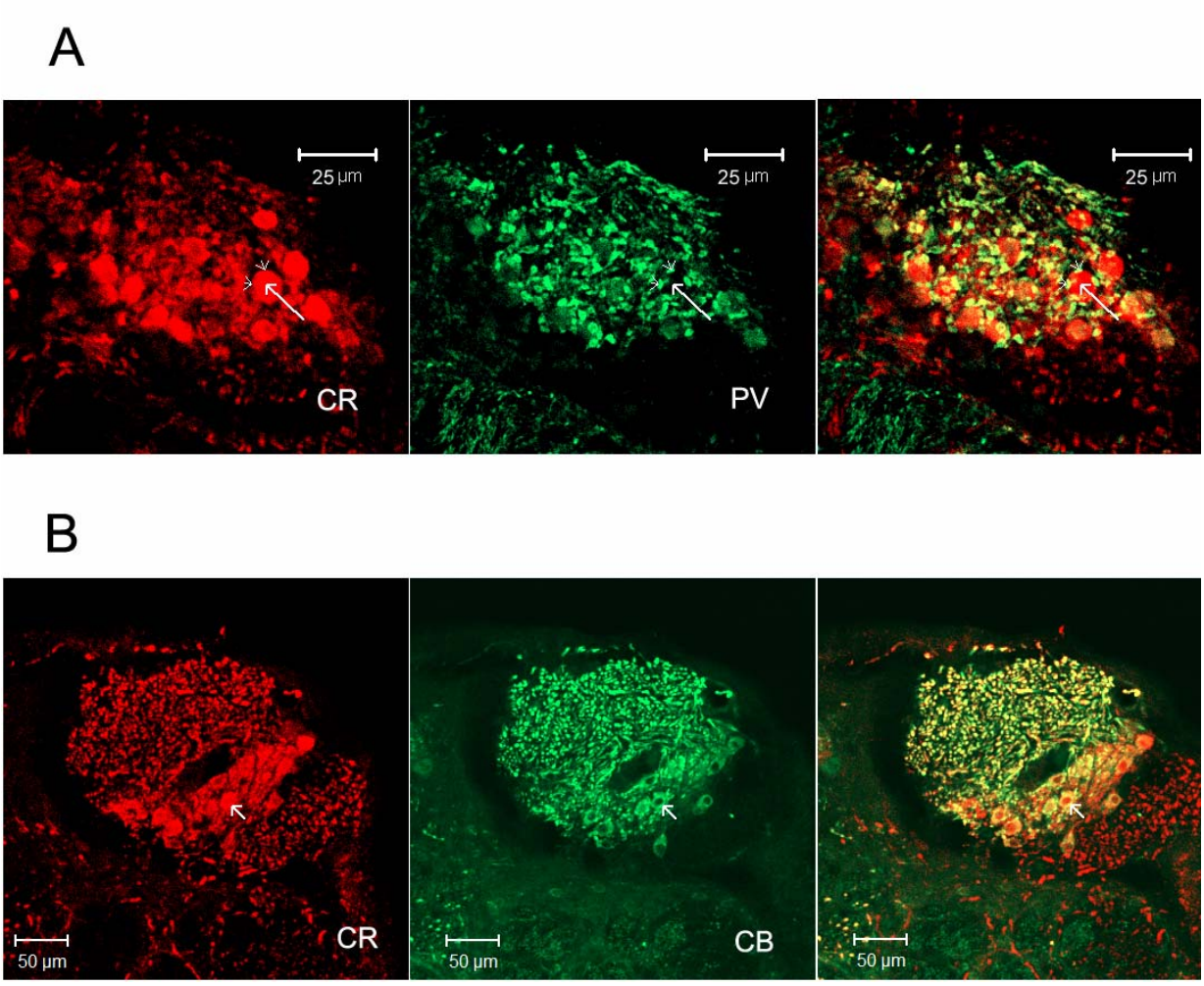


Figure 10. Fluorescence double labeling of calcium binding proteins in Nucleus Angularis. A. CR and PV. CR was present in the cell bodies and terminals in NA, PV was only localized at terminals around neurons. Many terminals co-expressed CR and PV. CR also labeled the efferent ascending fibers leaving NA and projecting to the ventral hindbrain (arrow) B. CR and CB. CR-ir cells in medial nucleus of NA. CB labeled the perisomatic cytoplasm of many neurons in NA. Arrows indicate some neurons were co-localized by both of the two proteins; some neurons only expressed one of them.

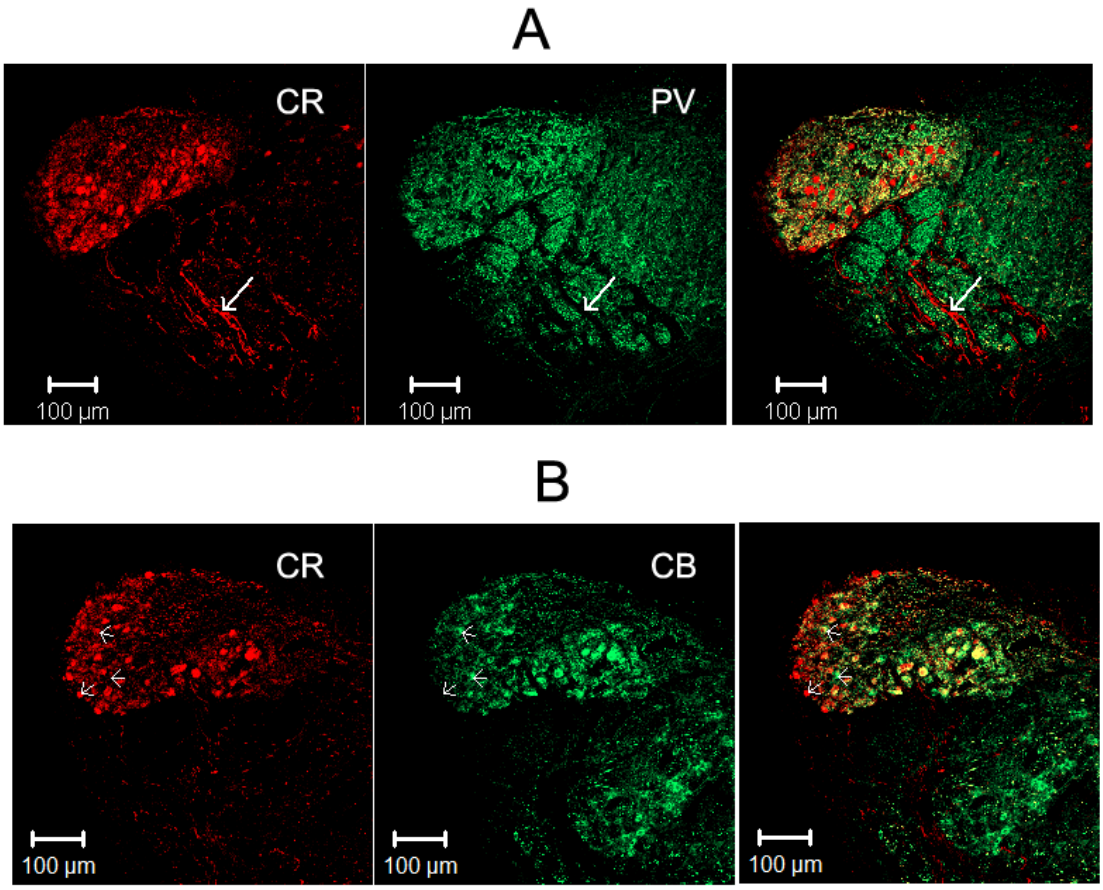
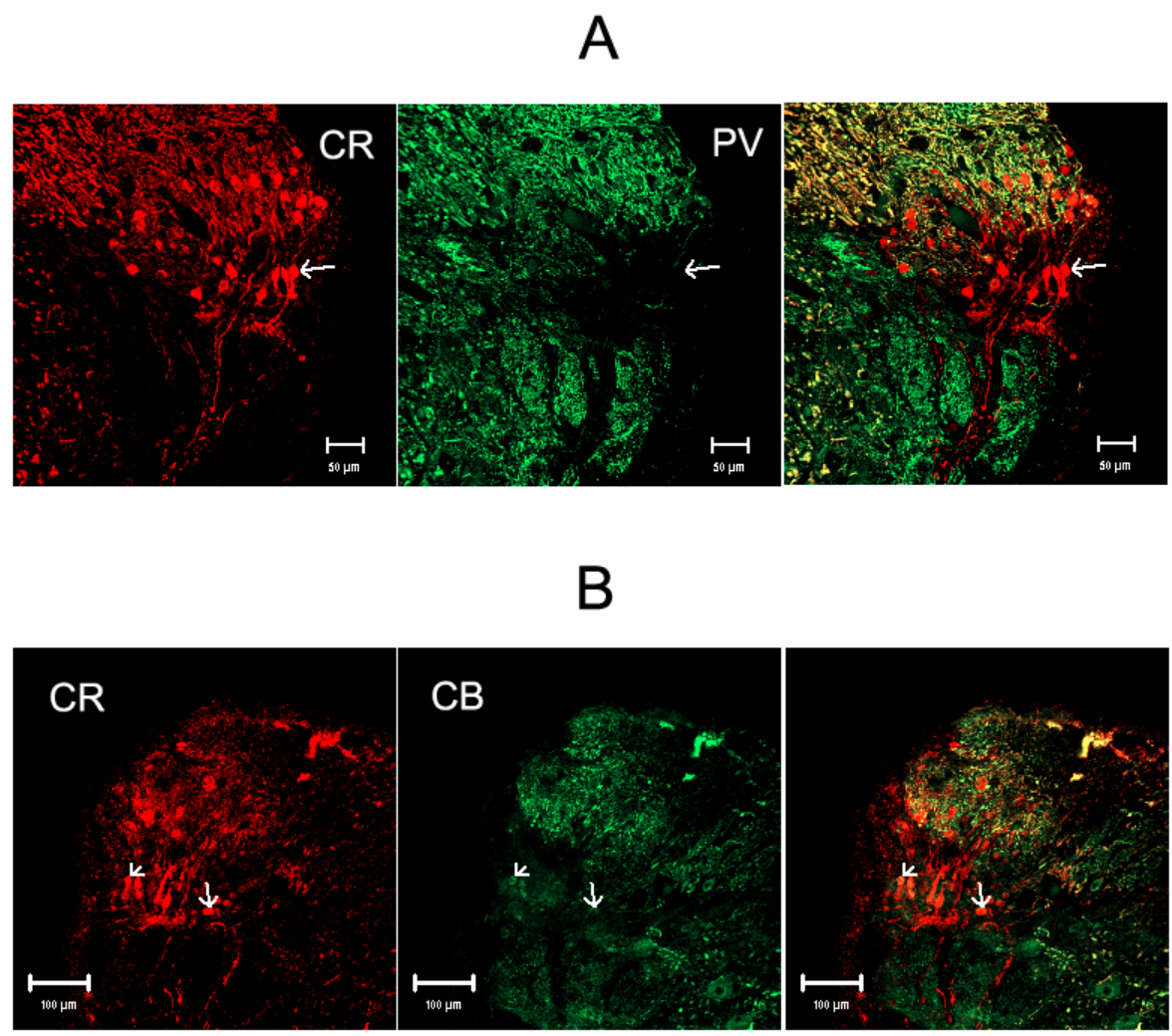


