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Perineuronal Nets and Subtypes of GABAergic Cells Differentiate Auditory and Multisensory Nuclei in the Intercollicular Area of the Midbrain

Nichole L Beebe¹, William A Noftz^{1,2}, Brett R Schofield^{1,2}

¹Northeast Ohio Medical University, Rootstown, OH

²Kent State University, Kent, OH

Abstract

The intercollicular region, which lies between the inferior and superior colliculi in the midbrain, contains neurons that respond to auditory, visual, and somatosensory stimuli. Golgi studies have been used to parse this region into three distinct nuclei: the intercollicular tegmentum (ICt), the rostral pole of the inferior colliculus (ICrp), and the nucleus of the brachium of the IC (NBIC). Few reports have focused on these nuclei, especially the ICt and the ICrp, possibly due to lack of a marker that distinguishes these areas and is compatible with modern methods. Here, we found that staining for GABAergic cells and perineuronal nets differentiates these intercollicular nuclei in guinea pigs. Further, we found that the proportions of four subtypes of GABAergic cells differentiate intercollicular nuclei from each other and from adjacent inferior collicular subdivisions. Our results support earlier studies that suggest distinct morphology and functions for intercollicular nuclei, and provide staining methods that differentiate intercollicular nuclei and are compatible with most modern techniques. We hope that this will help future studies to further characterize the intercollicular region.

Graphical Abstract

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

Intercollicular tegmentum; rostral pole of the inferior colliculus; nucleus of the brachium of the inferior colliculus; guinea pigs; VGLUT2; RRID: AB_2336875; RRID: AB_2336874; RRID: AB_2336881; RRID: AB_2278725; RRID: AB_2665454; RRID: AB_10807945; RRID: AB_2336132; RRID: AB_141607; RRID: AB_2534012; RRID: AB_2535710; RRID: AB_2735091; RRID: AB_2336066; RRID: SCR_001775

Introduction

In the midbrain, the inferior colliculus (IC) is a hub for auditory processing and the superior colliculus (SC) is a hub for visual and other sensory inputs to drive orienting or escape behaviors (Comoli, Das Neves Favaro, Vautrelle, et al., 2012; Pereira & Moita, 2016; Winer & Schreiner, 2005). An “intercollicular region” lies between the inferior and superior colliculi, and contains neurons that respond to auditory, visual, and somatosensory stimuli (Aitkin, Kenyon, & Philpott, 1981; Blomqvist, Danielsson, & Norrsell, 1990; Robards, 1979). The intercollicular region can be parsed into distinct areas, including the intercollicular tegmentum (ICt), the nucleus of the brachium of the inferior colliculus (NBIC) and the rostral pole of the inferior colliculus (ICrp). In Golgi studies, the ICt nuclei are described as surrounding a distinct ICrp nucleus that lies just medial to the NBIC (Meininger, Pol, & Derer, 1986; Morest & Oliver, 1984). The NBIC is divided into cell sparse (interstitial) and cell dense (core) nuclei. The NBIC is among the better understood of these areas. It projects to both the SC and the auditory thalamus, but the projections to the SC have received the bulk of the attention. The NBIC contains a rough map of auditory

space, and sends this information to the deep layers of the SC, where the information is used to guide orienting behaviors (King, Jiang, & Moore, 1998; Schnupp & King, 1997).

The ICrp and ICt are less understood. Few studies have focused on either of these nuclei, so information has to be extracted from studies of other regions that include data from the intercollicular region in their figures. These studies sometimes include borders between intercollicular areas but rarely describe how those borders were drawn. Early studies based on the Golgi method provide some of the most detailed descriptions of the borders, but the Golgi method is difficult to combine with modern neuroscience approaches. Differences in naming conventions add another layer of uncertainty. For example, the ICt can also be referred to as the intercollicular nucleus (although whether these two terms are strictly interchangeable is unclear), and occasionally the entire intercollicular region is referred to simply as the brachium of the IC. While it is generally straightforward in experimental tissue to distinguish these regions from the adjacent areas such as the superior colliculus and the periaqueductal gray, it has been more difficult to distinguish borders within the intercollicular area (between regions, such as the intercollicular tegmentum vs. IC rostral pole) and between intercollicular regions and the “traditional” subdivisions of the IC (such as the lateral cortex). Progress in understanding these intercollicular areas has been hampered by the lack of criteria that can be easily applied to modern experimental approaches.

In the course of studying perineuronal nets (PNs) and GABAergic cell subtypes in the IC, we noticed that several of the markers appeared to indicate clear borders among the intercollicular areas (Beebe, Mellott, & Schofield, 2018; Beebe & Schofield, 2018; Beebe, Young, Mellott, & Schofield, 2016; Foster, Mellott, & Schofield, 2014). We have also established criteria for distinguishing four subtypes of GABAergic cells in the IC based on staining for PNs and perisomatic rings of boutons that immunostain for vesicular glutamate transporter 2 (VGLUT2) (Beebe, Young, Mellott, & Schofield, 2016). The subdivisions of the IC that were previously examined (central nucleus, dorsal cortex, and lateral cortex) differ in the proportions of the four GABAergic cell subtypes they contain.

