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Multimodal inputs and modularity of the inferior colliculus prior to audition.

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by Joseph Anthony Balsamo
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PUBLIC PRESENTATION

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Disclaimer

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Multimodal Inputs and Modularity of the Inferior Colliculus Prior to Audition.

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ABSTRACT

A fully functional nervous system requires assimilation of sensory modalities to adequately interpret stimuli and determine appropriate motor responses. Two such regions responsible for multimodal integration are found in the auditory system, the cochlear nucleus (CN) and the inferior colliculus (IC). The lateral cortex of the IC (LCIC) in particular receives a diverse multimodal input array to discrete modular/extramodular zones. Staining for certain neurochemicals, including GAD, a marker for GABAergic neurons, reveal this compartmentalized LCIC organization. The present study utilizes fluorescent anterograde tract-tracing techniques to determine the development of a somatosensory brainstem projection from the spinal trigeminal nucleus (Sp5), to multimodal aspects of the CN and LCIC. The results indicate somatosensory innervation of these structures parallels that of developing auditory afferents and that auditory-somatosensory convergence described in the adult in these areas is likely set prior to acoustic experience. Immunohistochemical GAD staining and expression studies for molecular guidance molecules (EphA4, ephrin-B2, ephrin-B3) reveal early LCIC modularity during the period of bimodal projection shaping. Such findings suggest an emergence of LCIC domains early in development that may in part be guided by Eph-ephrin protein interactions. Understanding the neuronal development of converging auditory and somatosensory maps is essential for understanding their presumed roles in suppression of self-generated sounds. Furthermore, a sound foundation for mechanisms guiding multimodal input array formation is necessary to improve noninvasive interactions that aim to reset maladaptive map plasticity underlying debilitating conditions like tinnitus.

Keywords: inferior colliculus, GAD, somatosensory, auditory, multimodal
INTRODUCTION

The auditory system is primarily responsible for deciphering complex sound characteristics such as pitch, frequency, and intensity. Interpreting precise signal attributes and computation of complex tasks requires multiple processing centers and cross communication among different sensory modalities. The inferior colliculus (IC), located in the auditory midbrain, is one such processing area classically described as an auditory relay hub. The three subdivisions of the IC, the central nucleus (CNIC), lateral cortex (LCIC), and dorsal cortex (DCIC) (Faye-Lund and Osen, 1985; Loftus et al., 2008), have been investigated to varying degrees. Well characterized is the tonotopic input array to the CNIC from other auditory centers (Merzenich and Reid, 1974; Roth et al., 1978; Semple and Aitkin, 1979; Schreiner and Langner, 1988; Kandler et al., 2009) resulting in distinct laminar inputs that are frequency dependent.

Although such frequency mapping is ideal for purely auditory nuclei, interpreting coded information in characteristic frequencies, this model appears less appropriate for multisensory regions within the auditory pathway. For example, the cochlear nucleus (CN) receives auditory input from the cochlea (Nelken, 2008) as well as somatosensory afferents from trigeminal ganglion (TG), dorsal column nuclei: cuneate (Cu) and gracilis (Gr), and spinal trigeminal nucleus (Sp5) (Itoh et al., 1987; Wright and Ryugo, 1996; Zhou and Shore, 2004; Haenggeli et al., 2005). Similarly, the LCIC not only receives auditory innervation from auditory cortex (AC), CN and CNIC (Aitkin et al., 1981; Ryugo et al., 1981; Saldaña and Merchán, 1992; Malmierca et al., 1995; Saldaña et al., 1996; Zhou and Shore, 2006) but also somatosensory innervation from Cu, Gr, and Sp5 across a variety of adult species including rat, cat, guinea pig, and monkey (RoBards et al., 1976; Robards, 1979; Coleman and Clerici, 1987; Wiberg et al., 1987; Weinberg and Rustioni, 1989; Shore and Zhou, 2006; Zhou and Shore, 2006). The LCIC cytoarchitecture is
not defined by distinct frequency laminae like its CNIC counterpart, but rather as modular (layer 2) and extramodular (layers 1 & 3) zones as exhibited by a host of neurochemical expression patterns (Chernock et al., 2004; Hilbig et al., 2007, Lesicko and Llano, 2015).

