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Modular-extra modular organization in developing multisensory shell regions of the mouse inferior colliculus

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Abstract

The complex neuroanatomical connections of the inferior colliculus (IC) and its major subdivisions offer a juxtaposition of segregated processing streams with distinct organizational features. While the tonotopically layered central nucleus is well-documented, less is known about functional compartments in the neighboring lateral cortex (LCIC). In addition to a laminar framework, LCIC afferent-efferent patterns suggest a multimodal mosaic, consisting of a patchy modular network with surrounding extramodular domains. This study utilizes several neurochemical markers that reveal an emerging LCIC modular-extramodular microarchitecture. In newborn and post-hearing C57BL/6J and CBA/CaJ mice, histochemical and immunocytochemical stains were performed for acetylcholinesterase (AChE), nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), glutamic acid decarboxylase (GAD), cytochrome oxidase (CO), and calretinin (CR). Discontinuous layer 2 modules are positive for AChE, NADPH-d, GAD, and CO throughout the rostrocaudal LCIC. While not readily apparent at birth, discrete cell clusters emerge over the first postnatal week, yielding an identifiable modular network prior to hearing onset. Modular boundaries continue to become increasingly distinct with age, as surrounding extramodular fields remain largely negative for each marker. Alignment of modular markers in serial sections suggests each highlight the same periodic patchy network throughout the nascent LCIC. In contrast, CR patterns appear complementary, preferentially staining extramodular LCIC zones. Double-labeling experiments confirm that NADPH-d, the most consistent developmental modular marker, and CR label separate, nonoverlapping LCIC compartments. Determining how this emerging modularity may align with similar LCIC patch-matrix-like Eph/ephrin guidance patterns, and how each interface with, and potentially influence developing multimodal LCIC projection configurations is discussed.

Keywords

auditory; histochemistry; immunocytochemistry; mapping; mosaic; multimodal; patch-matrix; striosome; RRID: AB_11210186; RRID: AB_2619710

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1. Introduction

Neuroanatomical approaches often provide valuable insights concerning the fine structure organization or microarchitecture of brain regions that appear nondescript in classical stains or Golgi preparations. Histochemical and immunocytochemical methods are particularly useful for highlighting specific neuronal subsets against seemingly homogeneous backgrounds. Studies comparing various neurochemical markers within a given structure and their relative degree of overlap have proved important for defining functional zones or compartments, thereby providing a more comprehensive understanding of network complexities (Burwell, 2001; Gerfen, 1992; Ma, 1993; Mana & Chevalier, 2001; Soares et al., 2001; Wallace 1986a,b).

The inferior colliculus (IC) is typically portrayed as a midbrain relay hub in the ascending auditory system. This depiction, while fitting for its tonotopically-arranged central nucleus (CNIC), does not aptly describe its surrounding shell nuclei (the lateral cortex of the IC, LCIC, and the dorsal cortex of the IC, DCIC), that receive a rich array of multimodal inputs (Coleman & Clerici, 1987; Gruters & Groh, 2012; Lesicko, Hristova, Maigler, & Llano, 2016). Many of these projections specifically target the LCIC and exhibit highly compartmentalized terminal distributions in a variety of species (Stebbins, Lesicko, & Llano, 2014). Somatosensory and auditory afferents, in particular, appear largely segregated in the adult mouse LCIC (Lesicko et al., 2016). Somatosensory inputs ascending from the spinal trigeminal and dorsal column nuclei in the brainstem, along with descending projections from somatosensory cortex, terminate within a discontinuous patchy or modular LCIC layer 2 lattice. In contrast, auditory inputs from the CNIC and auditory cortex exhibit seemingly complementary terminal fields, preferentially targeting surrounding presumptive LCIC extramodular zones.

This recently described LCIC connectional modularity appears to correlate with previous reports of an underlying neurochemical framework. A distinct set of LCIC modules that span layer 2 have been most thoroughly described in the adult rat (Chernock, Larue, & Winer, 2004) and mouse (Lesicko et al., 2016; Stebbins et al., 2014) for a variety of markers, including acetylcholinesterase (AChE), nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), cytochrome oxidase (CO), glutamic acid decarboxylase (GAD), and parvalbumin (PV). Analogous markers that emphasize surrounding LCIC extramodular zones, however, have yet to be identified. Although not explicitly stated, data from a single study in rat suggests calretinin (CR) may be such a marker (Lohmann & Friauf, 1996).

Understanding how circuit arrangements interface with defined neurochemical and metabolic markers should provide insights concerning the functional significance of the patch-matrix-like LCIC configuration. Currently lacking is an appreciation for the development of its micro-organization and the mechanisms that shape early LCIC target zones and their multimodal connections. This study describes the emergence of neurochemically-defined LCIC modular fields, focusing exclusively on AChE, NADPH-d, CO, and GAD, as preliminary findings for PV suggest it is a weak modular marker in neonatal mice. CR is identified as a novel, highly specific extramodular marker and is

compared with LCIC modular patterns. Experiments pairing modular staining with CR-labeling in the same tissue reveal complementary LCIC zones that are clearly delineated early in development. The described developing neurochemical framework is discussed in light of previous findings from our lab that show similar modular-extramodular patterns revealed by Ephrephrin guidance molecule expression (Cramer & Gabriele, 2014; Gabriele et al., 2011; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). Implications regarding potential signaling mechanisms that may influence the specificity of early multimodal LCIC inputs are addressed.