Figure 11. Fluorescence double labeling of calcium binding proteins in nucleus laminaris. A. CR and PV. PV were completely absent in NL region. CR had the exact same distribution pattern in NL as in the HRP immunohistochemistry. There was no co-expression of CR and PV in NL. B. CR and CB. CB had low expression at perisomatic cytoplasm of NL that was co-expressed by CR (arrows). The neuropil of NL neurons were negative for CB staining but positive for CR.



3.1.3 Superior olivary nuclei

There were two superior olivary nuclei, one dorsal and one ventral. The ventral SO was located at the ventral portion of the brainstem, while the dorsal SO was larger and more ovoid shaped. The three calcium binding proteins delineated the superior olivary nuclei by different staining patterns.

Both of the superior olivary nuclei exhibited dense CR-ir fibers with large boutons (average diameter =3.65 ± 0.67μm, n=38) surrounding unlabeled neurons (Figure 12). CR-ir fibers from the first order cochlear nuclei projected to both the ipsilateral and contralateral olivary nuclei (arrows in Figure 12). The arrows in Figure 7B and colored lines in Figure 7A identified a CR-ir tract connecting the dSO and the first order nuclei. No postsynaptic elements in the olivary nuclei were CR-ir. The CB-ir elements in dSO consisted of lightly labeled cytoplasm and terminals (Figure 14). No CB-ir labeling was found in vSO. PV stained both cell bodies and neuropil in both vSO and dSO (Figure 14). There was also some PV-ir neurons and fibers in the area between dSO and vSO (Figure 14). PV was a useful marker to delineate the SO nuclei. Dense GAD-ir terminals were found in both nucleus of SO while approximate 30% neurons were GAD-ir in dorsal nucleus of SO. Fewer GAD-ir neurons were observed in vSO (Figure 14).

Figure 12. Calretinin-ir terminals in Superior Olivary nuclei with a diagram of SO projections. CR-ir was present in terminal boutons surrounding unlabeled somata in both the dSO and the vSO. CR-ir fibers also marked the projection from the cochlear nuclei to the ipsilateral dSO and vSO (green line and arrows), and to the contralateral vSO (red line and arrows). Scale bars for the bottom two figures = 50µm, others = 500µm.