While LCIC connectivity and neurochemical modularity has been described in adults, analogous studies regarding the emergence of modular fields and patterning of multimodal inputs are largely lacking. To date, there exist only a handful of studies that address auditory inputs to the LCIC. CN projects bilaterally to layer 2 LCIC modular zones extending into rostral extremes of the subdivision (Kandler and Friauf, 1993). Complementary to this pattern, descending inputs from AC (Torii et al., 2013) and intrinsic connections from the neighboring CNIC (Noftz et al., 2014) (Figure 1) project primarily to ipsilateral, extramodular LCIC domains and are established prior to hearing onset.

In terms of developing somatosensory afferents to the LCIC, even less is known. Adult data regarding somatosensory LCIC inputs have focused on projections arising from Sp5 (Zhou and Shore, 2004, 2006; Shore and Zhou, 2006), which encodes proprioceptive information from facial, oral and vocal cord structures (Romfh et al., 1979; Capra, 1987; Jacquin et al., 1989; Nazruddin et al., 1989; Takemura et al., 1991; Suemune et al., 1992), and dorsal column nuclei, responsible for proprioceptive contributions from the limbs (RoBards et al., 1976; Coleman and Clerici, 1987; Wiberg et al., 1987; Weinberg and Rustioni, 1989; Hilbig et al., 2007). Pathways from the aforementioned somatosensory nuclei in adult species are primarily contralateral and target discrete LCIC modular fields (RoBards et al., 1976; Coleman and Clerici, 1987; Wiberg et al., 1987; Weinberg et al., 1989; Zhou and Shore, 2006; Hilbig et al., 2007). These patchy, discontinuous layer 2 patterns in the adult appear to be segregated largely with regard to heavy auditory inputs from AC and CNIC that preferentially terminate in layers 1 and 3 (Lesicko and
The present study identifies an early somatosensory input from Sp5 to LCIC in newborn mouse. We discuss the timing of developing Sp5 inputs with that of emerging auditory afferents and LCIC modularity assessed by GAD staining and Eph-ephrin guidance protein localization.

MATERIALS AND METHODS

Animals

Experiments were performed on C57BL/6J control mice (Jackson Laboratories, Bar Harbor, ME) prior to hearing onset at various postnatal ages (n = 9) in keeping with previously described time points for topographic inputs to the IC (Gabriele et al., 2000, 2007, 2011; Henkel et al., 2005; Fathke and Gabriele, 2009; Wallace et al., 2013). All experimental procedures were performed in compliance with National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996) and received prior approval from the Institutional Animal Care and Use Committee.

NeuroVue Tracing of Sp5-IC Projections

Postnatal mice were given an overdose of ketamine (200 mg/kg) and xylazine (20 mg/kg) and perfused through the heart (physiological rinse followed by 4% paraformaldehyde solution, pH 7.4). Brains were removed from the skull and post-fixed overnight at 4°C in paraformaldehyde fixative solution. Post-fixed tissue was blocked in the coronal plane just rostral to superior colliculus (SC) and caudally at the spinal cord brainstem junction before embedding (5 ml 8% gelatin in dH2O/10 ml egg yolk). Brains were then sectioned caudal to rostral at 75 µm on a Vibratome until Sp5 could be identified with brightfield microscopy. Slivers of lipophilic dye-soaked filter paper (Molecular Targeting Technologies, West Chester, PA) were cut with Micro-
Vannas surgical scissors with the aid of a dissecting microscope and were then placed in Sp5. It should be noted even with the care and precision taken with each dye placement that variations exist among cases regarding precise location and amount of dye delivered. Tissue blocks were incubated at 37°C for 1 month in the dark to facilitate dye diffusion. Fixative solution was replaced every two weeks to maintain tissue integrity. After incubating, the remaining block of tissue was sectioned at 75 µm and counterstained with bis-benzamide nuclear counterstain (Hoechst 33258; Invitrogen, Carlsbad, CA) for 5 min to visualize Sp5 and IC cytoarchitecture. Sections were rinsed 3x for 5 min in PBS, mounted, and coverslipped while wet with GelMount (BioMeda, Foster City, CA).