2. Materials and Methods

2.1. Animals

Experiments were performed on C57BL/6J ($n = 59$) and CBA/CaJ ($n = 15$) control mice (total 74 mice, Jackson Laboratories, Bar Harbor, ME). Nearly equal numbers of males and females were utilized and included in the findings. Developmental ages were examined ($n = 3$ at each age for each marker) leading up to (postnatal day 0, 4, 8) and including the onset of hearing (postnatal day 12, P12), as well as a later stage nearing weaning (P20). All experimental procedures were performed in compliance with the National Institutes 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 (Protocol No. A14–15).

2.2. Tissue fixation and sectioning

Mice were injected intramuscularly with an overdose of ketamine (200 mg/kg) and xylazine (20 mg/kg), transcardially perfused with a physiological rinse (0.9% NaCl, 0.5% NaNO₂ in deionized water), followed sequentially by 4% paraformaldehyde and 10% sucrose in 4% paraformaldehyde solutions (pH 7.4). Brains were removed and post-fixed at 4 °C (4% paraformaldehyde with 10% sucrose) before transfer to a final cryoprotective solution of 30% sucrose in 4% paraformaldehyde. Fixed tissue was blocked in the coronal plane rostral to the superior colliculus (SC) and at the caudal extreme of the brainstem. A needle mark was placed through the ventral brainstem for orientation purposes. Coronal sections were collected in 0.1 M phosphate buffer (pH 7.4). For reconstruction cases requiring serial order, sections were collected systematically and processed in a grid.

2.3. Immunohistochemistry

Processing of free-floating sections began with three phosphate buffered saline (PBS) rinses for 10 min each, followed by a quench of endogenous peroxidase activity (0.6% H₂O₂ in PBS for 10 min), and then again three more PBS rinses. Next, sections were incubated for 30 min in a blocking solution (5% normal horse serum). Serial dilution experiments were conducted to determine optimal concentrations for each of the primary antibodies. Primary antibody solution for GAD (anti-GAD65/67 made in rabbit, 1:3,000, AB1511, Millipore, RRID: AB_11210186) or CR (anti-CR, made in rabbit, 1:5,000, CR 7697, Swant, RRID: AB_2619710) was applied and sections were agitated overnight or up to 48 hr at 4 °C. Following incubation, tissue was allowed to equilibrate to room temperature prior to three PBS rinses. Sections were then agitated in IMPRESS reagents (anti-rabbit IgG detection kit,

MP-7401, Vector Laboratories, Burlingame, CA) for 30 min. Sections were once again rinsed in PBS prior to detection with a 3,3'-diaminobenzidine peroxidase horseradish peroxidase (HRP) substrate kit (with or without nickel, SK-4100, Vector Laboratories). Tissue was then washed a final time in PBS, mounted on gel-subbed slides, and allowed to dry overnight. Slides were subsequently dehydrated, cleared, and coverslipped with DPX mounting media (06522, Sigma-Aldrich). Immunohistochemical control experiments entailed processing as stated above except for the omission of either primary antibody or ImmPRESS reagents from incubation solutions. Under each of these conditions, no positive reaction was observed.

2.4. Histochemistry

Tissue sections were processed for AChE, CO, and NADPH-d using procedures adapted from Karnovsky and Roots (1964), Wong-Riley (1979), and Scherer-Singler, Vincent, Kimura, and McGeer (1983), respectively. In cases marked for serial reconstructions, tissue was collected in grids maintaining order. Combinations of individual reactions were performed on alternative sections due to incompatibility of staining methods. Sections were mounted, dehydrated, cleared, and coverslipped as described above.

2.5. Combined histochemistry and immunohistochemistry

For double-labeling NADPH-d and CR experiments, animals were perfused and sectioned in the same manner previously described. Following three 10 min PBS rinses, tissue was reacted first according to NADPH-d protocol guidelines. After an incubation period of 60 min at 37 °C, sections were rinsed six times in PBS for 15 min prior to completion of the CR procedures detailed above.

2.6. Imaging, serial reconstructions, and quantification

Brightfield image capturing was performed using a Nikon Digital Sight Color Camera (Nikon, Melville, NY). Three-dimensional Z-stacks (Elements Software; Nikon) were flattened into two-dimensional images using an extended depth of field algorithm. Matching of montaged images and slight adjustments in brightness and contrast were made using Adobe Photoshop (San Jose, CA).