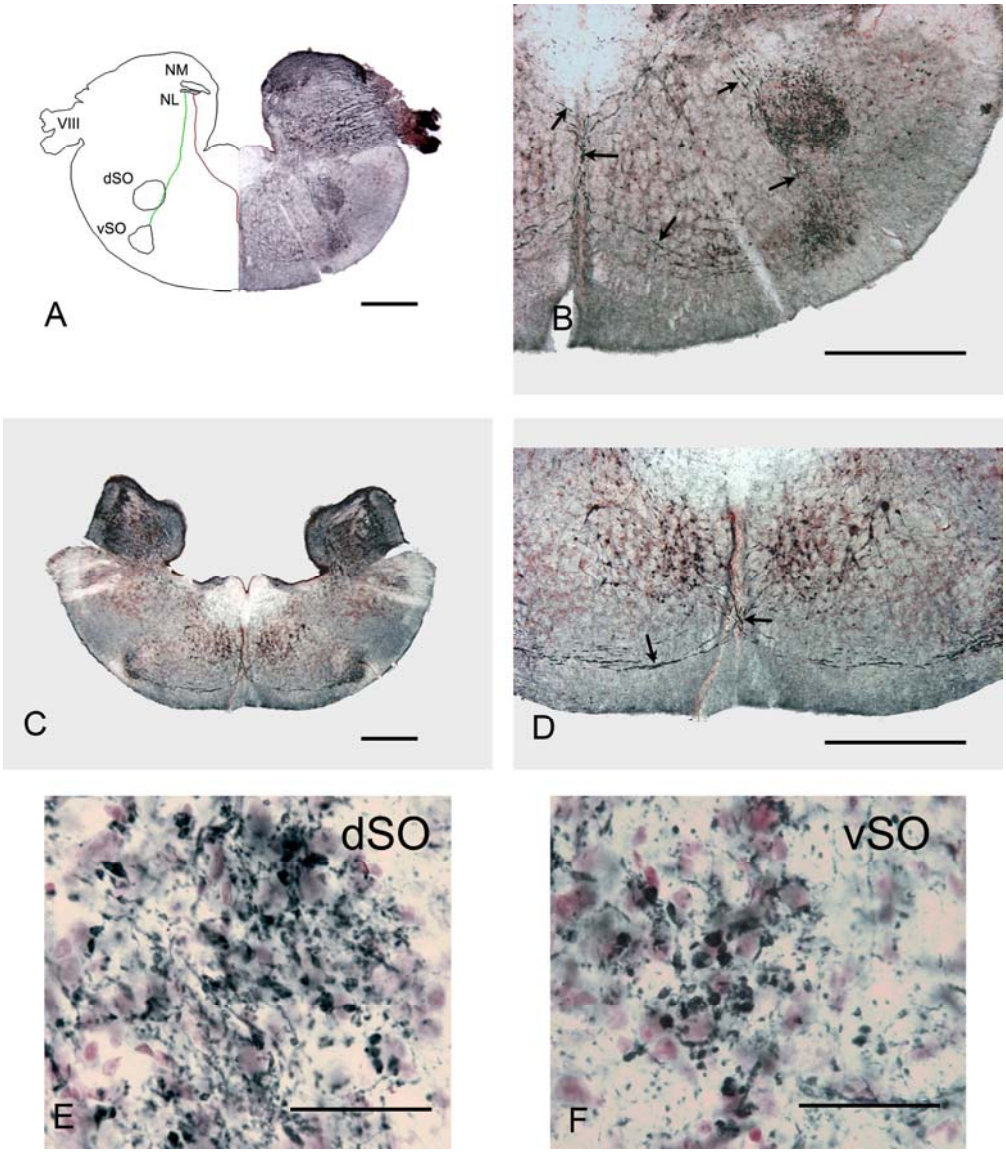


Figure 13. Calretinin and SV2 immunoreactivity in horizontal sections. A: Calretinin immunoreactivity. NM, NML and NA were well delineated, CR-ir auditory nerve fiber projected to first order nuclei. B and C: SV2 immunoreactivity. The auditory nerve input patterns were well identified due to the lack of SV2 material at the nerve tract. Bifurcated dorsal-caudal fibers of the auditory nerve innervated NM and NAL at the dorsal level (B), and ventral-rostral fiber approached the rostral NA at ventral level (C). D: SV2 immunoreactivity at NMM. Large perisomatic, “end-bulb” like structure were detected by SV2 in NMM. Scale bar for D=100µm; all others=400µm

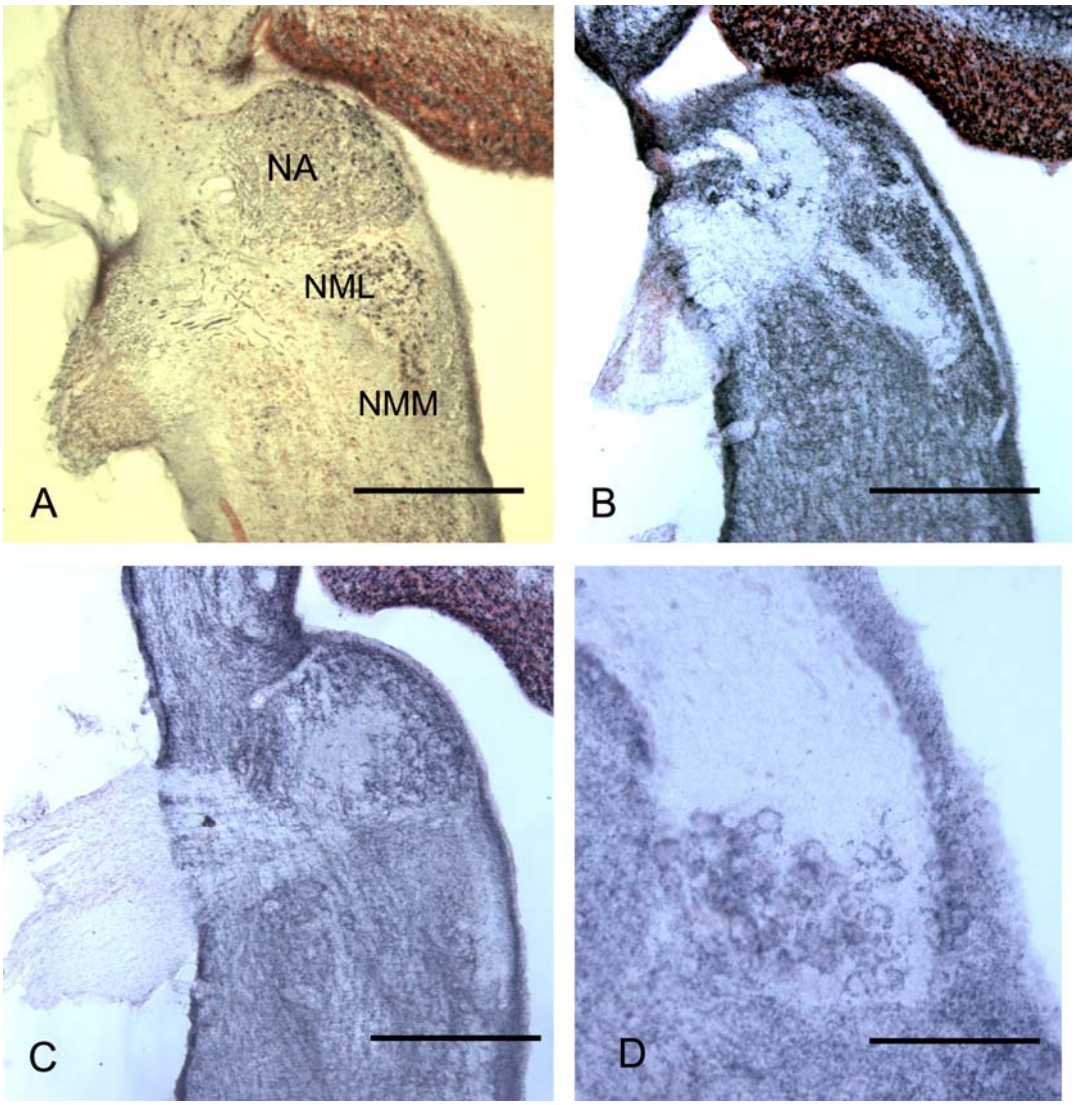
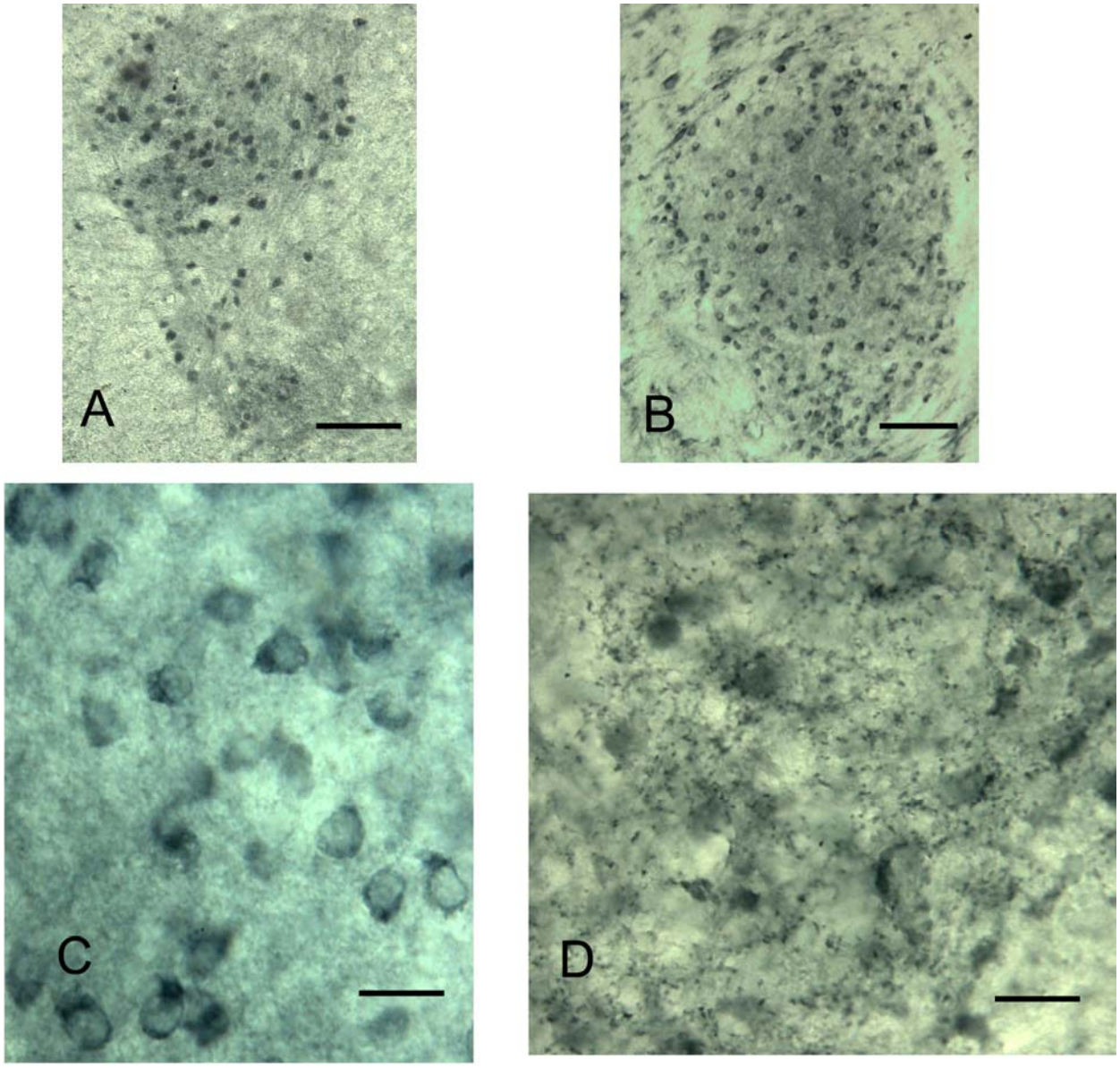