**GAD Immunohistochemistry**

Animals were perfused as described above with an additional fixation step using 4% paraformaldehyde, 10% sucrose solution (pH 7.4). Brains were then removed and post-fixed overnight in the same solution followed by an additional overnight cryoprotection in 4% paraformaldehyde, 30% sucrose solution (pH 7.4). 50 µm thick sections were taken on a sliding-freezing microtome in the coronal plane. Tissue processing began with an endogenous quench (3% H₂O₂ in PBS) of peroxidase activity for 5 min followed by three, 5 min PBS rinses. A 30 min protein-blocking step (2.5 % Normal Horse Serum) followed, conducted at room temperature. Primary rabbit anti-GAD (Millipore AB1511 1:3000) was applied overnight at 4°C. This antibody labels both cellular GAD isoforms (67 & 65 respectively). Tissue was then equilibrated to room temperature and rinsed 3x for 5 min in PBS. Sections were then incubated at room temperature in an anti-rabbit IMPRESS reagent kit made in horse (Vector Laboratories MP-7401 IMPRESS reagent kit). An additional three PBS rinses for 5 min preceded incubation with ABC-DAB reaction kit (Vector Laboratories SK-4100). Sections were then rinsed 3x for 5
min in PBS and mounted on gelatin-subbed slides. Sections were rehydrated briefly in dH$_2$O before being passed through a series of alcohol washes (50%, 70 %, 95%, 2x at 100%, for 10 min each) and two, 10 min xylene clearing steps. Slides were coverslipped with Vecta-mount mounting media (Vector H-5000).

**Microscopy and Image Capture**

A monochrome cooled CCD CoolSnap HQ digital camera (Roper Scientific, Tuscon, AZ) and a Nikon TE 2000 microscope (Nikon, Melville, NY) were used to capture fluorescent images. R and B Phycoeythrin (NueroVue) and DAPI (bis-benzamide) filter sets (ChromaTechnology, Brattleboro, VT) were used to visualize both molecules. Two monochrome channels were acquired, merged digitally, and pseudocolored (green: NeuroVue axonal labeling; blue: bis-benzamide nuclear counterstain).

Magnification series were collected throughout the rostrocaudal extent of the IC for Sp5 dye labeling. For images 10x and higher Z-stacks were acquired to capture focused labeling throughout the entire 75 µm thick section. Three-dimensional Z-stacks (Elements Software; Nikon) were flattened into two dimensions using a maximum projection algorithm. Images were saved as JPEG 2000 files. Brightness/contrast values were manipulated in some cases for illustration purposes or to emphasize the bis-benzamide channel to assist in drawing cytoarchitectural boundaries (Adobe Photoshop, San Jose, CA).

GAD image capture was performed using brightfield microscopy with a Nikon Digital Sight Color Camera using DSFi1 filter set (Nikon, Melville, NY). White balances were performed on image series captured at 4x, 10x, 20x, 40x, and 60x (Adobe Photoshop, San Jose, CA).
RESULTS

Sp5 Dye Placements

Sticks were localized in Sp5. Additionally, variation in dorsal or ventral placements were made in an effort to establish Sp5-LCIC topography; however, these attempts revealed no obvious trends in these early connections. As such, neither ventral nor dorsal dye placements show more robust input to LCIC or targeting of spatially distinct LCIC regions. Rather, dye placement size was the critical factor in the number of labeled inputs with larger placements revealing more axon fibers. It should be noted that in all cases, Sp5 dye placements resulted in backfilling of the spinal trigeminal tract (sp5), thereby confirming placement in Sp5 nucleus (Figure 2).

Sp5 Projections to CN

Anterograde experiments from Sp5 reveal inputs to anteroventral cochlear nucleus (AVCN) at P4 (Figure 3) and maintained at P8. At both ages inputs were ipsilateral dominant with heaviest terminal fields in AVCN marginal zones (Figure 3), as has been described in adult guinea pig (Zhou and Shore, 2004).