Alignments of various neurochemical modular markers in adjacent sections were performed using a MicroBrightfield Biosciences (MBF Bioscience, Williston, VT) neuroplotting system. Utilizing a serial section manager in Neurolucida software, section and modular contours were reconstructed in the coronal plane. Alignment of adjacent sections was performed using a four-point (minimum) match of easily identifiable fiduciary landmarks in the neighboring sections. Aspects of the microvasculature and midline structures, including the dorsal aspect of the IC commissure, dorsal and ventral aspects of the cerebral aqueduct, and the ventral midline, were utilized as reliable landmarks.

Raw uncompressed TIFF images were imported into ImageJ software (NIH, Bethesda, MD) to facilitate LCIC modular measurements and to generate brightness plot profiles of relative staining patterns. Digitized images in the middle rostrocaudal third of the LCIC where modular-extramodular patterning was most apparent were analyzed. Imported images were

converted to grayscale and the threshold function was used to facilitate clear delineation of modular or modular void boundaries. Modular location was defined via a numbering scheme, with one being the ventral-most position. After setting scale, a line measurement tool was used to quantify the short and long axes of distinct modules (for AChE, NADPH-d, GAD65/67, and CO), as well as CR-negative modular voids. A freehand tool was used to trace closed contours of modular domains for area quantification. Student *t*-tests (two-tailed) compare average areas between developmental stages (statistical significance, $p < .05$). For assessing the discontinuous periodic nature of LCIC staining patterns, the same freehand tool with a set line thickness of 20 pixels was used to sample along the LCIC contour, bisecting layer 2 modular fields. Sampling was performed from ventral-to-dorsal and for multiple sections along the rostrocaudal axis of the LCIC. Comparisons of modular and extramodular plot profiles in age-matched animals were highly suggestive of complementary patterns, and thus the impetus for the double-labeling experiments. Autocorrelation function maxima >0.6 (1.0 = perfect periodic signal, 0 = no detectable periodicity) served as objective criteria to verify the periodic nature of layer 2 modular-extramodular labeling.

3. Results

3.1. LCIC modular patterns in early postnatal mice

Neurochemical staining for AChE, NADPH-d, GAD, and CO revealed an easily discernable pattern of discontinuous LCIC layer 2 modules by the end of the first postnatal week. At P8 (Figure 1a–d), clearly defined patches or modules were readily distinguishable even at low magnification for each of the markers, and remained so at hearing onset (P12, Figure 1e,f) and up to the latest developmental stage examined (P20, Figure 1g,h).

Distinct intermittent clustering of positively-labeled somata and neuropil spanning the dorsoventral layer 2 contour was especially conspicuous given its contrast to surrounding zones that were largely negative. The characteristic patchy or modular appearance was most noticeable at mid-rostrocaudal regions, before seemingly blending and obscuring in deeper aspects of the rostral LCIC. While invariably certain modules were more uniform than others in their appearance along the rostrocaudal axis of the LCIC, qualitatively the overall patterning for each of the markers looked remarkably similar at all of the ages.

Modular shape varied with relative position along the ventral-to-dorsal extent of the LCIC in the coronal plane of mid-rostrocaudal sections. Modular morphology was ovoid or round in more ventral locations (defined as positions 1–3), in contrast to seemingly flattened dorsal domains (defined as positions 4, 5). This change was increasingly apparent with age. At P20, comparisons of short and long axes of ventrally- and dorsally-grouped modules confirm qualitative observations of variable morphology based on location (Figure 2). Dorsal modules (see inset positions 4, 5; Figure 2, filled diamonds) exhibited short axes that were statistically smaller (Student *t*-tests, two-tailed; $p < .0004$) than that of more ventral modules (see inset positions 1–3; Figure 2, open squares), while there were no significant differences between their long axes ($p > .10$).

3.2. Histochemical markers stain same set of LCIC modules

Adjacent sections throughout the rostrocaudal extent of the LCIC were stained to determine if the histochemical markers were in relative alignment, thus staining the same modular set. One previous experiment in P8 mice showed that GAD and CO modules are aligned (Lesicko et al., 2016). This study expands on this finding by examining the co-registry of NADPH-d with CO and AChE. Reconstruction studies focused on P8, P12, and P20 mice, when modules were most evident with readily discernible boundaries. NADPH-d and CO patterns at hearing onset are largely overlapping, with positive modules exhibiting a high degree of spatial registry throughout the rostrocaudal extent of the LCIC (Figure 3, top). Similar colocalization was observed for each of the examined marker pairings with comparable alignment precision at each of the stages. Representative mid-rostrocaudal pre- and post-hearing reconstructions are shown at P8 for NADPH-d and AChE (Figure 3, bottom left) and at P20 for NADPH-d and CO (Figure 3, bottom right).

3.3. NADPH-d most reliable modular marker

By P20, modular LCIC patterns were even more striking than at earlier ages, especially for NADPH-d (Figure 4). In caudal extremes, thin bridges of positive labeling often connected neighboring modules (Figure 4a). Modules became distinct entities mid-rostrocaudally, with normally five and sometimes as many as six patches being apparent in a single coronal section (Figure 4b–d). Modular size varied considerably depending upon the relative plane of sectioning and precise dorsoventral/rostrocaudal location. Clean modular arrangements collapsed in rostral extremes of the LCIC (Figure 4e,f), giving way to the honeycomb lattice-like architecture of the SC that has been previously described (Wallace, 1986b; Wallace & Fredens, 1989).

