Published in final edited form as:

Neuroscience. 2009 April 21; 160(1): 103–114. doi:10.1016/j.neuroscience.2009.02.036.

Sources of Cholinergic Input to the Inferior Colliculus

Susan D. Motts^{1,2} and **Brett R. Schofield^{1,2}**

¹ *Department of Anatomy and Neurobiology, Northeastern Ohio Universities Colleges of Medicine and Pharmacy*

² *Department of Biomedical Sciences, Kent State University*

Abstract

We combined retrograde tracing with immunohistochemistry for choline acetyltransferase to identify the source of cholinergic input to the inferior colliculus (IC). Injection of a retrograde tracer into one IC labeled cells in many brainstem nuclei. Retrogradely-labeled cells that were also immunoreactive for choline acetyltransferase were identified in two nuclei in the midbrain tegmentum: the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT). More PPT and LDT cells project ipsilaterally than contralaterally to the IC and, on both sides, there are more projecting cells in the PPT than in the LDT. Double-labeled cells were not found in any other brainstem nucleus.

A common feature of cholinergic cells in PPT and LDT is collateral projections to multiple targets. We placed different retrograde tracers into each IC to identify cells in PPT and LDT that project to both ICs. In both PPT and LDT, a substantial proportion (up to 57%) of the immunoreactive cells that contained tracer from the contralateral IC also contained tracer from the ipsilateral IC.

We conclude that acetylcholine in the IC originates from the midbrain tegmental cholinergic nuclei: PPT and LDT. These nuclei are known to participate in arousal, the sleep/wake cycle and prepulse inhibition of acoustic startle. It is likely that the cholinergic input to the IC is directly associated with these functions.

Keywords

Acetylcholine; pedunculopontine tegmental nucleus; laterodorsal tegmental nucleus; sensorimotor gating; acoustic startle; prepulse inhibition

The inferior colliculus (IC) is a large midbrain nucleus that integrates auditory and other information from brainstem nuclei and cortical regions and serves as the primary source of auditory projections to the thalamus. Considerable progress has been made in understanding how individual IC cells integrate the converging information to give rise to specific response properties (e.g., Pollak et al. 2003; Oliver 2005). There also has been growing interest in how modulatory neurotransmitters, such as serotonin and acetylcholine (ACh), can alter responses to sound (Farley et al. 1983; Habbicht and Vater, 1996; Hurley et al. 2002, 2008; Ji et al.

Contact Details: Brett R. Schofield, PhD, Department of Anatomy and Neurobiology, Northeastern Ohio Universities Colleges of Medicine and Pharmacy, 4209 State Route 44, PO Box 95, Rootstown, OH 44272 USA, Telephone: 330-325-6655, Fax number: 330-325-5916, Email address: E-mail: bschofie@neuoucom.edu.
Section Editor: Dr. Charles R. Gerfen

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2005). Serotonergic input arises from the raphe nuclei (Klepper and Herbert, 1991). Recent studies have begun to reveal both the physiological effects and the types of receptors associated with the serotonin inputs to IC cells (Peruzzi and Dut, 2004; Hurley, 2006; Wang et al. 2008).

Several types of data indicate that ACh also plays a major role in the processing of acoustic stimuli by IC neurons. Physiological recording combined with acoustic stimulation and application of cholinergic agonists or antagonists shows that a majority (80% or more) of IC cells are affected by ACh (Watanabe and Simada, 1973; Farley et al. 1983; Habbicht and Vater, 1996). Acetylcholinesterase, the degradative enzyme for ACh, and choline acetyltransferase (ChAT), a synthetic enzyme for ACh, are present throughout the IC (Shute and Lewis, 1967; Henderson and Sherriff, 1991) as are nicotinic and muscarinic receptors (Schwartz, 1986; Glendenning and Baker, 1988; Morley and Happe, 2000). Farley et al. (1983) suggested that ACh enhances responses to specific stimuli (i.e. improves signal/noise ratio). Habbicht and Vater (1996) concluded that ACh plays a neuromodulatory role by “setting the level of neuronal activity” in the IC. Ji et al. (2001) implicated ACh in some of the effects of corticofugal stimulation on IC responses, suggesting that ACh interacts with descending as well as ascending inputs to the IC. Habbicht and Vater (1996) pointed out that further speculation was hindered by lack of knowledge of the source of the ACh in the IC.

