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Complementary multimodal compartments in the developing inferior colliculus

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Complementary Multimodal Compartments in the Developing Inferior Colliculus

An Honors College Project Presented to
the Faculty of the Undergraduate
College of Science and Mathematics
James Madison University

By Roxana Behrooz
May 2017

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors College.

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Finally, I would like to thank my parents for their love and support.
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Complementary Multimodal Compartments in the Developing Inferior Colliculus

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ABSTRACT:

The auditory system is responsible for detecting, encoding, and deciphering hearing. The inferior colliculus (IC) is a major relay hub situated in the midbrain, that is subdivided into a central nucleus, and surrounding dorsal and lateral cortices. The central nucleus of the inferior colliculus (CNIC) is organized tonotopically based on a frequency gradient and strictly processes auditory information. In contrast, recent studies show that the lateral cortex of the inferior colliculus (LCIC) is actually multimodal, receiving inputs from not just auditory sources, but also somatosensory and visual structures. The precise organization of patterned inputs to the LCIC and their development has yet to be fully established. Mounting evidence suggests a modular LCIC framework with surrounding extramodular zones that provide an anatomical substrate for input-output arrays. Previously, a series of histochemical and immunocytochemical stains including acetylcholinesterase (AChE), cytochrome oxidase (CO), glutamic acid decarboxylase (GAD), nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), and parvalbumin (PV) were identified as discrete markers of LCIC layer 2 modular fields. The present study builds upon these findings and establishes calretinin (CR), a calcium binding protein, as a complementary extramodular marker. CR-specific labeling was observed in LCIC zones surrounding presumptive layer 2 modules at all ages, yet became increasingly more distinct at later developmental stages. This finding somewhat contrasts previous results in developing rat in which LCIC CR patterns were more evident prior to hearing onset (Lohmann and Friauf, 1996). NADPH-d and CR double-labeling confirms a complementary modular/extramodular LCIC substrate that is established during the early postnatal period. Similarly organized Eph-ephrin guidance expression patterns and developing multimodal projection patterns suggest that this arrangement is functionally important. Understanding the neuronal development of the
modular/extramodular architecture of the LCIC is crucial in future development of therapeutic
treatments regarding brain plasticity and tinnitus.

**INTRODUCTION:**

The auditory system is involved in detecting and deciphering auditory inputs, and it consists of
peripheral and central components. The peripheral system begins with sensory receptors and
includes structures of the ear such as the Organ of Corti, receptor hair cells, the spiral ganglion,
and the auditory nerve. The brain and spinal cord comprise the central nervous system, and
central auditory aspects include many complexes in the brainstem and midbrain, beginning with
the cochlear nucleus. The inferior colliculus (IC) is considered the crossroads of auditory
processing as it is situated in the midbrain and links lower brainstem centers with higher regions
of thalamus and cortex. The IC is subdivided into a central nucleus, lateral cortex, and dorsal
cortex (CNIC, LCIC, DCIC). The central nucleus is organized tonotopically on a frequency
continuum, much like most other parts of the ascending system. In contrast, the lateral cortex is
not strictly tonotopic, but rather exhibits a discontinuous, modular organization (Gruters and
Groh, 2012; Stebbings et al., 2014).

The development of this modular or patchy arrangement is not solely dependent on the
organism’s experience. The specificity of the synaptic connections within the IC develop largely
prior to the organism’s ability to process sound. This is in large part due to the guidance of
proteins in the brain that help steer developing axonal connections. For example, Eph-ephrins, a
family of signaling molecules, instruct specific axonal arrangements and appropriate mapping of
target regions (Flanagan et al., 1998). Eph-ephrin expressions previously studied in the Gabriele
laboratory appear similar to terminal field distributions of many tract-tracing studies that hint at a
modular LCIC organization (Wallace et al. 2016). Understanding the emergence of the micro-
organization of the LCIC should provide insights regarding multimodal functional
responsibilities of this structure.

A variety of staining methods can provide insights concerning the anatomical substrate of
a given brain structure. A series of neuro- and immunohistochemical approaches have suggested
a functional compartmentalization of the LCIC (Chernock et al., 2004). One marker, GABA, is
an inhibitory neurotransmitter synthesized by glutamic acid decarboxylase (GAD) enzymes. The
GAD65 isomer is located primarily in axon terminals and membranes, while GAD67 is localized
in neuronal somata (Burianova et al., 2009). GAD 65/67 staining reveals a network of discrete
LCIC modules in a variety of adult species. GABAergic LCIC staining is quite similar to other
markers including, parvalbumin (PV), cytochrome oxidase (CO), acetylcholinesterase (AChE),
and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d). Of these, NADPH-d
appears to be the most reliable and distinct modular marker in developing mouse (Dillingham
and Gabriele, 2016).