3.4. CR specifically labels LCIC extramodular fields

Despite a variety of markers for LCIC layer 2 modules, analogous approaches that highlight surrounding extramodular domains have yet to be determined. In both C57BL/6J and CBA/CaJ P20 mice, immunostaining for the calcium-binding protein CR yields a pattern of LCIC labeling that appears complementary to that of the characterized modular markers (Figure 5). CR label is most heavily concentrated in LCIC layers 1 and 3, and strikingly absent from presumptive layer 2 modular fields. Devoid CR patches mirror that of NADPH-d-positive domains, both in their relative location and in their morphological appearance along the LCIC rostrocaudal axis (Figure 5a–f). This CR matrix encompassing discontinuous layer 2 modular voids is most evident at mid-rostrocaudal levels where intermodular bridges of CR link prominent layer 1 and 3 labeling (Figure 5d,e).

3.5. Developmental progression of NADPH-d modular and CR extramodular patterning

While apparent at hearing onset and distinct by P20, an extended series of neonatal experiments examined the developmental progression of the neurochemically-defined LCIC compartmental organization. At birth, distinct NADPH-d modules were not readily apparent, although NADPH-d positive neurons were evident in appropriate intermediate layers of the LCIC (Figure 6a, brackets). In age-matched tissue stained for CR, CR-positive cell bodies are apparent and show initial signs of occupying regions surrounding that of probable

developing modular fields (Figure 6b). This primitive organization develops into identifiable modular-extramodular patterns by P4 (Figure 6c,d), and easily recognizable by P8 (Figure 6e,f). By hearing onset, boundaries delineating NADPH-d-positive patches and the CR-positive matrix were clearly defined (Figure 6g,h). Modular labeling was most striking at P20, as NADPH-d positive patches exhibited increased neuropil staining (Figure 6i). CR-staining at this post-hearing time point remained highly localized to domains consistent with LCIC extramodular fields (Figure 6j).

3.6. Complementary markers for LCIC modular-extramodular fields

Sampling along mid-rostrocaudal LCIC layer 2 contours at P20 when LCIC patterns are well established resulted in brightness plot profiles with strong periodic components. Both NADPH-d modular (Figure 7a) and CR extramodular (Figure 7b) sampling exhibited significant signal fluctuations. However, plots of NADPH-d and CR from matching dorsoventral aspects of adjacent LCIC sections appeared largely out of phase with one another (Figure 7a,b). These findings, together with qualitative observations (refer back to Figures 4–6), suggest a complementary modular-extramodular LCIC arrangement.

Double-labeling studies for NADPH-d and CR confirm such a complementary, patch-matrix like pattern. At P20, NADPH-d-positive modules and CR extramodular labeling was largely segregated and nonoverlapping (Figure 8a,b). Despite this clear distinction, NADPH-d neurons were occasionally observed outside of modular boundaries, as were a few CR-positive cells evident within patchy domains. Double-labeled neurons were absent throughout the full extent of the LCIC.

3.7. Developmental progression of LCIC modular-extramodular organization

Similar experiments combining NADPH-d histochemistry and CR immunocytochemistry were performed at birth and at P8 to assess any relative shaping that might occur during this early developmental period (Figure 9). At P0, there was some evidence of early segregation, although less intense staining and not fully organized NADPH-d-positive LCIC neurons made it difficult to clearly delineate patterns (Figure 9a,d,g,j). NADPH-d labeling by P8 is much more apparent making modules more easily discerned (Figure 9b,e,h,k). At this stage still several days prior to hearing onset, early definition of NADPH-d modular and CR extramodular zones is apparent. While CR-positive staining is present in layer 1 at this age, labeling in aspects of layer 3 and intermodular layer 2 bridges is modest. A clear, nonoverlapping patch-matrix-like arrangement is striking at P20 (Figure 9c,f,i,l), especially at mid-rostrocaudal levels (Figure 9f). At each of the stages, layer 2 NADPH-d labeling is somewhat more uniform caudally, before individual modules become distinct entities and ultimately converge in rostral extremes of the LCIC (Figure 9j,k,l).

Higher magnification examination of representative modular-extramodular labeling at P0, P8, and P20 suggests an increasingly clear LCIC compartmental arrangement (Figure 10a–c). Despite mostly complementary patterns, NADPH-d and CR cell populations are not entirely segregated. Similar to that previously described at P20 (refer back to Figure 8), there was no evidence of double-labeled LCIC neurons at any of the examined ages.

At any given age, there was substantial variation in individual LCIC layer 2 compartmental areas given different dorsoventral/rostrocaudal positioning and relative plane of sectioning. Across the developmental period examined there was a trend of increasing area for both NADPH-d modules and CR modular voids (Figure 10d), with significant differences between each of the ages (Student *t*-tests $p < .001$ for each comparison). Average modular area at birth was $4,368 \mu\text{m} \pm 1,453$, compared to significantly larger average areas of $13,755 \mu\text{m} \pm 4,293$ at P8, and $21,887 \mu\text{m} \pm 9,951$ by P20.