Figure 14. PV, CB and GAD immunoreactivity in Superior Olivary nuclei. A: PV immunoreactivity. PV-ir labeled cell bodies in both dSO and vSO. B and C: CB immunoreactivity. CB-ir labeled cell bodies in dSO; D: GAD immunoreactivity. GAD-ir terminals and a few GAD-ir cell bodies were observed in SO. Scale bars for A and B = 100µm, C and D = 20µm.

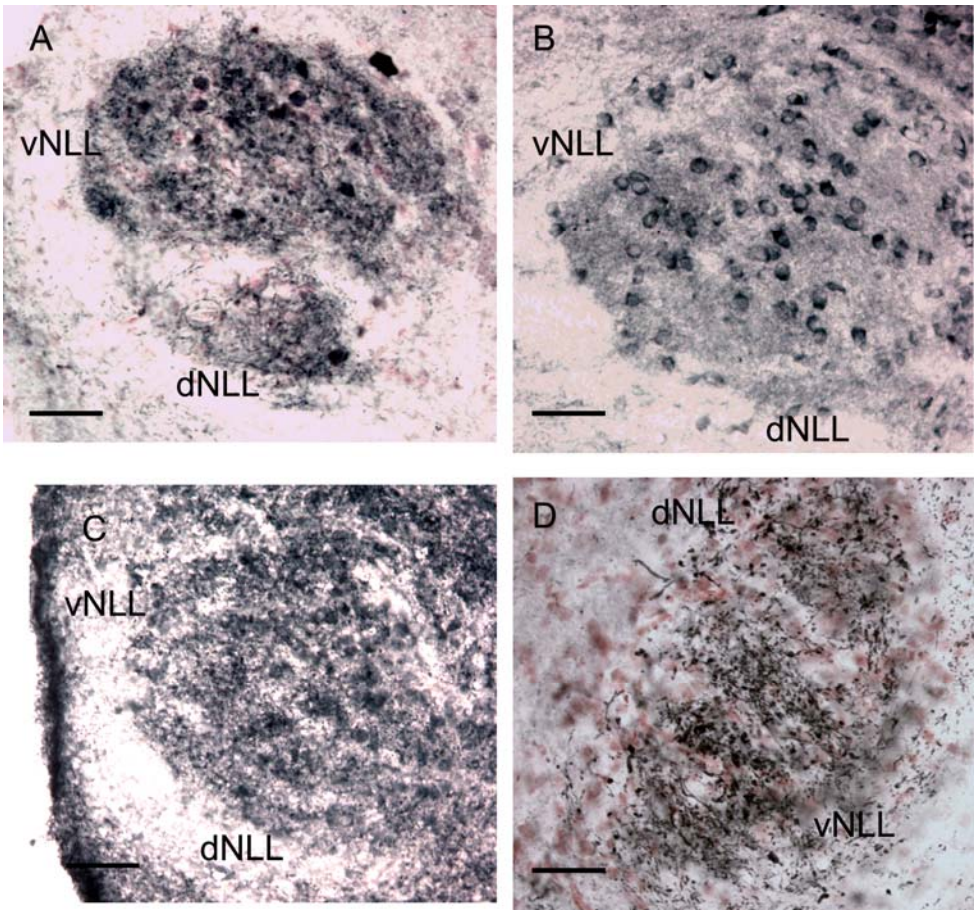


3.1.4 Nucleus of the lateral lemniscus

The lemniscal nuclei were located in lateral and rostral hindbrain, rostral to the SO and the first order cochlear nuclei. They were divided into a dorsal NLL (dNLL) and a ventral NLL (vNLL). The vNLL was ventral and rostral to dNLL. Moreover, it was larger and had more GAD-ir cell bodies and denser CR-ir terminals. Both NLL contained CR immunoreactive fibers, assumed to originate from the first order nuclei, and no CR-ir somata.

Labeled CR-ir axons marked the tract connecting the vSO and vNLL. Dense CR-ir terminals were distributed throughout vNLL and dNLL (Figure 15D). dNLL had denser CR-ir terminals. A clear border was detected between the two nuclei (Figure 15D). CR-ir fibers also marked the connection between the vNLL and vSO (figure not shown). Fine CB-ir perisomic terminals were distributed throughout the nuclei of NLL (Figure 15B). PV-ir somata and neuropil were present in the vNLL and dNLL. Most cell bodies in vNLL were GAD-ir and fewer in dNLL. They might be overlapped with PV-ir (Figure 15A). Both dorsal and ventral nucleus had widely distributed dense GAD-ir terminals, some of which was co-localized by CR and CB or PV (Figure 15C). SV2 labeled synaptic terminals throughout the lemniscal nuclei.

Figure 15. Calcium binding protein and GAD immunoreactivity in horizontal sections through the nuclei of the Lateral Lemniscus, vNLL (v) and dNLL (d). . A: PV-ir neurons and neuropil; top=rostral. B: CB-ir somata and fine terminals. C: GAD immunoreactivity in vNLL revealed many GAD-ir cell bodies, while dNLL had dense GAD-ir terminals and fewer GAD-ir cell bodies. D: Transverse section with CR-ir at NLL. CR-ir terminals were present at both dNLL and vNLL. vNLL had denser terminals. All scale bars= 50µm.



3.2 Auditory midbrain

The torus semicircularis is the homolog of the inferior colliculus in birds, and located beneath the optic tectum. It was previously divided into central, laminar, and superficial nuclei (Kennedy and Browner, 1981). The laminar nucleus may correspond to the avian intercolliclular nucleus (Díaz et al., 2000). The central and lateral nuclei of gecko torus semicircularis could be differentiated by distinct patterns of calcium binding proteins immunoreactivity. A field of CR-ir fibers and terminals was located at the lateral edge of the central nucleus (Figure 16 A and D). Also, CR-ir cell bodies were located in both the laminar nucleus of TS and the medial-dorsal region of the central nucleus of TS (TSc). The neurons in the dorsal region of the central TS had long CR-ir bipolar dendrites in contrast to CR-ir neurons with short dendrites in the laminar nucleus (Figure 16 B and C). Very few other CR-ir elements were found in the central TSc. CB-ir neurons were distributed thoughout the central nucleus and had a low expression in TSl (Figure 17 B). Some CB-ir neurons in the lateral region of the central nucleus were surrounded by the CR-ir terminals. CR and CB were rarely co-expressed in the lateral nucleus of torus (Figure 18B). Many intensely immunoreactive PV-ir neurons and neuropil were present at the ventral-lateral portion of the central nucleus (Figure 17 and 18 A). Some PV-ir neurons in lateral region of torus were surrounded by CR-ir terminals (Figure 18A). Very few PV-ir neurons were identified in TSl.

Figure 16. Calretinin-ir neurons and terminals in Torus Semicircularis. A: overview of CR distribution. CR-ir cell bodies were located at laminar nucleus of Torus (TSl) and at the boundary of central nucleus (TSc). B: CR in TSl. Neurons with short dendrites at TSl were CR-ir. C: CR at medial-dorsal portion of TSc. CR-ir neurons with long dendrites were found at medial-dorsal portion of TSc. D: CR at lateral portion of TSc. CR-ir terminal fields were observed at the lateral portion of TSc. Scale bar for A =200μm; others=50μm.