There is no evidence that there are cholinergic cells located in the IC, suggesting that the cholinergic innervation arises extrinsically (reviewed in Motts et al. 2008). One possibility involves projections from the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT), two large cholinergic nuclei in the brainstem tegmentum that are components of the ascending reticular activating system (Woolf and Butcher, 1989). The PPT and LDT are the major sources of cholinergic input to the thalamus (Steriade et al. 1988). In addition, many PPT cells respond to acoustic stimuli and influence the acoustic startle response (Reese et al. 1995a, b; Koch et al. 1993; Dormant et al. 1998). Projections from these nuclei to the IC could be important in arousal and in processing of sounds during different behavioral states (Pedemonte et al. 1994).

The second possibility for the source of ACh in the IC is projections from cholinergic cells in nuclei of the ascending auditory pathways. Among these nuclei, cholinergic cells are most numerous in the superior olivary complex (Motts et al. 2008). The majority of these cells (perhaps all) are considered the sources of cholinergic projections to the cochlea and cochlear nucleus; they are not believed to project to the IC (Sheriff and Henderson, 1994). Other auditory nuclei, such as the ventral nucleus of the lateral lemniscus and the sagulum, are known to project to the IC and also to contain cholinergic cells, but whether the latter cells project to the IC is unknown. Projections from cholinergic cells in these lower auditory nuclei would presumably serve a role quite different from projections that originate in the PPT or LDT.

For the current study, we used retrograde tracers to identify cells that project to the IC combined with immunohistochemistry to identify cholinergic cells. By using combinations of retrograde tracers we also identified individual immunoreactive cells that send branched axonal projections to both the left and right ICs.

EXPERIMENTAL PROCEDURES

All procedures were performed in accordance with the Institutional Animal Care and Use Committee and NIH guidelines. Eight albino (Charles River; Wilmington, MA) and ten pigmented (Elm Hill; Chelmsford, MA) adult guinea pigs of either gender weighing 250–600 grams were used. During all experiments, efforts were made to minimize suffering and the number of animals used.

Surgery

For surgery, the guinea pig was anesthetized with either halothane (3% for induction, 1.5–2.5% for maintenance) in nitrous oxide and oxygen or isoflurane (4–5% for induction, 1.75–2.25% for maintenance) in oxygen. The animal's head was shaved and disinfected with Betadine (Purdue Products L.P., Stamford, CT). Moisture Eyes PM ophthalmic ointment (Bausch & Lomb, Rochester, NY) was applied to each eye. The animal was given atropine sulfate (0.05 mg/kg, i.m.) to minimize respiratory secretions during anesthesia. The animal's head was positioned in a stereotaxic frame. Body temperature was maintained with a feedback-controlled heating pad. Sterile instruments and aseptic technique were used for all surgical procedures. An incision was made in the scalp. The incision edges were injected with Marcaine (0.25% bupivacaine with epinephrine 1:200,000; Hospira, Inc., Lake Forest, IL), a long-lasting local anesthetic. A small hole was drilled in the skull using a dental drill. Following the tracer injection, Gelfoam (Harvard Apparatus, Holliston, MA) was placed in the craniotomy site and the scalp was sutured. The animal was given an injection of Ketofen (ketoprofen 3 mg/kg, i.m.; Henry Schein, Melville, NY 11747) for post-operative pain control. The animal was then removed from the stereotaxic holder and placed in a clean cage. The animal was monitored until it was ambulatory and able to eat and drink without difficulty.

Retrograde tracers

Stereotaxic coordinates were used to guide injections of tracers into the brain. We used several fluorescent tracers (red beads [Luma-Fluor, Inc., Naples, FL], green beads [Luma-Fluor], Fast Blue [EMS-Chemie GmbH, Gross Umstadt, Germany], fluorescein dextran [Invitrogen, Carlsbad, CA] and FluoroGold [FluoroChrome, Inc., Englewood, CO]) for these experiments (Table 1). A 1 µl or 10 µl Hamilton microsyringe was used to inject one of the tracers into the IC. Each microsyringe was used only for a single tracer. In order to obtain large injection sites, each tracer was injected at 1–4 sites within the IC (total volume = 0.3–0.8 µl). In some cases, different tracers were injected into the left and right IC to allow us to identify individual cells that send collateral projections to both sides.