The present study shows that staining for PNs or for GABAergic cells can discriminate the major intercollicular areas from each other and their surrounding neighbors. The borders appear similar to those first described in Golgi studies and marked or implied in a variety of tracing and physiological studies. Such staining is readily combined with modern methods and should greatly facilitate comparison of results across studies. In addition, four subtypes of GABAergic cells are present in the intercollicular region, and the relative proportions of subtypes distinguish the intercollicular nuclei from one another and from adjacent subdivisions of the IC. The recognition of inhibitory cell subtypes has provided key insights into inhibitory function in many brain areas (Kubota, 2014; Lovett-Barron & Losonczy, 2014), but has yet to be addressed in the intercollicular region.

Methods

All procedures were conducted in accordance with the Northeast Ohio Medical University Institutional Animal Care and Use Committee and National Institutes of Health guidelines.

Results are described from seven adult pigmented guinea pigs of either gender (Elm Hill Labs). Animal weights ranged from 295 to 780 g. Efforts were made to minimize the number of animals and their suffering. Methods used here were described previously (Beebe, Mellott, & Schofield, 2018; Beebe, Young, Mellott, & Schofield, 2016).

Perfusion and tissue processing.

Each animal was deeply anesthetized with isoflurane until breathing stopped and corneal and withdrawal reflexes were absent. The animal was then perfused transcardially with Tyrode's solution, followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, then 250 ml of the same fixative containing 10% sucrose. The brain was removed and stored in fixative containing 25% sucrose at 4°C overnight. The following day, the cortex and cerebellum were removed, and the brainstem was frozen and cut into 40 or 50 µm sections in the transverse plane on a sliding microtome. Sections were collected in six series.

For one series, tissue sections were washed in phosphate-buffered saline (PBS; 0.9% NaCl in 0.01 M phosphate buffer, pH 7.4) and then permeabilized in 0.2% Triton X-100 in PBS for 30 minutes. Nonspecific staining was blocked by treating sections with 10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for one hour. After blocking, PNs were stained with either fluorescein-labeled *Wisteria floribunda* agglutinin (WFA; 1:100; Vector Laboratories FL-1351; RRID: AB_2336875) or with biotinylated WFA in PBS for one hour (1:100; Vector Laboratories B-1355; RRID: AB_2336874). If biotinylated WFA was used, it was labeled by incubating tissue in a solution of Alexa Fluor 405- or Alexa Fluor 488-conjugated streptavidin (1:100; Molecular Probes S-32351 and S-11223, respectively; RRID: AB_2336881 for S-11223) in PBS for one hour at room temperature. Sections were rinsed with PBS, and then a mixture of primary antibodies was applied in a solution containing 1% NGS and 0.2% Triton X-100. Sections were allowed to incubate at 4°C for 48 hours. The following antibodies were included in the mixture: anti-GAD67 to label GABAergic neurons (1:250; Millipore MAB5406; RRID: AB_2278725), anti-VGLUT2 to label VGLUT2-containing boutons (1:2500; Millipore AB2251; RRID: AB_2665454), and anti-NeuN as a neuron-specific stain (1:500; Millipore ABN78; RRID: AB_10807945). After primary incubation, if fluorescein-conjugated WFA was used, sections were rinsed in PBS and treated with a biotinylated anti-guinea pig antibody to label the anti-VGLUT2 primary antibody (1:100; Vector Laboratories BA-7000; RRID: AB_2336132). Omission of the anti-VGLUT2 primary antibody revealed some staining resulting from this anti-guinea pig secondary antibody; because this secondary staining was restricted to cell bodies and we examined only VGLUT2-labeled boutons here, it was ignored (Sergeeva & Jansen, 2009). Sections were then incubated in a mixture including an Alexa Fluor 488- or an Alexa Fluor 546- conjugated anti-mouse antibody (to reveal GAD67; 1:100; Molecular Probes A21202 and A10036, respectively; RRID: AB_141607 and AB_2534012, respectively), an Alexa Fluor 750- conjugated anti-rabbit antibody (to reveal NeuN; 1:100; Molecular Probes A21039; RRID: AB_2535710), and an Alexa Fluor 647-conjugated anti-guinea pig antibody if biotinylated WFA was used, or an Alexa Fluor 647-conjugated streptavidin if fluorescein-conjugated WFA was used (to reveal VGLUT2; each at 1:100; Molecular Probes A21450 and S-21374, respectively; RRID: AB_2735091 and AB_2336066, respectively) in PBS.

Sections were mounted on gelatin-coated slides from 0.2% gelatin solution, allowed to air dry, and then coverslipped with DPX mounting medium (Sigma).

Data analysis.