PV is one calcium-binding protein that is important for regulating intracellular calcium
levels. Calretinin (CR) has also been used effectively as stains of specific neuronal
subpopulations to provide insights regarding distinct organizational features. PV
immunoreactivity is more prevalent in the CNIC while also expressed in LCIC in a modular
fashion. In contrast, preliminary findings suggest CR is more prevalent in the lateral cortices of
rat (Ouda and Syka, 2012). Thus, PV is an effective modular marker, while CR may provide a
means for highlighting surrounding extramodular regions. In the rat auditory brainstem, PV
levels increase with age, while CR levels decrease (Lohmann and Friauf, 1996). Specifically, CR
IC expression is evident as early as E20 with decreased CR labeling up to P20.
CR expression in the IC has been examined as mentioned above in a variety of species, including rat and adult mouse (Zettel et al., 1997; Lesicko et al., 2016). However, its development and examination as a reliable extramodular LCIC marker had not previously been studied in mice. This project aims to describe the distribution of CR immunoreactivity in the lateral cortex of the IC in developing mice. Because CR appears to preferentially stain extramodular zones in rat, it is hypothesized that CR will serve as an extramodular marker in developing mouse. In addition, it is anticipated that double-labeling experiments combining CR staining with the best previously identified modular marker, NADPH-d, will result in a complementary organization. Such an arrangement might suggest segregation of inputs into unimodal compartments that are then integrated between modular and extramodular fields (Lesicko et al., 2016).

MATERIALS AND METHODS:

Animals

Experiments were performed on C57BL/6J control mice (Jackson Laboratories, Bar Harbor, ME). Developmental ages were examined leading up to (postnatal day 0, 4, 8) the onset of hearing (postnatal day 12) and to postnatal day 20. All experimental procedures were performed in compliance with the National Institute of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80-23, revised 1996) and received prior approval from the Institutional Animal Care and Use Committee. (IACUC Protocol No. A14-15)
**Tissue fixation and Sectioning**

At designated developmental stages, animals were perfused transcardially with a physiological rinse followed by a 4% paraformaldehyde fixative solution. Brains were harvested from the skull and placed in 4% paraformaldehyde and 10% sucrose followed by 30% sucrose for 24 hours at 4°C to ensure cryoprotection. A needle mark was placed through the ventral brainstem for orientation purposes. A block of brain tissue including the auditory brainstem and midbrain was cut at 50 µm on a sliding freezing microtome and collected in 0.1M phosphate buffer. For reconstruction cases that require maintaining serial order, sections were collected systematically and processed in a grid.

**Calretinin Immunohistochemistry**

A quench step for endogenous peroxide activity was performed with 0.6% peroxide in PBS for 10-15 minutes. Next, free floating sections were rinsed three times with PBS for 10 minutes each. Prior to incubation in primary antibody (rabbit anti-calretinin, 1:5000, SWANT Cat. No 7697; serial dilution experiments were conducted to determine the optimal CR concentrations) for 48 hours at 4°C, a blocking step was performed using 5% normal horse serum (NHS) in PBS. Following incubation, sections were allowed to equilibrate to room temperature for 20 minutes. After three 5 minute PBS rinses, an anti-rabbit Impress reagent kit (Vector Labs, Cat. No MP-7401) was applied at room temperature for 30 minutes. Sections were once again rinsed in PBS for three 5 minute intervals. Next, a DAB (diaminobenzidine) reaction was performed (Vector Labs, Cat. No SK-400) either with or without a nickel enhancement to visualize CR expression. Tissue was then rinsed for three 5 minute intervals in PBS, mounted on gel-subbed slides, and allowed to dry overnight. Slides were then coverslipped after dehydration and clearing steps utilizing alcohols and xylenes.
For NADPH-d and CR double-labeling, animals were perfused and sections of the brain were cut down in the same manner that was previously mentioned. Then, three 10 minute PBS rinses were completed. Afterwards, Tris Buffer, Malic acid sodium salt, NADPH, Nitroblue tetrazolium, and Triton-X were sonicated and stirred until they were dissolved. Sections were incubated at 37°C for 60 minutes. After this, six 20 minute PBS rinses were completed followed by the CR chemistry mentioned above.