Perfusion and sectioning

After 5–14 days, to allow for transport of the tracers, the animal was given an overdose of sodium pentobarbital (440 mg/kg, i.p.). After loss of withdrawal reflex and cessation of breathing, the animal was perfused briefly with Tyrode's solution followed by 250 ml of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 followed by 250 ml of the same fixative with 10% sucrose added. The brain was removed and stored in fixative with 25–30% sucrose. The following day the cortex and cerebellum were removed and the brainstem was frozen and cut in either the transverse or sagittal plane on a sliding microtome into 40–50 µm thick sections, which were collected serially in 6 sets. For identification of cytoarchitectural borders and landmarks, one series was stained with thionin. The remaining series were used for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry for choline acetyltransferase (ChAT) was performed on the tissue sections to label putative cholinergic cells (see Motts et al. 2008 for details). Briefly, the sections were exposed (1–3 days at 4°C) to goat anti-ChAT polyclonal antibody (Chemicon AB 144P, diluted 1:25 to 1:100). The sections were treated with 1% biotinylated rabbit anti-goat antibody (Vector BA-5000) and labeled with streptavidin conjugated to an appropriate fluorescent marker (AlexaFluor 350 [AF350; blue], AlexaFluor 488 [AF488, green], or AlexaFluor 647 [AF647; near-infrared], Invitrogen; Carlsbad, CA) that was easily distinguished from the retrograde tracers (Table 1). The sections were mounted on gelatin-coated slides, allowed to dry and coverslipped with DPX (Sigma).

We have previously reported the results of control experiments for the ChAT antibody, Chemicon AB144P, under the conditions used in the present experiments (Motts et al. 2008). Briefly, Western blot analysis of guinea pig brain tissue produced a band of staining at the expected 68–70 kDa. Staining in tissue sections was eliminated by pre-adsorption of the primary antibody with ChAT or by omission of primary or secondary antibody from the solutions.

Data analysis

Labeled cells and injection sites were plotted using a Neurolucida reconstruction system (MBF Bioscience, Williston, VT) attached to a Zeiss Axioplan II microscope. Sections were analyzed from the middle thalamus rostrally to the level of the dorsal column nuclei caudally. Borders of nuclei containing labeled cells were identified by comparison with adjacent thionin-stained sections or by removing the coverslips from plotted sections, staining the sections with thionin and re-applying coverslips. Adobe Illustrator was used to arrange the plots of sections, change the color of markers, adjust line thicknesses, and to add labels to the plots.

The cholinergic nuclei were identified as described previously (Leonard et al., 1995; Motts et al. 2008). The laterodorsal tegmental nucleus (LDT) is located primarily within the periaqueductal gray dorsomedial to the superior cerebellar peduncle (scp). The pedunculopontine tegmental nucleus (PPT) is pierced by the scp caudally and extends rostrally towards the substantia nigra. The borders of these nuclei cannot be discerned clearly using a Nissl stain, so we used the distribution of ChAT+ cells to define the extent of the nuclei.

Photomicrographs were obtained using either a Zeiss Axioskop fluorescence microscope and Magnafire camera (Optronics) or a Zeiss Imager Z1 fluorescence microscope and AxioCam HRm camera (Zeiss). Adobe Photoshop was used to add scale bars, size and crop images, erase background around tissue sections, and adjust intensity levels. Images of AF647, which fluoresces at infrared wavelengths, were taken in monochrome mode and pseudocolored with either the camera software (AxioVision, Zeiss) or with Photoshop. For quantitative summary, cases were selected in which the ChAT immunostain was bright and even across sections and in which there were at least 50 retrogradely labeled cells per bin (every sixth section) in PPT and LDT (left and right sides combined).

In some cases, we injected different tracers in the left and right IC (Table 1). When analyzing these data, each cell was examined for the presence of each tracer (as well as the immunolabel). Labeled cells were plotted with distinct symbols for each possible combination of labels. Cells that contained both retrograde tracers (“double-tracer”) were interpreted as sending axon branches to both ICs. Triple-labeled cells (double-tracer plus immunolabel) were interpreted as cholinergic cells that send axon branches to left and right IC. For quantitative summary of collateral projections, we selected cases in which each of the tracers used met the criteria described above.

RESULTS

We combined retrograde tracer injections and immunolabeling for ChAT to identify the source of cholinergic input to the IC. The various retrograde tracers yielded similar patterns of labeled cells. No differences in data from albino or pigmented guinea pigs were seen.