For quantitative analysis, two transverse sections through the intercollicular region were selected from each of three cases. Of the nuclei of interest, the ICrp has the smallest overall dimensions, so sections were chosen to ensure a good representation of this region. Two sections were selected from each animal to provide adequate sample sizes for quantitative comparison. Each section included parts of all four regions of interest, and typically included the caudal end of the superior colliculus. Note that the NBIC, unlike the other areas, continues well rostral to these sections, extending as far as the medial geniculate body of the thalamus. Our sampling was restricted to the caudal areas, where the NBIC borders the other regions of interest. Each section was outlined using a Neurolucida reconstruction system (MBF Bioscience; RRID: SCR_001775) attached to a Zeiss AxioImager Z2 microscope. A low-magnification photomontage of the GAD67 and WFA fluorescence was collected, and the merged image was used to draw borders between the SC, NBIC, ICt, ICrp, and the periaqueductal gray. The section was then remounted in the microscope, and a “virtual tissue” photomontage of NeuN immunoreactivity was collected at 2- μ m-depth intervals with a 63X oilimmersion objective (numerical aperture 1.4). The montage was displayed on a Cintiq 21UX interactive pen display (Wacom) attached to the Neurolucida system. The Cintiq stylus was used to manually trace the soma of every NeuN-reactive cell with a visible nucleolus within 4 μ m of one cut surface of each section. We chose this depth as a criterion for analysis because preliminary analysis showed that each of the fluorescent markers penetrated the section at least this far; thus, lack of staining with a given marker is unlikely to be attributable to inadequate penetration of the staining reagents (Mellott, Foster, Ohl, & Schofield, 2014). The section outline, with its associated NeuN-stained soma outlines, was then aligned to the original section, and each neuron was viewed with the appropriate fluorescence filters to identify expression of the stained markers (a PN, expression of GAD67, or a dense ring of axosomatic VGLUT2-expressing boutons). A soma was considered to have a dense ring of VGLUT2-expressing boutons if greater than approximately 75% of the perimeter was covered by VGLUT2+ puncta, a criterion set previously to guarantee consistency across studies (Beebe, Young, Mellott, & Schofield, 2016). Each neuronal outline was color coded to indicate the marker(s) with which it was associated. A total of 3518 neurons were outlined and coded. Data including each cell's location (e.g., NBICc), coded cell type (e.g., GAD67+), perimeter, area, X and Y coordinates of the centroid, and minimum and maximum Feret diameters for each soma outline were exported from Neurolucida into R v3.4.1 for Mac OS X (R Core Team, 2017) for all further analyses. Neurons with a profile that overlapped a subdivision outline were excluded from analysis, so as not to be counted twice.

Similarity in proportions of GABAergic and non-GABAergic cells and proportions of GABAergic subtypes between intercollicular areas were tested with G tests for goodness of fit. Source code for G tests was imported from <http://www.psych.ualberta.ca/~phurd/cruft/g.test.r>. Because six pairwise g-tests were conducted between intercollicular areas, the threshold for statistical significance was adjusted using a Bonferroni correction to 0.05/6 (or

0.008). The difference in average GABAergic soma profile area between intercollicular areas was tested with a linear mixed effects model. Case was included as a random factor in models to account for minor variations in soma size that could result from fixation differences. The “nlme” package was used for fitting of linear mixed effects models (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2017), and the “emmeans” package was used for pairwise comparisons (Tukey’s HSD; Lenth, 2018). Because soma profile area data were log-normally distributed, data were log-transformed before statistical testing. Bar graphs were generated in Microsoft Excel. Photomicrographs were taken using a Zeiss AxioImager Z2 microscope with an attached Apotome II (Zeiss) using Neurolucida software (MBF Biosciences). Photographs were taken using structured illumination microscopy with optical sectioning at 0.2 μ m depth intervals. Adobe Photoshop (CS6, Adobe Systems) was used to add scale bars, and crop and colorize images. Brightness and contrast levels were adjusted globally when necessary.

Results

The intercollicular region can be divided into four distinct areas based on staining for GAD and PNs

Figure 1 shows images of a coronal section through the intercollicular region stained for GAD (Fig. 1A, magenta, top) and PNs (Fig 1B, green, middle). Distinct areas can be identified in each channel as well as in the merged image (Fig. 1C, bottom). The ICt shows less staining for GAD and PNs when compared to the adjacent deep SC and periaqueductal gray, while more intense GAD and PN staining differentiate the NBIC core nucleus (NBICc) and ICrp from the surrounding NBIC interstitial nucleus (NBICi) and ICt. There were no obvious differences in GAD or PN staining between the NBICi and ICt, however previous studies differentiate the NBIC from the ICt (Morest & Oliver, 1984), so a line that encompasses the NBICc was drawn from the dorsal to ventral lateral surface of the brachium (the protrusion along the lateral edge of the section). While there were variations between individuals, the differences we describe allowed us to consistently identify and distinguish the intercollicular areas across animals.

Based on the above qualitative observations, we asked whether there are quantitative differences between intercollicular areas (the NBICi, the NBICc, the ICrp, the ICt) regarding the relative prevalence of GAD+ cells, their soma characteristics, and their association with extracellular markers such as PNs. Borders between the areas were identified as described above. In addition, the ventral boundary of the ICt abuts the midbrain reticular formation. The GAD stain provides little differentiation here, but PNs are more numerous in the reticular formation than in the ICt. Even so, the lateral ~1/3 of this border is difficult to discern because the density of PNs in the reticular formation drops off. For the purposes of quantitation, we arbitrarily extended this line to the point where the border of the NBIC meets the lateral edge of the section. Future studies with other criteria, perhaps including physiological or connectional data, may provide more clarity for this ventral border. Each of the quantitative differences we examined is shown in Table 1. Some especially relevant differences are presented in detail below.