**Image Capturing and Processing**

To assess the organization of CR LCIC staining hypothesized to be extramodular and periodic, brightness profiles were generated. A freehand tool in ImageJ set to a line thickness of 20 pixels was used to draw curved contours to sample acquired raw image data. Sampling was done from ventral to dorsal, bisecting presumptive Layer 2 modular fields. A minimum of three sections were sampled per case along the rostrocaudal extent of the LCIC. Data from brightness profiles were analyzed to confirm the presence of periodic trends in the CR staining. Averaged autocorrelation function maxima > 0.6 (1.0= perfect periodic signal, 0= no periodicity) served as an objective criteria to verify the presence of periodic LCIC staining, and maxima fitting this criteria was checked for biological significance by reexamining captured images used to generate brightness profiles (Wallace et al., 2013). Matching of montaged images and slight adjustments to brightness and contrast were made using Adobe Photoshop.

**3-Dimensional Reconstructions**

To reconstruct 3-dimensional images of neurochemical marker data, Neurolucida software was used as sections were aligned using a four-point (minimum) match of easily identifiable landmarks. Some landmarks that were used include the dorsal aspect of the IC commissure,
dorsal and ventral aspects of the cerebral aqueduct, section contour of the ventral midline, and other easily distinguishable vasculature and midline structures.

**RESULTS:**

**NADPH-d Is Most Reliable LCIC Modular Marker:**

There is distinct LCIC Layer 2 modular labeling for the neurochemical markers AChE, NADPH-d, GAD, and CO (Figure 1 and Figure 2). NeuroLucida reconstruction alignments at hearing onset using fiduciary landmarks show the strong spatial registry among modular markers (Figure 3). The modular organization, particularly of NADPH-d, is apparent at all time points, increases in clarity leading up to the onset of hearing, and is strikingly defined by postnatal day 20 (Figure 4). The Layer 2 modules are most distinct in the mid rostral-caudal coronal sections (Figure 4B-D), typically with 6-7 patches surrounded by Layer 1 and Layer 3 void regions. However, thin bridges of positive labeling often connect neighboring modules in caudal extremes, and modules appear to begin to converge together in rostral extremes (Figure 4E, F). The size of each module varies depending on the relative plane of the section and the anatomical location.

**Calretinin Specifically Labels LCIC Extramodular Fields:**

CR staining is used to highlight surrounding extramodular domains concentrated in Layer 1 and Layer 3 of the LCIC. Similar to that of NADPH-d, CR labeling increases in clarity leading up to the onset of hearing until it is fully defined at P20 (Figure 5 and Figure 6). Also mirroring the morphology of NADPH-d, CR extramodular zones are most apparent in mid rostrocaudal
sections, and the labeled extramodular areas appear complementary to NADPH-d positive modules across the rostral-caudal axis (Figure 5).

**Complementary Nature of NADPH-d Modular and Calretinin Extramodular Patterning:**

NADPH-d modular and CR extramodular labeled regions appear complementary in a variety of ways. In age matched tissue, CR positive cell bodies appear localized to regions surrounding developing modular fields. This pattern becomes increasingly evident by P4, easily recognizable by P8, clearly defined by hearing onset, and most striking at P20 (Figure 6). Sampling performed at P20 along LCIC Layer 2 contours generate brightness profiles that show strong periodic signals for both NADPH-d (Figure 7A) and CR (Figure 7B). Plots taken from matching rostrocaudal sections at consistent LCIC dorsoventral positioning yield NADPH-d and CR signal fluctuations that appear out of phase with one another, suggesting complementary modular and extramodular arrangement of the LCIC (Figure 7).

**Double-labeling Confirms NADPH-d and Calretinin as Complementary Markers for LCIC Patch-Matrix-like Arrangement:**

Double-labeling studies show similar developmental patterns and confirm previously discussed single-labeling studies. Evidence of early compartmental precision and clear segregation at P0 was lacking due to less intense NADPH-d staining at that age (Figure 8A, D, G, J). However, there is considerable separation of NADPH-d modular and CR extramodular staining by P8 (Figure 8B, E, H, K), and this organization becomes clearly discernible by P20 (Figure 8C, F, I, L). This non-overlapping arrangement is evident at all rostrocaudal levels of the LCIC, but is most clear at mid-rostrocaudal levels where Layer 2 modules are most distinct (Figure 9).
Although there are some NADPH-d labeled neurons outside the modules and some CR positive cells within the modular confines, double-labeling studies confirm the largely complementary nature of NADPH-d Layer 2 patches with encompassing domains. Interestingly, there was no evidence of double-labeled LCIC neurons throughout the developmental stages studied (Figure 10). Double-labeled neurons were only observed in aspects of the midbrain just rostral to the LCIC, particularly in the intercollicular nuclei and deep layers of the superior colliculus (Figure 11).