The injection sites were usually centered in the IC (Fig. 1) and frequently approached the IC borders. We excluded any case in which the injection spread ventrally into the tegmentum, rostrally into the superior colliculus or medially into the periaqueductal gray. None of the injections spread across the midline into the contralateral IC.

Retrogradely labeled cells were present in many areas, including the cochlear nuclei, superior olivary complex, sagulum and nuclei of the lateral lemniscus as well as the superior colliculus and thalamus. In most cases, the dorsal column nuclei and spinal trigeminal nuclei also contained retrogradely labeled cells. The damage caused by multiple injections made IC subdivisions difficult to discern with the thionin stain. However, given the large size of injections and the patterns of retrograde labeling, we conclude that the injections often included multiple IC subdivisions. Across cases, the injection sites included all major parts of the IC except the most dorsomedial regions.

ChAT-immunolabeled cells were present in many brainstem areas, as described in detail previously (Motts et al. 2008). We examined all areas of the brainstem that contained retrogradely labeled cells for evidence of cells that were labeled with both ChAT immunohistochemistry and retrograde tracer. We describe first the cells that contained ChAT immunolabel and a single retrograde tracer; i.e., cholinergic cells that project to the IC. We then describe presumptive cholinergic cells that send branching axons to both ICs.

Projections to ipsilateral or contralateral IC

Figure 2 shows examples of retrogradely labeled cells that were also immunopositive (“tracer/ChAT+” cells). Such cells were readily identified in the PPT and LDT (Fig. 2A–D). Overall, 18% – 89% of the retrogradely-labeled cells in these 4 nuclei (ipsilateral and contralateral PPT and LDT) were ChAT-immunoreactive (9 cases, 2472 total cells; average percentage = $51 \pm 8\%$ [s.e.m.]). Cells that were not labeled with the ChAT marker may be non-cholinergic or the immunostaining could have failed to label them (due perhaps to limited penetration of the antibodies). Incomplete immunostaining is likely to explain some but not all the ChAT-negative cells because we observed tracer-labeled/immunonegative cells in close proximity to tracer/ChAT+ cells (Fig. 2A, B, arrowheads). We conclude that some non-cholinergic cells in the PPT and LDT project to the IC. However, the observation that up to 89% of the tracer-labeled cells could be ChAT+ suggests that the majority of PPT and LDT cells that project to the IC are cholinergic.

Figure 3 illustrates the distribution of tracer/ChAT+ cells in the PPT and LDT after an injection of FluoroGold into the right IC. In the LDT, tracer/ChAT+ cells were present throughout the nucleus. In the PPT, the tracer/ChAT+ cells were located throughout the nucleus, although few cells were present in the caudal or rostral ends. For each nucleus, there were more cells on the ipsilateral side than the contralateral side (Fig. 4). On each side, the PPT contained more cells than the LDT.

No other brainstem nuclei contained tracer/ChAT+ cells. Figure 2(E) shows a typical result in the superior olivary complex. A single field of view from the lateral superior olivary nucleus contained both ChAT+ cells and retrogradely labeled cells in close proximity. Both types of label were found at similar focal planes, indicating that the immunochemicals penetrated the tissue sufficiently to reach the retrogradely-labeled cells. This argues strongly that the retrogradely-labeled cells are non-cholinergic. A similar negative finding characterized other nuclei, including the periolivary nuclei, cochlear nucleus, ventral nucleus of the lateral lemniscus, sagulum and nucleus of the brachium of the IC. Other areas that contained retrogradely labeled cells, such as the intermediate layers of the superior colliculus and areas in and around the medial geniculate nucleus, contained retrogradely-labeled cells but did not contain ChAT-labeled cells.

Collateral projections to left and right IC

Collateral projections are characteristic of cholinergic cells in both the PPT and the LDT (e.g. Woolf and Butcher, 1986; Jourdain et al. 1989; Shiromani et al. 1990; Semba et al. 1990). In

the present experiments, injections of different retrograde tracers into left and right ICs produced double-labeled (“double-tracer”) cells in several auditory nuclei (e.g. dorsal cochlear nucleus, superior paraolivary nucleus), consistent with previous reports in guinea pigs (Schofield and Cant, 1992; 1996). Double-tracer cells were also present in PPT and LDT. The majority of these cells (over 80%) were immunopositive (Fig. 5). These triple-labeled cells were more common in PPT than LDT (Fig. 6).