The percentage of neurons that are GAD+ differs between intercollicular areas

We counted NeuN+ neurons across four intercollicular areas, then characterized whether each NeuN+ neuron displayed staining for GAD, classifying it as GABAergic. In each area, both GAD+ and non-GAD neurons were observed (Figure 2A; magenta and yellow arrows, respectively). In each area, the majority of neurons were non-GAD, however areas differed in their relative proportions of GAD+ and non-GAD neurons (Figure 2B). On average, the NBICc had the highest proportion of GAD+ neurons (27%), while the ICt had the lowest proportion of GAD+ neurons (11%). G-tests for goodness of fit determined that the proportions of GAD+ and non-GAD neurons were significantly different between the ICt and NBICi ($G_{(1)} = 56.01$, $p = 7.21 \times 10^{-14}$), between the ICt and the NBICc ($G_{(1)} = 49.24$, $p = 2.26 \times 10^{-12}$), between the ICt and the ICrp ($G_{(1)} = 11.65$, $p = 0.00064$), between the NBICi and the NBICc ($G_{(1)} = 46.06$, $p = 1.15 \times 10^{-11}$), and between the NBICc and the ICrp ($G_{(1)} = 7.05$, $p = 0.0079$), but not between the NBICi and the ICrp ($G_{(1)} = 1.85$, $p = 0.17$).

The mean soma size of GAD+ neurons differs between intercollicular areas

Golgi studies of intercollicular areas have described differences in neuronal soma size (Meininger, Pol, & Derer, 1986; Morest & Oliver, 1984). Specifically, smaller cells are present in the ICrp and larger cells are present in the ICt. In this study, we did not see clear differences between regions in mean soma size when all NeuN+ cells were considered (Table 1). In the IC, soma size appears particularly important with regard to GABAergic cells (Schofield & Beebe, 2019). In the present study, GAD+ neurons showed a wide range of soma sizes so we compared the profile areas of GAD+ somas in each of the four regions (Figure 3). Average GAD+ soma size was largest in the NBICc (mean soma profile area of $223 \mu\text{m}^2$), and smallest in the ICt and the NBICi (both had a mean soma profile area of $161 \mu\text{m}^2$). A linear mixed effects model of the data was constructed, and a likelihood ratio test showed that there was a significant relationship between soma profile area and region for GAD+ cells ($\chi^2_{(3)} = 38.52$, $p < 0.001$). Pairwise comparison using a Tukey's HSD test showed significant differences in GAD+ soma profile area between the NBICi and the NBICc ($p < 0.001$) and between the NBICc and the ICt ($p < 0.001$).

The proportions of GAD subtypes differ between intercollicular areas

GABAergic neurons in the IC can be categorized into four subtypes based on the presence or absence of a perineuronal net and a perisomatic ring of VGLUT2+ boutons (Beebe, Young, Mellott, & Schofield, 2016). Individual GAD+ somas can have just a perineuronal net ("GAD-PN"), just a VGLUT2 ring ("GAD-VGLUT2 ring"), both a ring and a net (GAD-PN-VGLUT2 ring), or neither marker ("GAD-Only"). Furthermore, the four subtypes differ in average soma size, with GAD-Only cells being smallest and GAD-PN-VGLUT2-ring cells being largest. Given the qualitative differences in PN staining and quantitative differences in GAD+ soma size between the intercollicular regions, we examined whether the four intercollicular areas differ in the proportions of the four GAD subtypes. Examples of three subtypes are shown in Figure 4A, including GAD-Only cells from the ICt and the NBICi (light blue arrows), a GAD-PN cell from the NBICc (light green arrow) and a GAD-PN-VGLUT2 ring cell from the ICrp (dark green arrow). GAD-VGLUT2 ring cells, the least

common subtype, were observed in the ICt, the NBICi and the ICrp, but were very rare (Figure 4B). The most common subtype of GAD+ cells in each area was the GAD-Only subtype. This subtype made up a majority of GAD+ cells in each region except for the ICrp, where they made up an average of 45% of GAD+ cells. We noted that the variation in the proportions of GAD subtypes present was especially high in the ICrp. This could be due to a small sample size, or high variability in the proportions of GAD subtypes could be a characteristic of the ICrp. Because the sample size of GAD-VGLUT2 ring cells was small, these cells were combined with GAD-PN-VGLUT2 ring cells into a “VGLUT2 ring-surrounded” group for statistical analysis. G tests for goodness of fit showed that the proportions of GAD+ subtypes differed between the ICt and the NBICi ($G_{(1)} = 34.75$, $p = 2.85 \times 10^{-8}$), between the ICt and the NBICc ($G_{(1)} = 48.85$, $p = 2.47 \times 10^{-11}$), between the ICt and the ICrp ($G_{(1)} = 34.82$, $p = 2.75 \times 10^{-8}$), between the NBICi and the NBICc ($G_{(1)} = 130.02$, $p = 2.2 \times 10^{-16}$), and between the NBICi and the ICrp ($G_{(1)} = 195.13$, $p = 2.20 \times 10^{-16}$), but not between the NBICc and the ICrp ($G_{(1)} = 2.30$, $p = 0.317$).