Sp5 Projections to LCIC

Sp5 sends afferent projections bilaterally to LCIC during circuitry development (Figure 4). Fiber trajectory was consistent in all cases at all ages (Figure 5). Labeled contralateral fibers ascend for a short distance before crossing the midline and passing just dorsal to the lateral superior olive (LSO) (Figure 5, A). Fibers then enter the IC (Figure 5, B) and project to medial, lateral, or rostral LCIC regions (Figures 6 & 7). Interestingly, rostral regions of LCIC, caudal to intertegmental nucleus (ITN), receive the heaviest inputs in all cases and show increased axonal
branching compared to medial and lateral aspects of the LCIC (Figure 7).

While synaptic inputs are present at P0 (Figure 4), no clear organization of Sp5 fibers into LCIC modular fields was observed. Similarly, Sp5 projection patterns at P4, while substantial, still showed no obvious specificity regarding projection distribution. At P8, preliminary observations suggest some refinement, but discrete modular organization of axonal afferents remains lacking. Further experimentation at slightly older developmental stages should determine when Sp5 inputs to LCIC organize into distinct layer 2 modules.

**GAD Immunohistochemistry**

GAD immunohistochemistry reveals GABAergic modules at P4 in LCIC (Figure 8). The GAD-positive area present in layer 2 (Figure 8, B) contains positive soma and terminal puncta (Figure 8, C) indicative of GABAergic input and output. These findings suggest an early modular LCIC organization despite lack of patchy somatosensory inputs at these ages.

**DISCUSSION**

This study presents the first developmental time course for somatosensory innervation to the CN and IC. While these nuclei receive somatosensory and auditory input at similar developmental stages, the emergence of somatosensory patterns (i.e. modules) appears to be delayed relative to auditory pattern formation. In addition we show neurochemical evidence for GAD-positive zones during development suggesting adult neurochemical expression patterns form during the critical period prior to hearing onset.
Dye Placements and Fiber Trajectory

Placements in Sp5 reveal fiber trajectories consistent with mature circuitry in guinea pig (Zhou and Shore, 2004, 2006), cat (Wiberg et al., 1986), and monkey (Wiberg et al., 1987) albeit in dissimilar proportions. These adult trajectories are predominantly contralateral, whereas our developmental findings suggest bilateral pathways. It remains to be determined if aspects of the observed ipsilateral projections are subsequently refined at later developmental stages or with experience.

Of particular note is the contralateral fiber trajectory at the level of LSO (Figure 5, A). Axonal fibers pass dorsal to this nucleus before entering the lateral lemniscus (LL) en route to the IC. Previously, we described LCIC modular input from LSO (Wallace et al., 2013). Considering the fiber trajectory of somatosensory pathways and dye placements reviewed in Wallace (2013), the described modular input in LCIC was likely due to clipping of adjacent somatosensory fibers from Sp5 (Itoh et al., 1987; Wiberg et al., 1987; Jain and Shore, 2006; Zhou and Shore, 2006, present study) and perhaps dorsal column nuclei (Robards, 1979; Coleman and Clerici, 1987; Weinberg and Rustioni, 1987; Wiberg et al., 1987; Weinberg et al., 1989).

Also worth mentioning are the retrogradely labeled ipsilateral cell bodies of the mesencephalic nucleus (Me5) observed in all cases regardless of age (Figure 6, A). Me5 targets upper cervical spinal cord areas via sp5 tract (Veazey and Severin, 1980; Raappana and Arvidsson, 1993; Pombal et al., 1997). Based on the close proximity of sp5 tract to Sp5 nucleus, dye likely diffused into the sp5 tract labeling Me5 cells via their efferent axons.
Projections to CN