In order to judge the prominence of collaterals in the cholinergic projection, we counted the double-tracer/ChAT+ cells in each nucleus and expressed that number as a percentage of the tracer/ChAT+ cells that were labeled from one or the other IC (Fig. 7; Table 2). In PPT, the double-tracer/ChAT+ cells (i.e., those with collateral projections) constitute up to 11% of ChAT+ cells that project to the ipsilateral IC and up to 57% of the ChAT+ cells that project to the contralateral IC. These percentages reflect the large ipsilateral PPT projection to IC and the smaller contralateral projection. Compared to the PPT, there are fewer cells in the LDT that project to IC, but a similar projection pattern exists. In LDT, the double-tracer/ChAT+ cells constitute up to 16% of ChAT+ cells that project to the ipsilateral IC and up to 50% of the ChAT+ cells that project to the contralateral IC.

DISCUSSION

Our results show that the PPT and the LDT are the sources of cholinergic input to the IC. A small number of apparently non-cholinergic cells in each of these nuclei also project to the IC. In addition, some cholinergic cells send branching axonal projections to both left and right IC. First we will discuss technical issues for interpretation of our data. We then compare our findings to earlier studies and discuss some of the functional implications.

Technical Considerations

ChAT, the synthetic enzyme for acetylcholine, is considered a specific marker for cholinergic cells (Levey and Wainer, 1982; Armstrong et al. 1983; German et al. 1985; Maley et al. 1988). We used the antibody (Chemicon AB144P) and the fixation and processing parameters that were validated previously for use in guinea pig brain (Motts et al. 2008). We conclude that all of the immunolabeled cells are likely to be cholinergic.

It can be difficult to interpret the absence of immunolabel. Based on our results, we conclude that the majority of PPT and LDT cells that project to the IC are cholinergic but that a small number of the projecting cells use a different neurotransmitter. Other neurotransmitters identified in PPT and LDT cells include GABA, glutamate and peptides such as substance P and corticotropin-releasing factor (Lavoie and Parent, 1994; Ford et al. 1995; Leonard et al. 1995; Vincent et al. 1986; Jia et al. 2003). It remains to be determined whether any of these transmitters are associated with the ChAT-negative cells that we labeled from the IC.

An important consideration in tracer studies is the possibility that axons of passage are labeled, whereby cells are labeled because their axons traverse an injection site even though they do not terminate there. The PPT and LDT give rise to several large bundles of cholinergic axons (Hallanger and Wainer, 1988). However, none of these bundles traverse the IC, so axons of passage are unlikely to have affected our results.

The identification of collateral projections with multiple retrograde tracers is known to underestimate the number of collaterals in a pathway (Schofield et al. 2007). The underestimation is compounded when identifying cells labeled with immunohistochemistry because of possible incomplete immunolabeling. We describe the triple-labeled cells (i.e., those with collaterals) as a percentage of the populations that contained either tracer. Because of the underestimation of the total cells due to incomplete labeling of each component of the pathway

(to each IC), the maximum percentage across cases may be a more appropriate representation of the pathway than, for example, the average percentage. Our data suggest that PPT cells with collateral projections to both ICs represent up to 11% of the projection to the ipsilateral IC and 57% of the projection to the contralateral IC. The percentages differ because of the larger total number of cells that project to the ipsilateral IC. Bilaterally-projecting cells clearly make up a larger part of the contralateral projection; in fact, it is impossible to know from our data whether any contralaterally-projecting cells do *not* also project to the ipsilateral IC. The same conclusions apply to projections from the LDT, for which the bilaterally-projecting population constituted up to 16% of the ipsilateral projection and 50% of the contralateral projection.

Comparison with earlier studies

There have been several broad studies of the ascending or descending projections from the PPT and LDT (e.g. Rye et al, 1987; Hallanger and Wainer, 1988; and Woolf and Butcher, 1989). The list of neuronal regions innervated by these nuclei is extensive, but the IC is rarely mentioned. It is not denied as a target, but is generally not discussed. Shute and Lewis (1967) suggested that PPT and LDT project to the IC, but the neurotransmitter was uncertain (Eckstein and Sofroniew, 1983). In a more restricted study, Sherriff and Henderson (1994) identified cholinergic projections to the cochlear nucleus in rats. As part of a control experiment, they injected True Blue or horseradish peroxidase into the IC of a rat (a single animal with each tracer). They observed retrogradely-labeled cells in the PPT and LDT that were double-labeled with ChAT immunohistochemistry and suggested that these nuclei are the sources of cholinergic input to the IC. Our conclusions are consistent with those of Sherriff and Henderson (1994), and provide further details on the relative contributions from each of the nuclei.