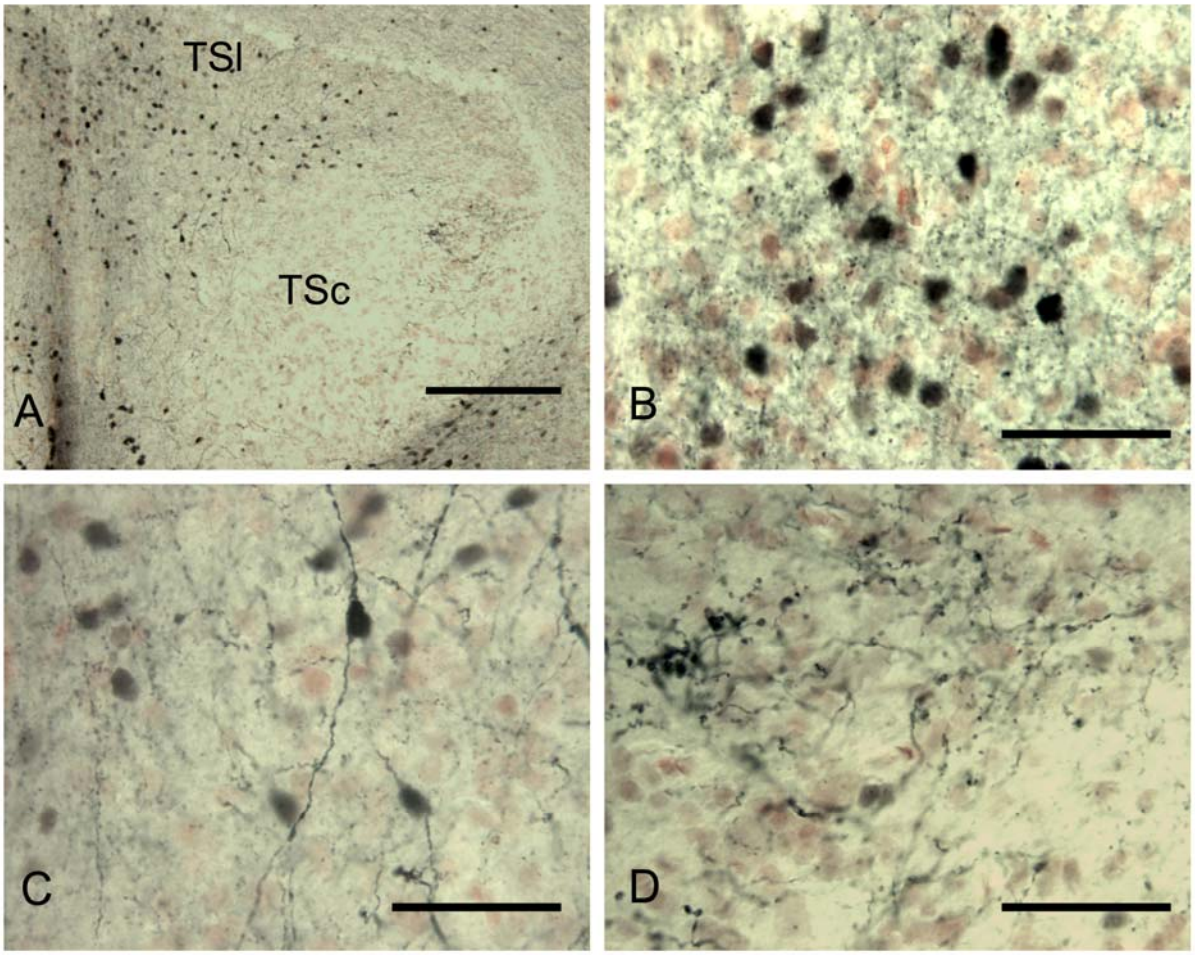
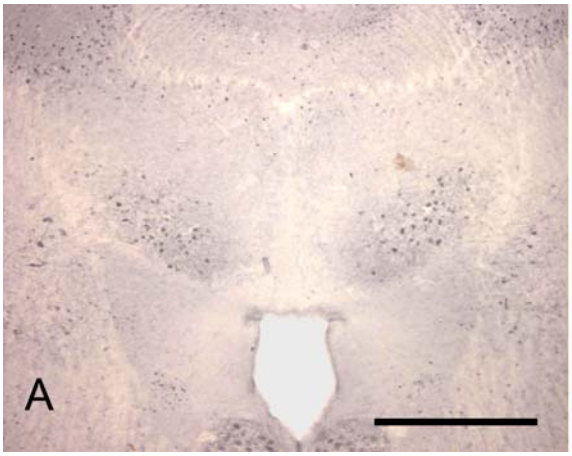


Figure17. Calbindin and Parvalbumin immunoreactivity in the Torus Semicircularis. A: PV-ir cell bodies and neuropil mainly occupied the ventral-rostral portion of TSc with a few labeled neurons present at TSl. B: CB at Torus. CB-ir neurons were distributed extensively in TS. Scale bars = 500 μm



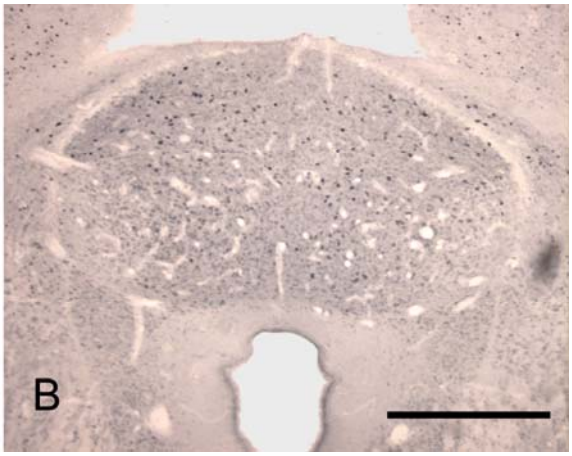
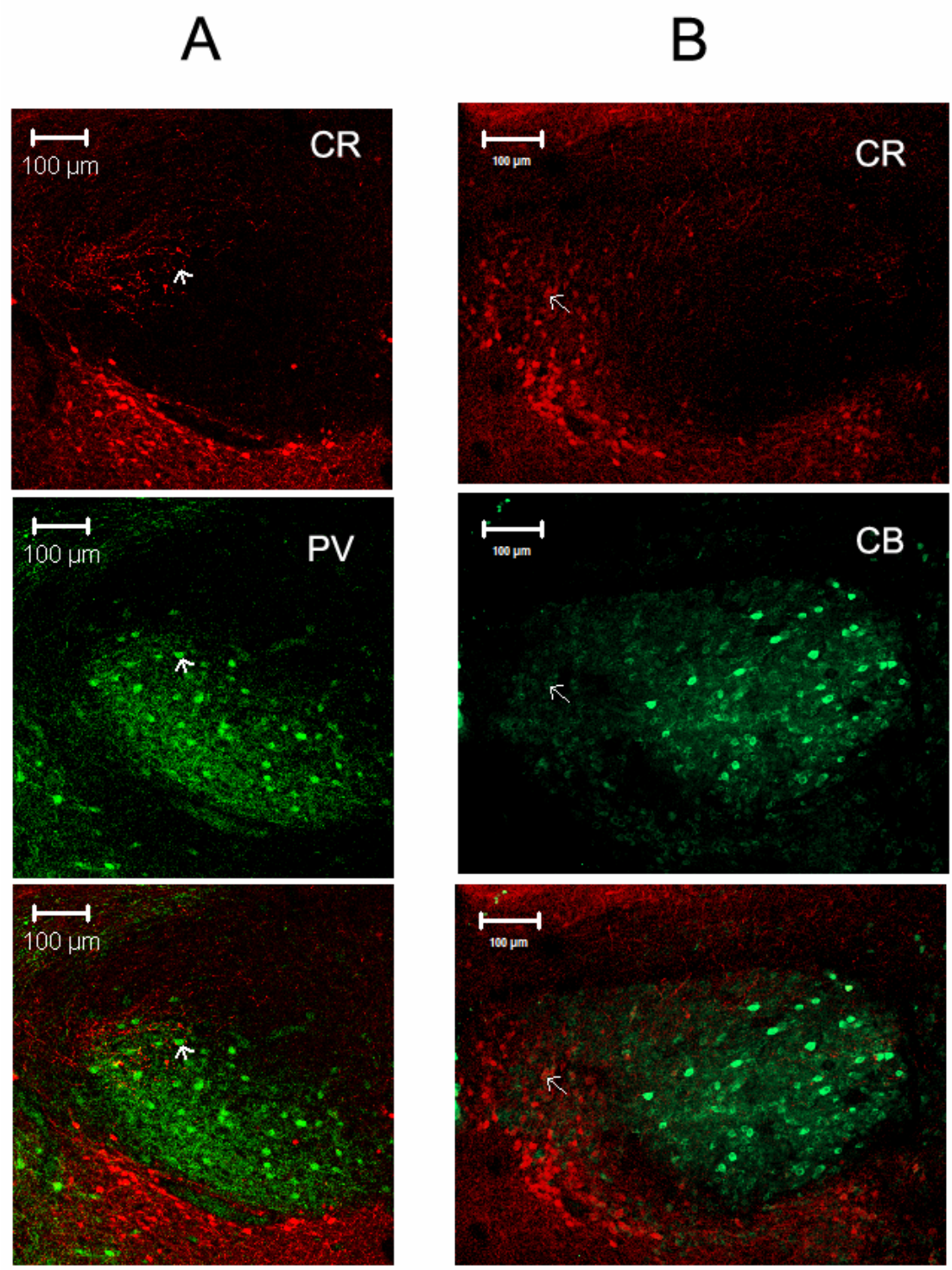