Discussion

The high level of variability in the way intercollicular areas are identified has hampered comparisons between studies and insights into the functions of these areas. Here, we describe histochemical markers that can distinguish intercollicular areas. We found that staining for either GABAergic cells or PNs can be used to divide intercollicular areas. The borders indicated by these two stains correspond to one another and appear to be directly comparable to the borders described in the earlier Golgi studies (Meininger, Pol, & Derer, 1986; Morest & Oliver, 1984). To a large extent, they can also be related to past tracing studies. Quantitatively, intercollicular areas differed in many of the metrics examined, including in their proportions of GABAergic and non-GABAergic cells, in the average soma size of GABAergic cells, and in the proportions of four subtypes of GABAergic cells. Interestingly, differences between the ICt and NBICi were less dramatic, raising the possibility that these areas represent a single structure. Differences in inputs and outputs may distinguish these areas, but interpretation of the data is hindered by lack of details in many connectional studies (see discussion below). We conclude that staining for GABAergic cells or for PNs can be used to differentiate intercollicular areas in future studies, which will allow more precise study of the circuitry and function of each individual intercollicular area. Further, the discovery of subtypes of GABAergic cells could provide a basis for future dissection of the inhibitory circuits within these areas.

The intercollicular areas are distinct from the IC proper

Because this study used identical methods and the same species as our previous study examining subtypes of GABAergic cells in the three main subdivisions of the IC (i.e., dorsal cortex, lateral cortex and central nucleus), we have a unique opportunity to directly compare data between intercollicular areas and the IC proper (Beebe, Young, Mellott, & Schofield, 2016; Figure 5). Generally, intercollicular areas had lower proportions of GABAergic cells than the three main subdivisions of the IC. When the proportions of subtypes of GABAergic cells are considered, a pattern emerges where the NBICc and the ICrp are more similar to the central nucleus of the IC, while the NBICi and the ICt are more similar to cortical areas

of the IC. Despite their similarities, the ICrp appears unlikely to be a rostral extension of the ICc. Based on Golgi studies, Meininger and colleagues describe the ICrp as lacking fibrodendritic laminae, a defining characteristic of the ICc (Meininger, Pol, & Derer, 1986). The same authors go on to say that the ICrp in both mouse and cat is physically separated from the ICc, concluding that the ICrp is distinct anatomically, and presumably functionally, from the ICc and from the surrounding ICt. That the NBIC is distinct from the IC proper has never really been disputed, but the status of the remaining areas is less clear. Numerous studies have combined the IClc with the ICt (and possibly the ICrp) into an “external nucleus (or cortex) of the IC (e.g., Aitkin, Dickhaus, Schult, & Zimmermann, 1978; Aitkin, Webster, Veale, & Crosby, 1975; Björkeland & Boivie, 1984). Reasoning for this includes the prevalence of somatosensory inputs across these areas as well as projections from each of these areas to the SC (Aitkin, Kenyon, & Philpott, 1981; Björkeland & Boivie, 1984; Chernock, Larue, & Winer, 2004; King, Jiang, & Moore, 1998; Mellott, Beebe, & Schofield, 2018). In addition, some species possess “GABA modules” in the lateral IC that can also be found in intercollicular areas (Chernock, Larue, & Winer, 2004; Lesicko, Hristova, Maigler, & Llano, 2016; Dillingham, Gay, Behrooz, & Gabriele, 2017); our interpretation is that such modules, which are not distinct in guinea pigs, likely occur in the ICt but not in the ICrp (see Malmierca, Blackstad, & Osen, 2011 for further discussion of the rostral pole and intercollicular region in rats).

The intercollicular areas are distinct from each other

As described above, data regarding the circuitry of intercollicular areas is available but interpretation can be complicated by differences in terminology and uncertainties about how borders were distinguished. Generally, the pattern of inputs to the intercollicular region seems to reflect the same borders described in the present study. The dorsal column nuclei project broadly across the entire intercollicular region, whereas spino-tectal and spinal trigeminal inputs are concentrated in the ICt (Aitkin, Kenyon, & Philpott, 1981; Björkeland & Boivie, 1984; Hand & Van Winkel, 1977; Itoh, Kaneko, Kudo, & Mizuno, 1984; Jane & Schroeder, 1971; Schroeder & Jane, 1976; Wiberg & Blomqvist, 1984a, b; Wiberg, Westman, & Blomqvist, 1986, 1987). Other inputs that may be focused on the ICt come from cochlear root neurons, the ventrolateral tegmentum, the sagulum, the periaqueductal gray, and the insula (Henkel & Schneiderman, 1988; Herbert, Klepper, & Ostwald, 1997; López, Saldaña, Nodal, Merchán, & Warr, 1999; Wiberg, 1992; Winer, Larue, Diehl, & Hefti, 1998). In the superior olivary complex, the superior paraolivary nucleus may send inputs to each intercollicular area, while there is evidence for lateral superior olivary inputs only to the ICrp and the NBIC (Cant, 2013; Cant & Benson, 2006; Saldaña, Aparicio, Fuentes-Santamaría, & Berrebi, 2009; Shneiderman & Henkel, 1987). Both the ICt and the ICrp receive inputs from the dorsal and ventral nuclei of the lateral lemniscus, while there is less evidence for inputs from these areas to the NBIC (Cant & Benson, 2006 [injections 403, 407, 421, and 429]; Kudo, 1981; Shneiderman, Oliver, & Henkel, 1988; Whitley & Henkel, 1984). There is evidence for inputs from the SC to each intercollicular area, although evidence is strongest for the NBIC (Doubell, Baron, Skaliara, & King, 2000; Redgrave, Mitchell, & Dean, 1987; Sato & Ohtsuka, 1996; Van Buskirk, 1983). The intercollicular region in general is also a target of descending projections from the auditory cortex, but the pattern may be nuanced, with individual cortical areas targeting specific intercollicular areas