Anterograde tracing studies from Sp5 reveal ipsilateral dominant projections to AVCN (Figure 3) consistent with findings in adult guinea pig (Zhou and Shore, 2004). The described projection pattern through the sp5 tract is also conserved for fibers terminating in AVCN marginal zones (Zhou and Shore, 2004) suggesting mammalian continuity in pathway formation and that such pathways are established early in development. Zhou and Shore (2004) also presents terminal puncta labeling in dorsal cochlear nucleus (DCN) and posterior ventral cochlear nucleus (PVCN). Such projection patterns are lacking in our developmental mouse cases and require additional trials to identify such pathways. CN also receives descending auditory input from the superior olivary complex (SOC) and CNIC (Shore et al., 1991) as well as reciprocal connections with the opposing CN (Shore et al., 1992). Thus, auditory and somatosensory convergence may provide a multimodal center allowing for better sound source localization based on proprioceptive information.

Projections to LCIC

Early developmental bilateral terminal fields in LCIC lack defined modular input arrangements. Axon bundles approach the CNIC, before deferring to medial, lateral, or rostral zones. This preference for termination in LCIC regions is consistent in adult species (Wiberg et al., 1986, 1987; Zhou and Shore, 2006), yet inconsistencies arise when comparing projection laterality. Adult studies in cat (Wiberg et al., 1986), monkey (Wiberg et al., 1987), and guinea pig (Zhou and Shore, 2006) yield contralateral projections from Sp5 to LCIC forming conserved modular distribution patterns in layer 2. At birth (Figure 4), and as late as P8, substantial ipsilateral LCIC inputs from Sp5 persist. It remains to be seen whether ipsilateral inputs are pruned in later
developmental stages as contralateral terminations elaborate and organize into discrete modular distributions.

The heaviest labeling at all developmental ages was present in rostral LCIC just caudal to ITN (Figure 7) in line with findings in monkey (Wiberg et al., 1987). This transitional region is a pathway for a variety of sensory afferents to multimodal regions of SC. Therefore fibers from Sp5 may not only be terminating in LCIC but also passing through this nucleus en route to SC. However, clear varicosities on Sp5 axonal labeling in the LCIC suggest, at the very least, synaptic collaterals terminating in multimodal aspects of the LCIC.

The presence of auditory and somatosensory innervation of the LCIC supports a multisensory integration theory. In adult species ascending, horizontal, and descending auditory inputs terminate in layers 1 and 3 of LCIC (Ryugo et al., 1981; Saldaña and Merchán, 1992; Malmierca et al., 1995; Saldaña et al., 1996; Lesicko and Llano, 2015) with the exception of CN terminal zones in layer 2 (Zhou and Shore, 2006). The investigations regarding these same pathways during development reveals similar results (Kandler and Friauf, 1993; Gabriele et al., 2000; Torii et al., 2013, Noftz et al., 2014). Evidence of a multimodal topographic map in LCIC becomes evident when combining auditory and somatosensory input arrays (Figure 9). Moreover, developmental studies of auditory connectivity coupled with the results of this study imply this map forms before hearing onset.

The exact developmental time course for this proposed multimodal map remains to be established. Evidence presented here indicates somatosensory afferents from Sp5 are present at birth but are not completely refined as late as P8. It is likely continued modification of these inputs occurs between P8 and P12 based on fine-tuning of auditory inputs to the CNIC during this developmental window (Fathke and Gabriele, 2009). After discovering the post natal age at
which developmental Sp5 inputs to LCIC matches adult anatomy, double-labeling studies of auditory and somatosensory innervation of LCIC may reveal spatial and temporal relationships of the multisensory contributions to this region. This proposed study could potentially reveal if one sensory modality plays a role in coordinating the inputs of another.

**Eph-ephrin Expression Patterns and Axon Terminal Fields**

Our lab has also characterized modular/extramodular expression of Eph-ephrin signaling proteins reminiscent of reported adult neurochemically defined LCIC modules. This family of receptor tyrosine kinase signaling proteins utilize bidirectional signaling to encode place maps for complex sensory and motor systems prior to experience (Flanagan and Vanderhaeghen, 1998; Wilkinson, 2001; Cowan and Henkemeyer, 2002; Kullander and Klein, 2002). Within the LCIC EphA4 and ephrin-B2 expression patterns are isolated in layer 2 modules while ephrin-B3 demonstrates extramodular expression in layers 1 and 3 (Noftz et al., 2014) (Figure 10).