3.8. Transitional zone between LCIC compartmental mosaic and SC lattice-like arrangement

Largely complementary NADPH-d and CR patterning extended rostrally throughout the LCIC and into regions of the intercollicular nuclei and deep SC (Figure 11a). This organization of high levels of one marker associated with low levels of the other is consistent with that previously reported in the deep SC for NADPH-d and AChE (Wallace, 1986b). Unlike the LCIC, double-labeled neurons were abundant in aspects of the intercollicular nuclei (Figure 11b,c), as well as certain regions of the SC. There were no detectable differences in any findings concerning strain (C57BL/6J vs. CBA/CaJ) or gender (male vs. female).

4. Discussion

The present findings provide a developmental timeframe for the establishment of neurochemically-defined LCIC compartments. Results confirm that identified modular markers label the same set of layer 2 patches, and that their spatial registry is in place prior to hearing onset. Beyond this overlapping modularity, the data presented identify CR as a second network marker that specifically highlights the surrounding LCIC extramodular fields. Simultaneous labeling of modular (NADPH-d) and extramodular (CR) markers provides the first evidence of a clearly delineated patch-matrix-like organization in the nascent LCIC. Such distinct neurochemical compartments established early in development likely underlie an early specificity of unique sets of topographically organized multimodal connections (Figure 12).

4.1. Neurochemical modularity resembles LCIC Eph-ephrin guidance expression

Both the modular patterning revealed through AChE, NADPH-d, GAD, and CO staining, as well as the highly specific CR extramodular labeling, is reminiscent of previous reports from our laboratory in neonatal mouse and rat highlighting early LCIC guidance expression patterns (Cramer & Gabriele, 2014; Gabriele et al., 2011; Wallace, Kavianpour, & Gabriele, 2013; Wallace et al., 2016). Results from these immunocyto-chemistry studies and X-Gal staining experiments in *lacZ*Eph-ephrin mutants are strikingly consistent with the neuroanatomical patterns described here. EphA4 and ephrin-B2 expression at similar developmental time points is patchy and concentrated in presumptive LCIC layer 2 modules, while ephrin-B3 expression is extramodular and seemingly complementary (Wallace et al., 2016). One notable difference is that Eph-ephrin patterns are most apparent early on, before being downregulated with experience, while the described neurochemical markers and their respective patterns are even more striking past hearing onset. Given the conspicuous

similarities in their patterns during the neonatal period, we suspect that Eph-ephrin signaling drives the initial patterning of compartmentalized inputs to the LCIC and helps shape its early patch-matrix-like organization. As the early LCIC compartmental blueprint matures and sharpens, it may rely less on certain guidance mechanisms and more on relative activity levels in neighboring, modality-specific circuits. Ongoing experiments in our laboratory aim to directly test these contentions.

The precise registry of Eph-ephrin and neurochemical LCIC patterns remains unaddressed. Fundamental studies that demonstrate their relative alignment, as well as classify specific neuronal subsets localized within this LCIC micro-organization will be pivotal in our understanding of this brain structure and its processing capabilities. As a first step, we plan to confirm the notion that EphA4 and ephrin-B2 domains colocalize with the described neurochemical modular framework. We further expect ephrin-B3 expression to overlap with defined CR extramodular zones. Beyond these more global perspectives of compartmental markers and how they interface with each other and modality-specific projection streams, many questions also persist on a cellular level that should yield insights concerning potential subcircuits within defined LCIC compartments. For example, neuronal populations with unique guidance and neurochemical signatures may provide clues for finer-scale levels of processing within and between LCIC compartments, and perhaps implicate certain signaling proteins not only in the establishment of LCIC modular units, but also in the preferential targeting of certain cell populations (e.g., GABAergic or cholinergic) within such units.

4.2. LCIC connectional modularity and developing afferent systems

Evidence in a variety of adult species suggest that the diverse array of inputs to the LCIC recognize and adhere to its characteristic modular-extramodular framework (Bajo, Nodal, Bizley, Moore, & King, 2007; Lesicko et al., 2016; Saldaña, Feliciano, & Mugnaini, 1996; Saldana & Merchán, 1992; Stebbings et al., 2014; Torii, Hackett, Rakic, Levitt, & Polley, 2013; Wiberg & Blomqvist, 1984; Winer, Larue, Diehl, & Hefti, 1998; Zhou & Shore, 2006). Despite initial findings (Chernock et al., 2004) that a neurochemical modularity is present in some, but not all species (including mouse), it has since been verified that such an LCIC arrangement is in fact reliably observed across species. Furthermore, in adult mouse, this modular architecture is consistent with that of described multimodal LCIC afferent patterns. Ascending and descending inputs to LCIC modular fields arise from somatosensory sources, while similar multi-level auditory connections preferentially target surrounding extramodular zones (Lesicko et al., 2016; Stebbings et al., 2014). These seminal studies clearly demonstrate that projections from somatosensory brainstem nuclei and somatosensory cortex align with identified LCIC modular markers. Inputs from auditory cortex and the IC itself (arising from CNIC), on the other hand, target LCIC fields complementary to the neurochemically-defined layer 2 modules. We hypothesize that the described matrix-like auditory terminal fields would colocalize within the LCIC with the extramodular marker identified here, CR. The multimodal LCIC patterns described in the adult by Lesicko et al. (2016) appear highly specific and complementary in nature, although definitive experiments are still needed that simultaneously label modular (somatosensory) and extramodular (auditory) inputs in individual animals. In any case, it is likely that the LCIC receives largely segregated multisensory afferent streams. Such modality-specific