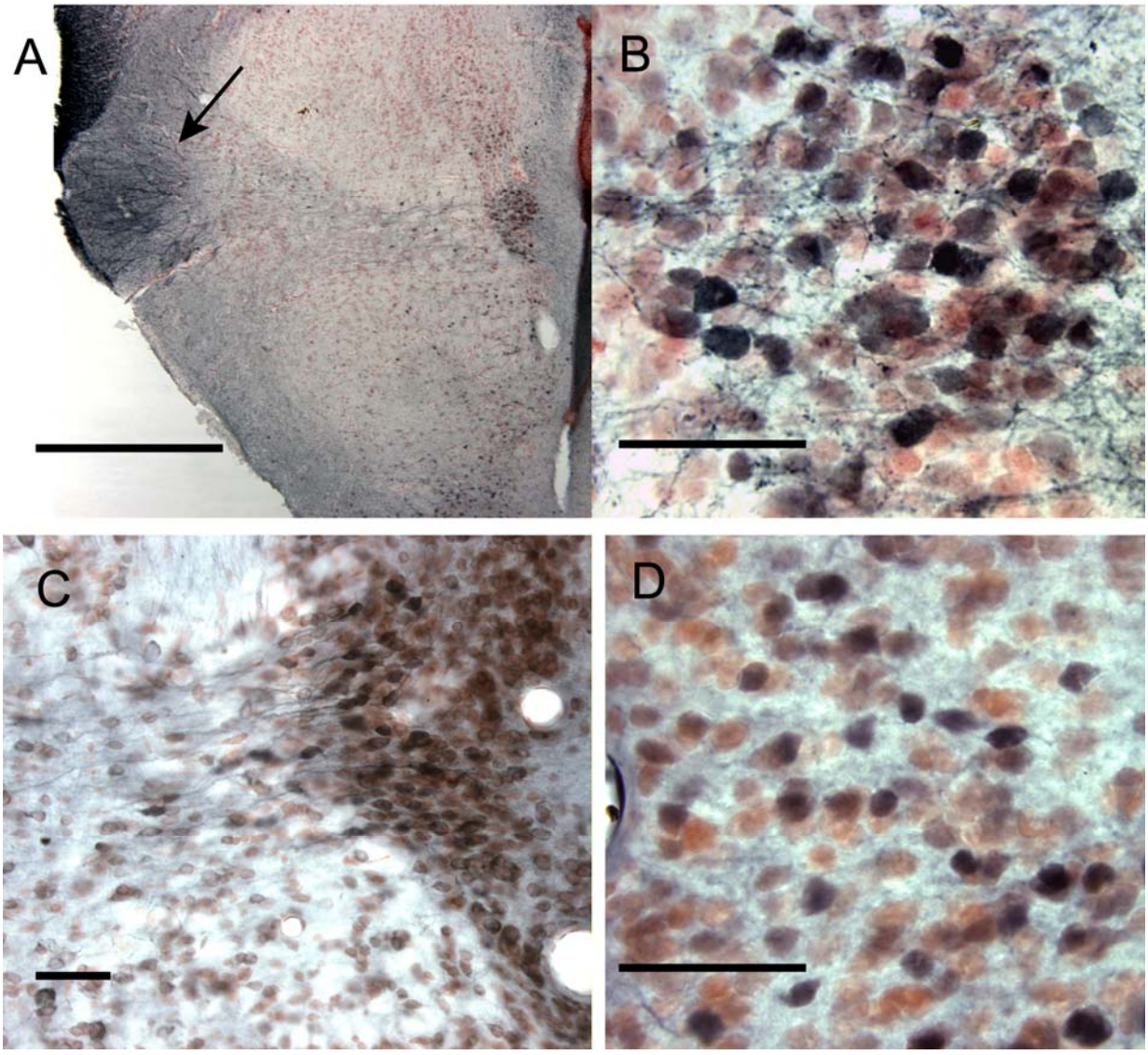
Figure 18. Fluorescence double labeling of calcium binding proteins in the Torus semicircularis. A. CR and PV in torus. CR labeled many cell bodies in laminar nucleus of Torus (TSl) and terminals in the lateral portion of TSc. PV-ir neurons were located ventral and medial to the CR-ir terminal region with little overlapping. Some CR-positive terminals were detected to attach the surface of the PV positive cells (arrow). B. CR and CB in torus. CB was present extensively in neurons of TSc and had a low expression in TSl. A few CR-ir terminals surrounded CB-ir neurons in the lateral portion of TSc and several neurons in TSl were slightly co-localized by CR and CB (arrow).


A
B
100 µm
CR
100 µm
CR
100 µm
PV
100 µm
CB
100 µm
100 µm

3.3 Auditory thalamus

Nucleus medialis is the auditory target in thalamus. It is adjacent to the midline. All calcium binding proteins labeled cell bodies in medialis. CR-ir ovoid neurons were found in nucleus medialis and the CR-ir positive fibers projected to the lateral regions of thalamus (Figure 19 A and B). CB-ir neurons and fibers were observed in both the nucleus medialis and the interconnected lateral region of thalamus (Figure 19 C). Neurons in nucleus medialis were also PV-ir, however the CR-ir and CB-ir fibers were not (Figure 19 D). Many but not all neurons co-expressed all three calcium binding proteins. The DVR is the auditory region of forebrain, and contained no calcium binding proteins and GAD immunoreactivity.

Figure 19. Calcium Binding Proteins in Nucleus Medialis of thalamus. A and B: CR distribution at MM; C: CB distribution at MM; D: CB distribution at MM. Cell bodies in the nucleus medialis were CR-ir, PV-ir and CB-ir. CR-ir fibers and CB-ir neuropil projected laterally to more lateral regions of thalamus (arrow). Scale bar for A = 500µm, all others = 50µm.



Chapter 4: Discussion

Calcium binding proteins may be important to maintain the normal functions of the ascending auditory pathways and they are good markers to delineate the auditory system of the Gecko (Figure 20).

4.1 Calcium binding protein distribution in the auditory system of vertebrates

Calcium has a crucial role as a second messenger in regulating many neural functions, ranging from the control of neurotransmitter release and neuronal excitability to the regulation of neuronal viability in development and disease. Calcium binding proteins CB, CR and PV use helix-loop-helix EF-hand calcium binding motif to bind free cytosolic calcium ion (Grabarek, 2006; Miller, 1995). The mechanisms underlying their roles in neuronal excitability, development and neurodegeneration are not fully understood, although it has been suggested that they modulate intrinsic neuronal excitability (Baimbridge et al., 1992).