**DISCUSSION:**

This study establishes CR as an extramodular marker in the LCIC of mice, presents the development of CR labeled neurons in mice, and demonstrates the complementary nature of CR zones with the previously identified modular marker, NADPH-d. CR is present in the LCIC at birth, becomes increasingly more evident with postnatal development, and exhibits variable patterning throughout the rostrocaudal dimension of the LCIC that is keeping with modular changes along this same axis. These findings somewhat contrast previous literature in developing rat which showed CR labeling decreasing in the midbrain with increasing age (Lohmann and Friauf, 1996). Further experimentation examining the spatial overlap of these neurochemical stains with Eph-ephrin signaling molecules and afferent-efferent patterns should provide insights concerning LCIC functional zones and circuit development.

**LCIC Functionality, Form and Function:**

The LCIC is a multimodal structure, processing primarily to auditory and somatosensory information (Lesicko et al., 2016). The physical structural organization of the LCIC perhaps mirrors its physiological function. Auditory signaling from areas such as the auditory cortex and
CNIC primarily terminate in the extramodular regions of the LCIC. In contrast, somatosensory inputs from structures such as the somatosensory cortex and dorsal column nuclei terminate in the LCIC modular regions (Lesicko et al., 2016). Future tract-tracing experiments are planned in conjunction with previously described histochemical and immunocytochemical staining to further examine the nature of LCIC compartments and the potential for multisensory integration between modality-specific subregions.

**Eph-ephrin modularity:**

Certain members of the Eph-ephrin signaling family exhibit the same patterning in the LCIC as the modular/extramodular neurochemical markers presented here (Wallace et al., 2016). Ephrin-B2 and EphA4 expression is localized to LCIC Layer 2 modular regions, while ephrin-B3 is concentrated within Layer 1 and Layer 3 extramodular zones (Figure 12). The development of the overall modular/extramodular pattern in the LCIC is likely due to Eph-ephrin guidance mechanisms, but certainly this notion requires considerable subsequent experimentation.

**Tinnitus and Neuroplasticity:**

Tinnitus is the perception of a phantom auditory stimulus, typically characterized by a perceived ringing in the ears, during which there is an increase in spontaneous firing in the central system. It can be caused by aging, disease, and trauma. Understanding the structure of the LCIC and the overall relationship between the auditory and somatosensory systems can help with the development of therapies to improve the quality of life of those with this debilitating condition. For instance, the interconnected nature of the sensory systems has helped establish Multimodal Synchronization Therapy, during which multiple sensory systems are stimulated in a non-invasive manner, as a possible treatment option for those with tinnitus (Markovitz et al., 2015). Somatosensory stimulation in particular has been shown to be fruitful in decreasing the abnormal...
firing patterns of certain auditory neuronal populations associated with tinnitus. For instance, when patients with tinnitus underwent physical therapy for 6 weeks, 53% experienced substantial improvement (Michiels et al., 2017). Better understanding of the anatomy of the multimodal LCIC where different modalities are present and likely influence one another could help determine LCIC’s role in this process.

CONFLICTS OF INTEREST STATEMENT:

The authors have no conflicts of interest.

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FIGURE LEGENDS:

**Figure 1.** Low magnification photomontages during the early postnatal period showing distinct LCIC Layer 2 modular labeling (*arrowheads*) for AChE (A), NADPH-d (B), GAD (C), and CO (D). Scale bars = 500 μm.

**Figure 2.** Higher magnifications of inset boxes in Figure 1 highlighting distinct LCIC layer 2 modular labeling (*dashed contours*) for AChE, NADPH-d, GAD, and CO. Scale bars = 500 μm.

**Figure 3.** Serial reconstruction of adjacent coronal sections stained for NADPH-d (blue) and CO (orange) at hearing onset.

**Figure 4.** Caudal-to-rostral distribution (A-F) of NADPH-d modular staining in LCIC Layer 2 in the coronal plane at P20. Scale bars = 500 μm.

**Figure 5.** Caudal-to-rostral distribution (A-F) of CR staining at P20 showing extramodular staining surrounding modular devoids (*arrowheads*). Scale bars= 500 μm.

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**Figure 10.** High magnification of progressively more defined and complementary NADPH-d/CR patterns at birth (A), P8 (B), and P20 (C). Despite some CR positive neurons being evident within modular confines, as well as some NADPH-d cells in extramodular zones, no double-labeled neurons were observed throughout the LCIC. Scale bars = 50 µm.

**Figure 11.** Double-labeled NADPH-d and CR positive neurons rostral to the LCIC in the intercollicular nuclei and deep layers of the superior colliculus. Scale Bar in A= 300 µm, B= 50 µm.

**Figure 12.** Summary figure of present neurochemical findings taken together with known input-output patterns and modular and extramodular Eph-ephrin LCIC expression.
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