Considering known developmental roles in other systems it is plausible Eph-ephrins present in the LCIC coordinate formation of discrete multimodal maps within this IC subdivision. When comparing expression data with differing sensory terminal fields it appears dissimilar sensory inputs localize to specific Eph-ephrin territories. Experiments consisting of Eph-ephrin mutant animal studies could support this theory thus providing valuable insights into mechanisms underlying the establishment of LCIC architecture.

**GAD Expression**

Neurochemical markers have proven helpful in revealing LCIC layers and modular/extramodular fields. GAD expression patterns characterized in adult rat (Chernock et al., 2004) and mouse (Lesicko and Llano, 2015) reveal distinct layer 2 modules. Here, we show GAD-positive zones
as early as P4 in LCIC (Figure 8). Intriguingly, GAD-positive cell bodies and terminal puncta are apparent (Figure 8, C) in agreement with findings in guinea pig (Nakamoto et al., 2014).

GAD-positive modules at P4, while present, are not yet completely distinct entities. It is likely that separation of modules occurs after P4 as observed for Eph-ephrin modular expression (Gabriele et al., 2011). Furthermore, it is not known whether Eph-ephrin and GAD-positive manifestations colocalize, interdigitate, or overlap. Multiple labeling studies for both neurochemicals can provide useful perception regarding the compartmentalization of the LCIC and whether they serve as a substrate for input-output arrays.

LCIC Functionality

The LCIC responds to complex auditory stimuli across a broadly tuned spectrum (Aitkin et al., 1975, 1981, 1994; Aitkin and Zimmermann, 1978; Syka et al., 2000; Suta et al., 2003; Ota et al., 2004) in addition to somatosensory stimuli (Aitkin and Zimmermann, 1978; Aitkin et al., 1981). Direct electrical stimulation to Sp5 (Zhou and Shore, 2004) also induces LCIC responses. Even so, understanding the functional significance of Sp5 projections to LCIC requires an appreciation for known Sp5 inputs. Facial and oral structures, such as the vocal tract, tongue, and jaw convey proprioceptive information (Romfh et al., 1979; Capra, 1987; Jacquin et al., 1989; Nazruddin et al., 1989; Takemura et al., 1991; Suemune et al., 1992) to Sp5 via the TG (Dehmel et al., 2008). Combining these findings with LCIC innervation from Sp5 provides a polysynaptic pathway that may be responsible for proprioceptive sensory input to the LCIC from body regions responsible for sound production. Therefore, Sp5 inputs to LCIC GABAergic zones, originating from intra-oral structures, could act as a priming mechanism for the presence of self-made vocalization, which may in turn activate inhibitory pathways that selectively suppress certain primary circuits.
tuned to voice-specific characteristics consequently increasing attentiveness to non-self auditory stimuli.

**Potential for Tinnitus Treatment**

Tinnitus, or the perception of a non-existent auditory stimulus, is a debilitating disease affecting millions of Americans. In addition to age related tinnitus onset, somatic tinnitus can occur due to plastic changes in neural circuitry. This particular type of tinnitus is not marked by aging but rather changes in somatosensory inputs affecting the central gain in auditory pathways (Levine, 1999; Eggermont, 2005; Saunders, 2007). Such changes in mapping can be attributed to whiplash, temperomandibular joint syndrome, or bruxism. Understanding the normal anatomy behind properly functioning sensory circuits can provide insights to anatomical deviation in tinnitus disease states. At the same time, knowing somatosensory circuitry can cause auditory dysfunction offers potential to exploit somatosensory pathways to alleviate symptoms. For example, stimulation on the skin at the level of C2 vertebrae activates a polysynaptic circuit that passes through Sp5 and onward to LCIC. Consistent stimulus at C2 could therefore change affected topography back to a normal conformation ultimately achieving tinnitus relief. Finding other somatosensory regions that innervate auditory modalities could aid in field progression of tinnitus treatment.