Calcium binding proteins are valuable markers of neuronal subpopulations for anatomical and developmental studies (Resibois and Rogers, 1992). Calretinin is particularly abundant in auditory neurons with precisely timed discharges (Rogers, 1987). Calcium binding protein expression characterizes the central auditory system of mammals such as rat (Celio, 1990; Lohmann and Friauf, 1996; Pór et al., 2005) and guinea pig (Caicedo et al., 1996). These proteins are also expressed in chicks (Parks et al., 1997; Rogers et al., 1989) and barn owls (Kubke et al., 1999; Takahashi et al., 1987). There are also reports of calcium binding protein expression in the torus

semicircularis of turtle and in diencephalon of lizard (Belekhova et al., 2004; Dávila et al., 2000).

The auditory system has been characterized by many special features in order to phase lock the sound accurately. These include large calycine axosomatic endings in the cochlear nuclei and calcium-permeable AMPA receptors (Otis et al., 1995; Parks, 2000; Raman et al., 1994; Trussell, 1999). Through buffering internal calcium, regulating Ca2+-sensitive ion channels and activation of second-messenger systems (Trussell, 1999), many potential roles for calcium binding proteins have been suggested, particularly in the auditory system where calcium binding proteins could be involved in temporally precise responses such as sharpening action potentials and modulating neurotransmitter release (Baimbridge et al., 1992; Rogers, 1987). These proteins might also buffer calcium to protect neurons from calcium mediated excitotoxicity (Lukas and Jones, 1994) .

I investigated the expression of three calcium binding proteins, CR, CB and PV, in the auditory system of the gecko. The auditory nerve and its presynaptic terminals surrounding the neurons of first order auditory nuclei expressed all three Calcium binding proteins. In mammalian spherical bushy cells and the cells of the Medial Nucleus of the trapezoid body and in the avian NM, a single stimulus generates considerable transmitter quanta from each axon terminal in order to drive the resulting EPSP to threshold quickly and reliably (Borst and Sakmann, 1996; Zhang and Trussell, 1994). Calcium binding proteins in the presynaptic auditory nerve terminals and nerve branches may facilitate precisely timed neurotransmitter release (Rogers, 1987). The neurons of NM have action potentials locked to the phase of

sound wave and a high rate of spontaneous activity at the presence of postsynaptic calcium permeable AMPA receptors (Otis et al., 1995; Raman and Trussell, 1995; Raman et al., 1994; Trussell, 1999). The presence of CR and CB in the cytoplasm of NM suggests a role in restricting high calcium levels during synaptic activation of NM neurons. The somata of first order auditory nuclei in gecko also showed no parvalbumin expression. These complementary calcium binding protein distributions may indicate possible different mechanisms of regulate calcium in different regions of the auditory brainstem. CR might play a role in calcium regulation in the first order nuclei, since it is expressed in NM and NA of gecko. Moreover, CR expression is also found in the somata of first order auditory nuclei of many other vertebrates, including birds (chick (Parks et al., 1997; Rogers et al., 1989) and owls (Kubke et al., 1999) and mammals (rat and guinea pig (Caicedo et al., 1996; Lohmann and Friauf, 1996)).

CB-ir was also observed in auditory nerve terminals and in the cytoplasm of the first order nuclei, while Pv-ir only labeled the auditory nerve terminals. In the chick NM, the distribution of calretinin changes during development from diffuse to highly concentrated beneath the plasma membrane (Hack et al., 2000). Its location in older chicks is very similar to that of CB in gecko NM, and it has been suggested that it might buffer the large calcium influx underneath the plasma membrane during high discharge rate (Hack et al., 2000).

CR, CB and PV had different distribution patterns in the torus semicircularis. Most CB-ir neurons in central nucleus of TS were not CR-ir. PV and CR were both expressed in the central nucleus, but in different sub-cellular locations.

A diagram summarizing the CaBP expression in the auditory system of gecko was provided in Figure 20. In summary, calcium binding proteins may be important to maintain the normal functions of the ascending auditory pathways and they are good markers to delineate the auditory system of *gecko*.

4.2 Central projection of cochlear nerve and first order nuclei in Gekko gecko

The gecko is the only lizard that uses vocalizations in intraspecific communication and aggressive warning sounds (Marcellini, 1977). It has unique peripheral auditory structures (Christensen-Dalsgaard and Manley, 2005; Manley, 2002; Manley et al., 1999). It has two types of hair cells in different areas along the auditory papilla, which are unidirectional and bidirectional types. The unidirectional hair cells respond to lower frequency sound and they are covered by a tectorial membrane. The bidirectional hair cells respond to higher frequency sound.

The middle ear cavities of Tokay *geckos* are connected through the mouth cavity, which could enhance the directionality of the ear by allowing sound access to both sides of each tympanic membrane, either canceling or enhancing its motion. This results largely in directional responses so that the signal from one ear has higher or lower amplitude than the other ear (Christensen-Dalsgaard and Manley, 2005). Geckos appear to have similar patterns of cochlear nerve central projection to alligator lizards. In alligator lizards, the tectorial fibers that contact unidirectional type hair cells, low-frequency region (<1kHz), project to three of the four divisions of the first order nuclei, which are NMM, NML and NAL (Szpir et al., 1990); the free-standing auditory nerve fibers that innervate the bi-directional high-frequency hair cells, project only to the NAM (Szpir et al., 1990). This organization is also in

agreement with the structure we found in the gecko. Our immunohistochemistical studies of calcium binding proteins, GAD and SV2 suggest there is a population of dorsal-caudal auditory nerve fibers that enter the auditory turbucle and bifurcate caudally to the NM and rostrally to lateral NA. The second population in a ventral-rostral fiber bundle was located more dorsally and consisted of a single branch that projected rostrally to medial NA. Again, those findings are consistent with the results of studies done in alligator lizard (Szpir et al., 1990). Our immunohistochemical studies combined with previous studies in alligator lizard support the hypothesis that there were two populations of auditory nerve fiber, one which contact unidirectional hair cells (low-frequency) and the other the bi-directional hair cells (high-frequency). Note that we still lack direct electrophysiological and anatomical evidence that these two nerve populations associate with the two types of hair cells and correspond to different range of frequencies.

The first order nuclei of the lizard show a great deal of interspecific variation. In many lizards, four nuclear populations that receive primary auditory inputs have been recognized: a medial and lateral nucleus magnocellularis and a medial and lateral angularis (Miller, 1975). Alligator lizard also have a distinct NMM, NML, NAM and NAL (Szpir et al., 1995). Only some lizards have a distinct NL structure (Ten Donkelaar et al., 1987). Conflicting reports exist in the literature because of the variation in the development of the nucleus magnocellularis and laminaris. Based on our present studies, NMM, NML, NAM, NAL and NL were recognized in *Gekko gecko* although an NL was not previously observed in this species (Miller, 1975). There were large ovoid cell bodies in NMM surrounded by perisomatic auditory

nerve endings labeled with CR, PV and SV2. Those terminals resembled the end-bulbs in the avian NM and the mammalian AVCN (Grothe et al., 2005). These similarities suggest that there is a homologous population corresponding to the NM of birds and turtles (Carr and Code, 2000). Whether the bushy cells in the mammalian AVCN are derived from the same ancestral population as the reptilian NM is unknown.

In gecko, NL was composed of two strands of CR-ir bipolar neurons with long dorsoventrally directed dendrites located just ventral to NM. This NL region merged laterally into NM. The location and CR-ir nature of the NL cells support the hypothesis that NL may be derived from NM. This property can also be observed in birds (Grothe et al., 2005). The *gecko* NL resembles the previously described NL in *Varanus exanthematicus* (Ten Donkelaar et al., 1987). Finally, our unpublished data indicates that the contralateral NL receives labeled fibers after injected tracer in NM (Tang et al. unpublished). The origins of NL are controversial; is NL common to all Reptilia, or is the lizard NL independently derived with respect to NL in birds? It is possible that the lepidosaur NL developed before and the split with archosaurs and is therefore homologous to the avian NL. At present I define it as NL according to its chemical neuroanatomy, position, structure, connection and the similarity to NL in *Varanus exanthematicus*. More physiological and developmental data are needed to confirm that this structure in *gecko* corresponds to the NL described in archosaurs.