(Bajo, Nodal, Bizley, Moore, & King, 2007; Budinger, Heil, Heiss, & Scheich, 2006; Cant & Benson, 2006; Coleman & Clerici, 1987; Hofstetter & Ehret, 1992; Kimura, Donishi, Okamoto, & Tamai, 2004; Mellott, Foster, Ohl, & Schofield, 2014; Rouiller, Hornung, & De Ribaupierre, 1989; Winer, Larue, Diehl, & Hefti, 1998). In addition, there is evidence for descending projections from the globus pallidus to the ICt and the NBIC (Shammah-Lagnado, Alheid, & Heimer, 1996; Shinonaga, Takada, Ogawa-Meguro, Ikai, & Mizuno, 1992). There is also a small amount of information about projections *between* intercollicular areas, with NBIC providing bilateral input with an ipsilateral dominance to the ICt and the ICrp, as well as a possible projection to the contralateral NBIC (Coleman & Clerici, 1987; Kudo, Tashiro, Higo, Matsuyama, & Kawamura, 1984).

Intercollicular areas also appear to differ in their output patterns. There has been much more study of NBIC output pathways compared to the ICrp and the ICt so these “differences” may be partially due to lack of study of ICrp and ICt outputs. With this caveat in mind, evidence exists for projections from the NBIC to the IC, the thalamus (including lemniscal and non-lemniscal parts of the auditory thalamus), and the auditory cortex (Budinger, Heil, Heiss, & Scheich, 2006; Cai, Yue, Su, et al., 2018; Calford & Aitkin, 1983; Kudo, Itoh, Kawamura, & Mizuno, 1983; Kudo, Tashiro, Higo, Matsuyama, & Kawamura, 1984; Mellott, Foster, Ohl, & Schofield, 2014; Mitani, Itoh, & Mizuno, 1987; Rouiller, Hornung, & De Ribaupierre, 1989; Senatorov & Hu, 2002; Winer, 1984; Winer, Chernock, Larue, & Cheung, 2002). The ICrp also sends a projection to the auditory thalamus, including non-lemniscal areas, and preferentially targets the posterior intralaminar thalamic nucleus and the peripeduncular nucleus over the medial geniculate body (Cai, Yue, Su, et al., 2018). There is extensive evidence documenting a projection to the SC from both the NBIC and the ICrp (Appell & Behan, 1990; Baldwin, Wong, Reed, & Kaas, 2011; Bednářová, Grothe, & Myoga, 2018; Doubell, Baron, Skalióra, & King, 2000; Harting & Van Lieshout, 2000; King, Jiang, & Moore, 1998; Kudo, Tashiro, Higo, Matsuyama, & Kawamura, 1984; Mellott, Beebe, & Schofield, 2018; Nodal, Doubell, Jiang, Thompson, & King, 2005; Thiele, Rübsamen, & Hoffman, 1996; Wallace & Fredens, 1989). Each intercollicular area, including the ICt, also appears to send a projection to the pretectum and the midbrain reticular formation, although different areas seem to project to different regions of these nuclei (Kudo, Itoh, Kawamura, & Mizuno, 1983). The ICt may also send projections to the lateral posterior thalamus, in addition to reciprocal projections with the PAG and the ventrolateral tegmentum (Herbert, Klepper, & Ostwald, 1997; Itoh, Kaneko, Kudo, & Mizuno, 1984; Wiberg, 1992).

All in all, these patterns of inputs and outputs seem to roughly match what has been documented in electrophysiological recordings from these areas: while each area can respond to visual, somatosensory, and auditory stimuli, the NBIC and the ICrp are more “auditory”, and the ICt is more “somatosensory” (Aitkin, Kenyon, & Philpott, 1981; Blomqvist, Danielsson, & Norrsell, 1990; Robards, 1979). Interestingly, PNs were most prominent in the NBIC and the ICrp and were less prominent in the ICt; this is consistent with the fact that, in the brainstem, auditory structures are heavily associated with PNs (Beebe & Schofield, 2018; Sonntag, Blosa, Schmidt, Rübsamen, & Morawski, 2015). Patterns of inputs and outputs also hint at distinct functions for each intercollicular area, which is supported by the literature. The NBIC clearly plays a role in sound localization; its neurons show sharp spatial tuning, a rough map of auditory space, and a clear preference for

agreement among sound localization cues (Schnupp & King, 1997; Slee & Young, 2014; Yao, Bremen, & Middlebrooks, 2015). Projections from the NBIC to the SC shape the map of auditory space in the deep SC. Recent studies have established that NBIC input to the SC can be excitatory or inhibitory, and that excitatory input can drive feedforward inhibition within the SC, providing possible insight into the mechanisms behind the auditory map in deep SC (Bednárová, Grothe, & Myoga, 2018; Mellott, Beebe, & Schofield, 2018). There is less evidence to support hypotheses about functions of the ICrp and the ICt, although some studies suggest a possible role in vocalization (Jürgens, Ehrenreich, & De Lanerolle, 2002; Dujardin & Jürgens, 2005) and roles in multisensory integration are supported by their patterns of inputs and outputs. The least distinct areas in our study were the NBICi and the ICt, between which we did not find many qualitative or quantitative differences. There is not extensive literature supporting a separation of the NBICi and the ICt, however this could be due to lack of information generally about the ICt, as well as the lack of separation between NBICi and NBICc in most studies. Based on current evidence, we cannot discount the possibility that the NBICi and the ICt represent a continuous region surrounding both the NBICc and the ICrp.