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REFERENCES


Figure Legends

**Figure 1. A.** Anterograde, ipsilateral labeling at P8 from CNIC to LCIC layers 1 and 3. Modular zones in layer 2 (*dashed contours*) are devoid of input and are known to receive somatosensory input in the adult. Scale bar = 500 µm.

**Figure 2. A.** Dye placement in left Sp5. Retrograde filling of axons in the sp5 tract (*lateral region, dashed contour*) confirm accurate placement in Sp5 nucleus. Scale bar = 500 µm.

**Figure 3. A.** Projections from Sp5 to ipsilateral AVCN are present at P4. Terminal fibers form connections in marginal zones (*inset arrow*). Scale bar = 500 µm.

**Figure 4.** Bilateral terminations of Sp5 inputs to LCIC at P0. Arrows indicate terminal axons. **A.** Fibers enter medial and lateral regions of the LCIC, yet the majority project to lateral LCIC aspects. **B.** Contralateral labeling is present but not as robust as the ipsilateral side. Terminations still prefer medial and lateral areas of the LCIC. Scale bars = 200 µm.

**Figure 5.** Contralateral fiber trajectory for projecting fibers from Sp5 en route to LCIC. **A.** Fibers cross the midline and pass dorsal to the LSO (*inset arrows*). **B.** Ascending Sp5 fibers entering IC via LL (*inset arrows*). Scale bar in A = 500 µm, B = 200 µm.

**Figure 6.** Bilateral LCIC terminal fields present at P4. **A.** Ipsilateral fibers approach the rostral CNIC before deviating laterally or medially. Terminal zones are not yet modular in appearance but rather enter as a distinct fascicle. Me5 cells are retrogradely labeled on the ipsilateral side due to Me5 efferents present in the sp5 tract projecting to spinal cervical regions. **B.** Contralateral fibers are also present in medial (*inset arrows*) and lateral zones. Some axon collaterals were also observed in the CNIC predominantly towards the rostral pole. **C, D** are
higher magnifications of inset boxes in A, B respectively. Scale bar in A, B = 500 µm; Scale bar in C, D = 200 µm.

**Figure 7.** Bilateral rostral LCIC terminal fields just caudal to ITN. Axon collaterals are most abundant in these rostral zones. Modular fields, however, remain absent. **A.** Ipsilateral projections appear more punctate and could potentially be traversing the tissue en route to ITN or SC. **B.** Contralateral fibers show full axons with branching collaterals. **C, D** are higher magnifications of inset boxes in A, B respectively. BIC = brachium of IC nucleus, bIC= brachium of IC tract. Scale bar in A, B = 500 µm; Scale bar in C, D = 200 µm.

**Figure 8.** GAD-positive modules present in layer 2 of LCIC. **A.** GAD-positive zone in LCIC (**inset arrow**). **B.** Higher magnification of inset box in (A). Distinct GAD-positive module is apparent in LCIC layer 2. Discrete modules have not yet fully separated from one another. **C.** High magnification image of ventral most GAD-positive patch in (B). Both cell bodies and terminal puncta are positive for GAD suggesting both GABAergic input and output at the level of LCIC. Scale bar in A = 200 µm; Scale bar in B = 100 µm; Scale bar in C = 30 µm.

**Figure 9.** Summary schematic comparing multimodal inputs to the IC during early development. This diagram combines data from the present study as well as Fathke & Gabriele (2009), Gabriele, Brunso-Bechtold, & Henkel (2000), Gabriele et al. (2011), and Kandler & Friauf (1993). DCN terminal zones are similar to Sp5 in their distribution to lateral and rostral regions of LCIC, however these fibers do not appear in medial aspects. DCN also projects bilaterally to laminar layers in CNIC along with other previously described inputs (e.g. LSO and DNLL).
**Figure 10.** Summary of ephrin-B2, EphA4, and ephrin-B3 protein expression. **A, B.** Ephrin-B2 and EphA4 positive (*dashed contours*) LCIC modules, respectively. **C.** Ephrin-B3 extramodular labeling (*negative modules, dashed contours*). Scale bars = 200 μm.
Figures

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