compartmentalization may well provide the staging necessary for subsequent multisensory integration locally between LCIC zones (Lesicko & Llano, 2016), and with known projection outputs to the intermediate and deep SC (Harting & Van Lieshout, 2000; Huffman & Henson, 1990; Knudsen & Knudsen, 1983).

Very little is known concerning the developmental timing and shaping of multimodal axonal patterns in the LCIC. Since compartmental Eph-ephrin LCIC expression patterns correlate temporally with early CNIC guidance gradients (Gabriele et al., 2011; Wallace et al., 2013, 2016), we anticipate a similar time course for LCIC modular-extramodular circuit assembly as has been previously documented for developing CNIC afferent layers (Fathke & Gabriele, 2009; Gabriele, Brunso-Bechtold, & Henkel, 2000a,b; Gabriele, Shahmoradian, French, Henkel, & McHaffie, 2007; Gabriele et al., 2011; Wallace et al., 2013). Preliminary studies from our laboratory suggest that like the CNIC, multimodal pioneer fibers are present in the LCIC by birth. During the first postnatal week, somatosensory brainstem inputs arising from the spinal trigeminal nucleus are evident bilaterally in the LCIC and exhibit significant branching (Balsamo & Gabriele, 2015). Similarly, labeled auditory afferents from the adjacent CNIC are abundant in the nascent LCIC, with clear extramodular terminal patterns emerging prior to hearing onset (Noftz, Gray, & Gabriele, 2014). It remains to be determined, however, whether compartmentalized LCIC inputs exhibit early projection specificity, or if such an arrangement results through the refinement of initially diffuse and overlapping projection patterns. Future experimentation examining the precise alignment of Eph-ephrin and neurochemical stains with developing multimodal projection patterns should provide mechanistic insights concerning the emergence of the LCIC microarchitecture and its functional connections.

4.3. Comparisons with SC lattice and striatal patch-matrix arrangement

Other brain structures, like the LCIC, also exhibit modular organizational features that are neurochemically and connectionally distinct. Appreciation of architectural compartments and corresponding networks has been key for elucidating specific functional roles in other areas. The intermediate and deep layers of the SC perhaps provide the most analogous arrangement to that described here for the LCIC. Both the LCIC and SC receive a rich array of multimodal inputs that originate from a variety of cortical and subcortical sources (Huerta & Harting, 1984). It is well established that multisensory layers of the SC exhibit a honeycomb-like neurochemical network or lattice consisting of a series discontinuous patches, reminiscent of the described LCIC modularity. In the intermediate SC, AChE, NADPH-d, and CO lattices are present and colocalized, yet in the deep SC, the pattern for AChE is offset and not in register with the other markers (Illing 1996; Wallace, 1986a,b). This organization of the multimodal SC into a mosaic of functional modules serves as the substrate for the orderly segregation of a variety of afferent and efferent systems (Illing & Graybiel, 1985; Mana & Chevalier, 2001; Wallace & Fredens, 1989). Considering that most of the described LCIC afferents to date display modular or extramodular terminal patterns that appear consistent with its defined neurochemical framework, it is likely the LCIC employs SC-like compartmentalized units for handling multisensory information streams. Despite its functional significance, very little is known about the ontogeny and shaping of patchy inputs to the intermediate and deep SC. We have shown previously in neonatal cat that

basal ganglia influences on the SC via nigrotectal projections are present by birth and exhibit an established patchy or lattice-like distribution pattern (Gabriele, Smoot, Jiang, Stein, & McHaffie, 2006). Similar tracing studies that focus on LCIC afferents and their relative alignment with neurochemical and guidance patterns are ongoing in our laboratory.