In reviewing the literature, it becomes clear that, in addition to being adjacent, the deep layers of the SC and the PAG are closely tied to the intercollicular areas described here (e.g. reciprocal projections between the ICt and the PAG [Wiberg, 1992], or projections to the SC from both the ICrp and the NBIC [Mellott, Beebe, & Schofield, 2018]). While we saw qualitative differences in GAD and PN staining between the ICt and deep SC/PAG, given these ties, a valuable future study might examine the potential quantitative differences. Additionally, any future studies of the intercollicular region might benefit from treating the NBIC, ICrp, ICt, PAG, and deep SC as a related set of multisensory nuclei.

Functional implications for differences in proportions of GABAergic subtypes

One of the more dramatic differences we observed between intercollicular areas was in the proportions of subtypes of GABAergic cells present. The possible functional consequences for differences in GABAergic subtypes have been discussed extensively elsewhere (Beebe, Mellott, & Schofield, 2018; Beebe, Young, Mellott, & Schofield, 2016; Foster, Mellott, & Schofield, 2014; Schofield & Beebe, 2019). The presence of VGLUT2 rings in the IC proper appears to support fast, reliable processing of temporal information (Geis & Borst, 2013; Ito, Bishop, & Oliver, 2009); a similar role in temporal processing may be associated with the VGLUT2-ringed cells in the intercollicular areas. PNs also carry functional implications. One function of PNs has to do with fast temporal processing; in the superior olivary complex, the removal of PNs disrupts firing rate and spike timing (Balmer, 2016; Blosa, Sonntag, Jäger, et al., 2015). However, for PN-surrounded cells that are not associated with VGLUT2 rings, the PN may be serving a different role. PNs can inhibit structural plasticity, affect synaptic plasticity, and protect neurons from oxidative stress (Bukalo, Schachner, & Dityatev, 2001; Cabungcal, Steullet, Morishita, et al., 2013; Corvetto & Rossi, 2005; de Vivo, Landi, Panniello, et al., 2013; Suttkus, Rohn, Jäger, Arendt, & Morawski, 2012). PNs seem to be most associated with lack of plasticity; specifically, studies show changes in behavior that seem to indicate increased plasticity following PN removal in many areas of the brain (e.g., Gogolla, Caroni, Lüthi, & Herry, 2009; Happel, Niekisch, Castiblanco Rivera, et al.,

2014; Lee, Leamey, & Sawatari, 2012; Pizzorusso, Medini, Landi, et al., 2006). Overall, the presence or absence of PNs and VGLUT2 rings are likely to confer different properties on the subtypes of GABAergic neurons. Thus, differences in the proportions of GABAergic subtypes support the idea that intercollicular areas differ in function.

Conclusion

We conclude that the intercollicular region contains numerous nuclei which are distinct from each other and from the more caudal “traditional” IC subdivisions. These nuclei differ in their staining properties and their complements of GABAergic cell subtypes, supporting earlier studies that suggest distinct morphology and functions for these nuclei. We further conclude that staining for GABAergic cells or for PNs can provide a way to distinguish these areas that is compatible with many modern methods. We believe that this will aid future studies in further characterization of intercollicular nuclei.

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Figure 1. The intercollicular region can be divided into four distinct areas based on staining for GAD and PNs.

Images show staining for GAD (a, magenta), and PNs (b, green). The bottom panel (c) shows the two images merged. This study describes four intercollicular regions: the NBICi, NBICc, ICrp, and ICt. Scale = 1 mm. Aq - cerebral aqueduct, ICrp - rostral pole of the IC, ICt - intercollicular tegmentum, NBICc - core nucleus of the brachium of the IC, NBICi - interstitial nucleus of the brachium of the IC, PAG - periaqueductal gray, SC - superior colliculus.

Figure 2. The percentage of neurons that are GAD+ differs between intercollicular areas.
a. Photographs showing NeuN+ cells that are GAD+ (magenta arrows) or GAD-negative (yellow arrows). Each row shows examples from a different intercollicular area. Scale = 50 μ m. **b.** A bar graph showing the percentage of NeuN-stained neurons that are GAD+ (magenta) and GAD-negative (yellow) in each intercollicular area. The highest percentage of GAD+ neurons was present in NBICc, and the lowest percentage of GAD+ neurons was present in ICt. Error bars represent SEM of the GAD+ groups; $n = 3518$ NeuN-stained neurons across three cases. A bracket with an asterisk indicates statistical significance between the indicated areas.

Figure 3. The mean soma size of GAD+ neurons differs between intercollicular areas.

A boxplot showing the median and range of soma sizes for each intercollicular area. The median soma is smaller in the NBICi and the ICT, and larger in the ICrp and the NBICc. A bracket with an asterisk indicates statistical significance between the indicated areas.