4.3 The organization of auditory midbrain in Gekko gecko

The torus semicircularis (TS) is the auditory midbrain target in reptiles. TS of *gecko* was divided into the central (TSc) and the laminar (TSl) nucleus without a

obvious superficial nucleus being observed in our immunohistochemical studies. The central nuclei of TS is the main recipient zone of ascending inputs from the lower brainstem and projects to the nucleus medialis of the dorsal thalamus (Foster and Hall, 1978; Kennedy and Browner, 1981; Ten Donkelaar et al., 1987). The reported auditory responses in previous studies were probably recorded in the TSc in *Gekko gecko* (Kennedy, 1974; Manley, 1981).

The distribution of calcium binding protein immunoreactivity in the central nucleus of the TS was similar to the expression patterns in lower brainstem auditory structures. The CR-ir terminals at rostral-lateral portion of TSc resembled the pattern of CR staining in SO and NLL. I hypothesize that the CR-ir terminals in the olivary and lemniscal nuclei and the lateral portion of TSc all originate from the CR-ir first order nuclei. This assumption needs to be tested by both physiological and tract tracing studies.

Whether the organization of the *gecko* TS is tonotopic remains unknown. The auditory midbrain of mammals and birds is tonotopically organized, though the pattern and frequency range are different (Grothe et al., 2005). Caiman and *Tupinambis nigropunctatus* exhibit tonotopic organization of the central nucleus of the auditory midbrain (Browner and Rubinson, 1977; Manley, 1971), but Tokay *geckos* exhibit a unique reversed tonotopic organization of their basilar papilla (Manley et al., 1999). The unidirectionally oriented hair cells (below 1kHz) lie in the basal region, while the apical region consists of bidirectionally-oriented hair cells with best frequencies between 1kHz to 5kHz (Manley et al., 1999). Also, the auditory nerve is tonotopically organized. Low-CF fibers were found at the posterior edge of

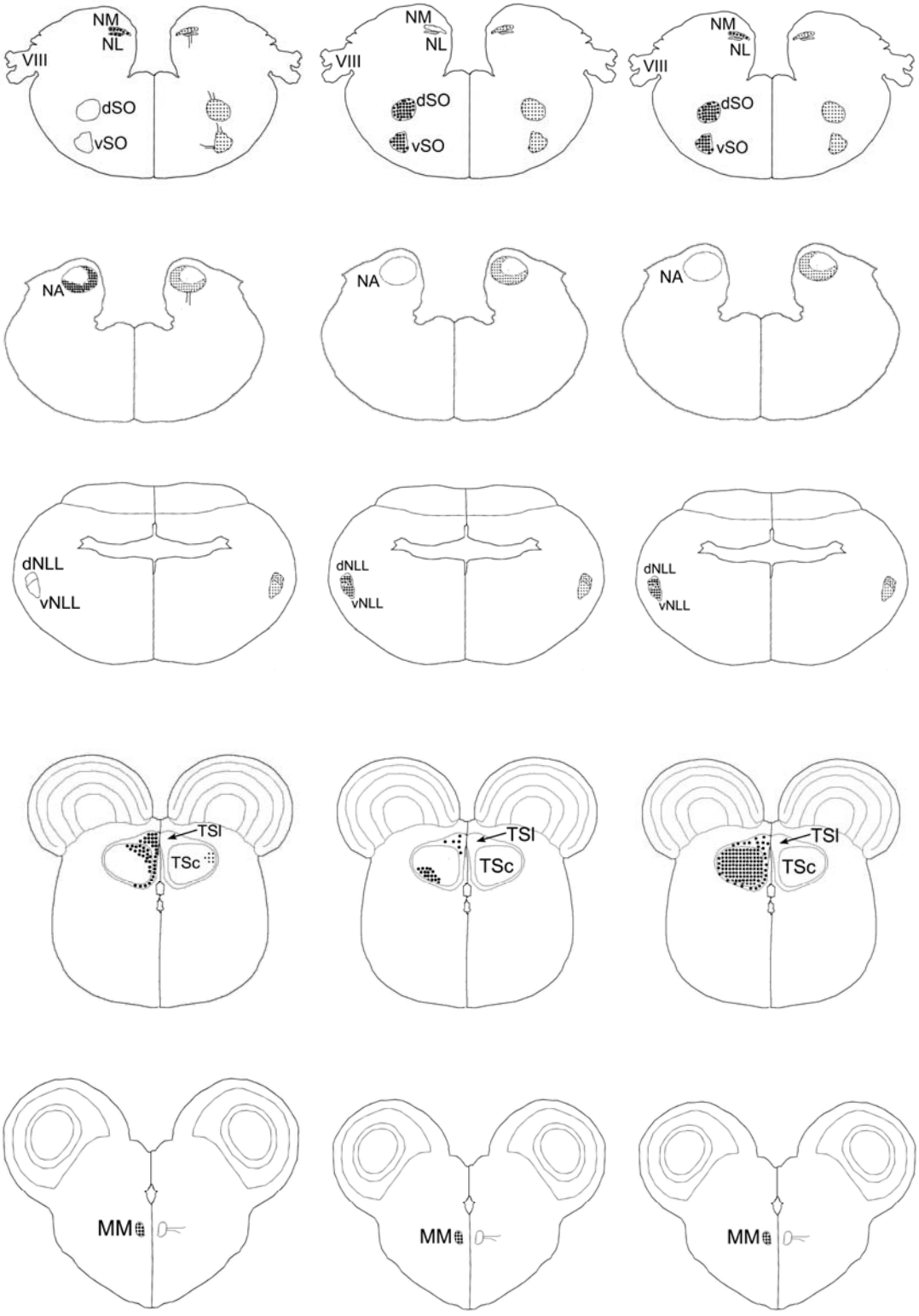
the posterior branch of the eighth nerve, and high CFs lay deep in the anterior edge (Eatock et al., 1981). It is hard to determine the complete tonotopic organization in NM of Tokay *gecko* due to its small size, although Manley reported a rough tonotopic organization in tokay's cochlear nuclei (Manley, 1972). Low CFs are represented caudomedially, and high CF rostrolateral. Hence, the tonotopic organization of the basilar papilla, nerve and cochlear nuclei suggests that tonotopic organization should remain in torus.

Finally, the immunoreactivity for CR, PV and CB displayed distinct distribution patterns in gecko central nucleus of TS. This suggests the presence of subdivisions within the central nucleus, and different recipient zones for olivary and lemniscal input. Tract tracing and electrophysiology experiments are being performed in parallel to answer these questions about the organization of gecko TS.

The laminar nucleus of TS in *gecko* has been implicated in control of vocalization (Kennedy, 1975). It may correspond to the avian intercolliclular nucleus because of its connection and role in vocalization (Díaz et al., 2000). All of the three calcium binding protein were expressed in the laminar nucleus.

Figure 20. Schematic drawings summarize the distribution of calcium binding proteins in the auditory nuclei of brainstem, midbrain and thalamus of *Gekko gecko*. The distribution of cell bodies is shown at left with cell body distribution shown as large dark dots and terminals at the right half sections with fine dots.

Calretinin Parvalbumin Calbindin



Chapter 5: Conclusions and future research

Calcium binding proteins were highly expressed in the auditory system of the gecko. All midbrain and hindbrain auditory nuclei were labeled with antibodies against Calretinin, Parvalbumin, Calbindin and GAD. Two first order nuclei, NM and NA, and NL, olivary and lemniscal nuclei were identified. Calretinin immunoreactivity delineated the projections between the first order nuclei, NL and the superior olivary nuclei, the nuclei of the lateral lemniscus and the torus semicircularis. In the auditory midbrain, the distribution of calretinin, parvalbumin and calbindin bear different patterns. Nucleus Medialis of the thalamus was delineated by calcium binding protein immunoreactivity.

Based on the present preliminary data about *gecko*'s auditory structures and connections along with previous comparative studies (Carr and Code, 2000), geckos appear to share similar auditory structures and connections with other land vertebrates. The auditory nerve projects to the first order nuclei, NM and NA in birds and lizards, or the cochlear nucleus in mammals. The NM connects to the second order nuclei nucleus laminaris. Also, the first order nuclei had connections to the superior olive.

Many unanswered questions about the auditory system of the gecko remain. Little is known about the cytoarchitecture of the auditory nuclei, their connections and their physiological organization. The present preliminary studies on the gecko auditory structures provided us with a framework for future tract tracing and molecular studies in the gecko.

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