Beyond the noteworthy similarities with the SC, the striatum with its complex mosaic arrangement is another structure that begs comparison to the LCIC. Its well-established patch (striosome)-matrix compartments were also first described using a variety of neurochemical approaches, and later functionally characterized on the basis of its highly segregated, input-output organizational scheme (Gerfen, 1992; Jiménez-Castellanos & Graybiel, 1989). Patchy striatal expression of μ opiate receptors (Herkenham & Pert, 1981), together with its surrounding matrix expression of AChE, calbindin, and somatostatin (Gerfen, 1985; Gerfen, Baimbridge, & Miller, 1985), parallel the complementary patterning presented for the LCIC with its set of modular markers and CR-positive extramodular expression. Just as afferent-efferent relationships of striatal compartments have been charted with respect to neurochemical (Gerfen 1989) and Eph-ephrin guidance markers (Janis, Cassidy, & Kromer, 1999; Richards, Scheel, Wang, Henkemeyer, & Kromer, 2007; Tai, Cassidy, & Kromer, 2013; Tai & Kromer 2014), similar studies are needed that reveal the functional significance and developmental shaping of LCIC modular-extramodular fields. If LCIC development follows a similar progression as that of the striatum, modular-extramodular neurons may be initially intermingled prior to subsequent segregation into appropriate micro-domains during the late embryonic period, as has been shown for striosome-matrix cells (Fishell, Rossant, & van der Kooy, 1990; van der Kooy & Fishell, 1987). While it is clear that somatosensory and auditory inputs target segregated zones of the adult LCIC mosaic (Lesicko et al., 2016; Stebbings et al., 2014), it remains to be seen how this arrangement interfaces with major LCIC output pathways, and whether this modality-specific segregation fully emerges early in development prior to experience.

4.4. Concluding remarks

Ever since seminal findings reporting bimodal response properties in the LCIC (Aitkin, Dickhaus, Schult, & Zimmermann, 1978), mounting anatomical and physiological evidence suggests a pivotal role of the LCIC in multisensory and other non-auditory processing. In addition to somatosensory inputs (Jain & Shore, 2006; Zhou & Shore, 2006), LCIC activity is clearly affected by visual cues, eye movements/position, certain vocalizations, self-generated sounds, as well as behavior context and reward anticipation (Gruters & Groh, 2012). With such a variety of influences, the LCIC is increasingly viewed as an integrative hub, uniquely positioned for synthesizing higher order tasks like auditory perception and behavior. It is therefore vital to gain a better understanding for its organization and specific processing capabilities to determine its influence on a multitude of other structures through its colliculofugal connections. Observed multisensory LCIC characteristics may be a combination of those imparted from other subcortical and cortical multimodal afferent systems, such as the cochlear nucleus (Basura, Koehler, & Shore, 2012; Shore & Zhou, 2006), as well as those generated locally within the LCIC through modular-extramodular field crosstalk (Lesicko & Llano, 2016). Relating regional physiological LCIC properties to the modular-extramodular framework presented here, and cataloging their registry with

throughput connectivity patterns, will serve to better identify functional micro-domains within its compartmentalized architecture. Finally, appreciating the guidance mechanisms at play in instructing its modularity should inform the influences that converging systems may exert on one another and perhaps the types of network mapping plasticity that one might anticipate with experience.

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Figure 1.

Neurochemical and immunostaining of lateral cortex of the inferior colliculus (LCIC) layer 2 modules in developing mouse. Representative low magnification photomontages at P8 (a, c), P12 (e), and P20 (g) highlighting the periodic, discontinuous patchy LCIC patterning for acetylcholinesterase (AChE), nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d), glutamic acid decarboxylase (GAD), and cytochrome oxidase (CO) (arrowheads). Inset boxes shown at higher magnification in (b), (d), (f), and (h). Dashed contours demarcate discrete modular boundaries for each of the markers. Scale bars in (a), (c), (e), and (g) = 500 μ m, in (b), (d), (f), and (h) = 100 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Figure 2.

Plot of modular shape at P20 with respect to relative position in representative mid-rostrocaudal LCIC sections. Modular locations were defined with a numbering scheme from ventral to dorsal (inset schematic). Based on qualitative observations that the two most dorsal LCIC positions exhibited a more flattened morphology, comparisons of short and long axes were made against more ventral positions exhibiting rounded or more ovoid appearances. Short axis measurements for dorsal modular locations (4, 5; filled diamonds) were statistically smaller ($p < .0004$) than that of more ventral modules (1–3, open squares). In contrast, long axes of modular groupings were not statistically different ($p > .10$)

Figure 3.

Serial reconstructions showing markers stain same set of LCIC modules. Neighboring sections were aligned using a four-point minimum match of identified fiduciary landmarks. At hearing onset (top), adjacent sections stained for NADPH-d and CO throughout the rostrocaudal extent of the LCIC have similar patterning with regards to number of patches, modular shape, and relative LCIC location. In rostral extremes where LCIC wraps around the central nucleus (CNIC), modular domains take up a deeper, more ventral position. Similar registry was observed for marker pairings at pre- and post-hearing stages (bottom). Representative reconstructions are shown for P8 (NADPH-d and AChE) and P20 (NADPH-d and CO) pairings at mid-rostrocaudal levels where modular patterning was most distinct [Color figure can be viewed at wileyonlinelibrary.com]

Figure 4.