Figure 4. The proportions of neurons of different GAD subtypes differ between intercollicular areas.

a. Zoomed in photographs of GAD+ cells from Figure 2, now showing staining for PNs (green, first column) and VGLUT2 puncta (cyan, second column). Examples of the three most common subtypes of GAD cells are shown: GAD-Only (light blue arrows, lacking a PN or a VGLUT2 ring; first two rows), GAD-PN (light green arrow, surrounded by a PN but lacking a VGLUT2 ring; third row), and GAD-PN-VGLUT2 ring (dark green arrow, surrounded by both a PN and a VGLUT2 ring; fourth row). Each row shows an example from a different intercollicular area. Scale = 10 μ m. **b.** A bar graph showing the percentages of GAD+ cells of each subtype in each intercollicular area. The ICrp and NBICc contain a lower proportion of GAD-Only neurons and a higher proportion of GAD-PN and GAD-PN-VGLUT2 ring neurons, while the NBICi and ICt are more heavily dominated by GAD-Only neurons. Error bars represent SEM of the GAD-Only groups; n = 594 GAD+ neurons across

three cases. A bracket with an asterisk indicates statistical significance between the indicated areas.

Figure 5. Intercollicular areas differ from “traditional” IC subdivisions in the percentage of neurons that are GAD+, and in the proportions of GAD+ subtypes.

a. A bar graph showing the percentage of NeuN-stained neurons that are GAD+ (magenta) and GAD-negative (yellow) in each intercollicular area (left group) and each of the main IC subdivisions (right group). Data for “traditional” IC subdivisions was taken from a previous study in guinea pig using the same methodology (Beebe, Young, Mellott, & Schofield, 2016). The three layers of the IClc are represented separately (IClc I, II, and III). Error bars represent SEM of the GAD+ groups. **b.** A bar graph showing the percentage of GAD+ cells of each subtype in each intercollicular area (left group) and in the three main IC subdivisions (right group). Error bars represent SEM of the GAD-Only groups.

Table 1.

Quantitative measures were taken across four intercollicular areas. For percentages and density calculations, the values are expressed as mean across three cases $\pm$ standard deviation. For soma profile areas, the values are expressed as mean of the entire data set (including data from all three cases) $\pm$ standard deviation. n = 924 cells from the ICt, 1896 cells from the NBICi, 421 cells from the NBICc, and 277 cells from the ICrp

		ICt	NBICi	NBICc	ICrp
Percentage of cells that are GAD+		11% $\pm$ 3%	19% $\pm$ 7%	27% $\pm$ 9%	20% $\pm$ 8%
Percentage of cells that are PN-surrounded		4% $\pm$ 1%	4% $\pm$ 2%	18% $\pm$ 1%	17% $\pm$ 6%
Percentage of cells surrounded by VGLUT2+ ring		2% $\pm$ 0.4%	1% $\pm$ 0.4%	4% $\pm$ 2%	4% $\pm$ 1%
GAD+Subtype Makeup	GAD-Only	78% $\pm$ 7%	86% $\pm$ 6%	55% $\pm$ 5%	45% $\pm$ 25%
	GAD-PN	9% $\pm$ 7%	10% $\pm$ 3%	31% $\pm$ 4%	35% $\pm$ 3%
	GAD-VGLUT2 ring	5% $\pm$ 4%	1% $\pm$ 1%	0% $\pm$ 0%	5% $\pm$ 4%
	GAD-PN-VGLUT2 ring	9% $\pm$ 5%	3% $\pm$ 2%	14% $\pm$ 3%	15% $\pm$ 27%
	NeuN+ cells	279 $\pm$ 31	291 $\pm$ 118	224 $\pm$ 59	249 $\pm$ 66
Cell Density (cells per mm ²)	GAD+ cells	32 $\pm$ 12	53 $\pm$ 24	57 $\pm$ 14	46 $\pm$ 9
	Non-GAD cells	246 $\pm$ 24	238 $\pm$ 102	167 $\pm$ 62	203 $\pm$ 74
	PN-surrounded cells	12 $\pm$ 2	12 $\pm$ 8	40 $\pm$ 8	44 $\pm$ 26
	VGLUT2 ring-surrounded cells	6 $\pm$ 1	3 $\pm$ 2	8 $\pm$ 1	9 $\pm$ 5
	NeuN+ cells	131 $\pm$ 64	124 $\pm$ 67	157 $\pm$ 91	133 $\pm$ 75
Soma area (μ m ²)	GAD+ cells	161 $\pm$ 84	161 $\pm$ 100	223 $\pm$ 128	194 $\pm$ 114
	Non-GAD cells	127 $\pm$ 60	117 $\pm$ 55	136 $\pm$ 63	119 $\pm$ 55
	PN-surrounded cells	210 $\pm$ 101	255 $\pm$ 143	246 $\pm$ 139	187 $\pm$ 99
	Non-PN-surrounded cells	128 $\pm$ 60	119 $\pm$ 56	138 $\pm$ 62	122 $\pm$ 64
	VGLUT2 ring-surrounded cells	219 $\pm$ 122	312 $\pm$ 206	403 $\pm$ 198	289 $\pm$ 130
	Non-VGLUT2 ring-surrounded cells	129 $\pm$ 61	123 $\pm$ 61	148 $\pm$ 72	127 $\pm$ 65