Periodic network of NADPH-d modules (arrowheads) in a P20 mouse. Caudal-to-rostral distribution (a–f) of NADPH-d LCIC layer 2 staining in the coronal plane. In caudal extremes, neighboring patches are linked by varying degrees of intermodular staining. At mid-rostrocaudal regions, LCIC modules appear as distinct entities before merging and assuming a deeper position in rostral portions. Scale bars = 500 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Figure 5. Calretinin (CR) immunostaining of extramodular LCIC domains in a P20 mouse. Caudal-to-rostral distribution (a–f) of CR labeling, in contrast to that observed for modular markers, is most concentrated in LCIC layers 1 and 3, leaving clear layer 2 modular voids (arrowheads). CR labeling appears complementary to that described for NADPH-d in age-matched mice. CR extramodular staining is most striking at mid-rostrocaudal regions (d, e) where thin strands of labeling connects layers 1 and 3, making layer 2 modular voids most apparent. Scale bars = 500 μ m

Figure 6.

Developmental progression of discrete NADPH-d modular and CR extramodular domains from matching rostrocaudal levels. NADPH-d modules at P0 (a), P4 (c), P8 (e), P12 (g), and P20 (i). The presence of layer 2 NADPH-d-positive neurons at birth (a, brackets) develops into increasingly discernible modules over the early postnatal period (c, e, g, i, dashed contours). CR labeling is nearly organized by birth (b) and clearly extramodular at later stages (d, f, h, j). Note CR modular voids (dashed contours). Scale bar in (a) 520 μm , (b–j) 50 μm [Color figure can be viewed at wileyonlinelibrary.com]

Figure 7.

Periodicity of established modular and extramodular LCIC patterning at mid-rostrocaudal levels. Representative brightness profile plots of NADPH-d (a) and CR (b) layer 2 staining at P20. LCIC sampling was performed on matching rostrocaudal sections at consistent LCIC dorsoventral positions (see schematics and inset boxes showing sampling contours in a, b). Note that troughs or modules (a, arrows) are seemingly complementary to peaks or modular voids (b, arrows). Autocorrelation function maxima suggest highly periodic signals for both NADPH-d and CR labeling (0.71 and 0.77, respectively) [Color figure can be viewed at wileyonlinelibrary.com]

Figure 8. Double-labeling experiments showing distinct complementary NADPH-d/CR patterning at P20. Photomontages at mid-rostrocaudal sections (a, b) where the largely segregated LCIC modular-extramodular arrangement is most evident. NADPH-d modular fields (blue, dashed contours), CR extramodular fields (brown). Scale bars = 100 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Figure 9. Development of complementary modular-extramodular LCIC microarchitecture. Caudal-to-rostral progressions of NADPH-d (blue) and CR (brown) LCIC labeling at P0 (a, d, g, j), P8 (b, e, h, k), and P20 (c, f, i, l). Presumptive modular boundaries denoted by dashed contours. Scale bars = 150 μ m [Color figure can be viewed at wileyonlinelibrary.com]

Figure 10.

Increasing size and clarity of NADPH-d (modular) and CR (extramodular) zones with postnatal age. High magnifications 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. Plot of layer 2 compartmental areas including all defined locales (ventral-to-dorsal, 1 through 5) with respect to postnatal age (d). Each data point represents a given area for an individual NADPH-d module (open blue circles) or CR modular void (open brown circles) from the mid-rostrocaudal third of the LCIC. Range of areas at any given age reflects small differences in planes of sectioning and described changes in morphology at variable dorsoventral positions. Areas throughout the range correlate closely with relative compartmental location. Data points from upper extremes coincide most often with modular positions 1 and 2, while the middle of the range is dominated by position 3, and lower extremes by positions 4 and 5 that frequently exhibit a flattened morphology. Linear regression analysis of the compiled data shows a clear trend of increasing LCIC individual compartmental area with postnatal age. For each of the ages, there was a statistically significant difference in area (asterisks; $p < .001$) [Color figure can be viewed at wileyonlinelibrary.com]

Figure 11.

Segregated LCIC modular-extramodular markers blend rostrally into previously described superior colliculus (SC) neurochemical lattice (a). Single- (NADPH-d, black arrowheads; CR, white arrowhead) and double-labeled (NADPH-d and CR, arrows) neurons are present rostral to the LCIC in the intercollicular nuclei (InCo) and deep layers of the SC (b). At high magnification, both markers are clearly distinguishable in individual neurons (c). Inset boxes in (a, b) shown at higher magnification in (b, c). Scale bar in (a) = 300 μm , (b) = 50 μm , (c) = 20 μm [Color figure can be viewed at wileyonlinelibrary.com]

Figure 12.

Summary figure of present neurochemical findings taken together with known multimodal input-output patterns and modular-extramodular/Eph-ephrin LCIC expression [Color figure can be viewed at wileyonlinelibrary.com]

Table 1
Antibody information

Antibody name	Structure of immunogen	Manufacturer info.	Concentration used
Anti GAD65/67	Rat glutamate decarboxylase (GAD65; C-terminus residues)	Millipore, AB1511, RRID: AB_11210186, rabbit, polyclonal	1:3,000
Anti calretinin	Recombinant human calretinin containing a 6-his tag at the N-terminal	Swant, CR 7697, RRID: AB_2619710, rabbit, polyclonal	1:5,000