Several brainstem auditory nuclei contain cholinergic cells. The largest population of such cells is in the superior olivary complex, where cholinergic cells are known to project to the cochlea and/or the cochlear nucleus (reviewed by Warr, 1992). Previous studies show that very few olivary cells send axon branches to both the IC and the cochlea (Aschoff and Ostwald, 1988; Riemann and Reuss, 1998) or to the IC and the cochlear nucleus (Schofield, 2002). This still leaves a possibility of a third population of olivary cholinergic cells that projects to the IC, but the present results argue against this conclusion; we found no cholinergic cells in the superior olivary complex that project to the IC. A second population of presumptive cholinergic cells is in the sagulum, another source of projections to the IC. In our experiments we found both retrogradely labeled cells and ChAT+ cells in the sagulum, but we never found tracer/ChAT+ cells in the sagulum. The ventral nucleus of the lateral lemniscus contains a small number of cholinergic cells that we suggested previously correspond to olivocochlear cells (Motts, et al. 2008), which are present in this nucleus in guinea pigs (Robertson et al. 1987). The present data support this interpretation. We found no cholinergic cells in the ventral nucleus of the lateral lemniscus that project to the IC. We conclude that the cholinergic cells found in the “traditional” nuclei of the ascending auditory pathways do not project to the IC.

Functional Implications

Physiological studies of ACh in the IC have been few. Application of ACh or related agents has a direct effect on the spontaneous activity of relatively few IC cells (about 10%; Curtis and Koizumi, 1961). In contrast, cholinergic agents do affect the responses of most IC cells to acoustic stimuli (Watanabe and Simada, 1973; Farley et al. 1983; Habbicht and Vater, 1996). Farley et al. (1983) suggested that ACh enhances unit responses to specific stimuli (i.e., improves signal/noise ratio). Habbicht and Vater (1996) concluded that ACh plays a neuromodulatory role by “setting the level of neuronal activity” in the IC. Our results suggest that these hypotheses should be examined in light of the known functions of the PPT and LDT.

The PPT and the LDT have been associated with a wide range of functions. These nuclei are part of the ascending reticular activating system and, along with other nuclei such as the locus coeruleus and raphe nuclei, play important roles in arousal and control of the sleep/wake cycle (e.g. Reese et al. 1995a, b; Vincent, 2000; Miller and O'Callaghan, 2006; Steriade, 2004; Jones, 2008). The PPT has also extensive connections with the basal ganglia and has been associated with motor function (e.g., Jackson and Crossman, 1983; Scarnati et al. 1987; Lee et al, 2000; Mena-Segovia et al. 2004, 2005; Winn, 2006). Many of these connections may involve the non-cholinergic cells in the PPT area more than the cholinergic cells, but there is evidence for cholinergic involvement in motor function. The discussion of PPT function has also extended into the cognitive domain, with growing evidence for a contribution of the PPT to attention (e.g., Garcia-Rill, 1991; Steckler et al. 1994; Inglis et al. 2001; Winn, 2006). We discuss a few of these topics in relation to studies on IC physiology.

There have been only a few studies of IC cell physiology during the sleep/wake cycle. Velluti and colleagues (Peña et al. 1992, 1999; Pedemonte et al. 1994; Goldstein-Daruech et al. 2002) have recorded from unanesthetized guinea pigs and shown that, at many levels of the auditory system including the IC, the responses to acoustic stimuli change as an animal passes from waking to slow-wave sleep to paradoxical sleep. The changes are likely to be related to gating of auditory information and the ability of acoustic stimuli to arouse or awaken an individual. It is likely that cholinergic projections from the PPT and/or LDT are involved in this process (perhaps in combination with noradrenergic and/or serotonergic inputs to the IC).

Fear conditioning (e.g., in which a tone of a specific frequency is associated with a leg-shock) can alter the responses of IC cells to acoustic stimuli (Ji et al. 2001). These alterations can be blocked by application of atropine to the IC, and thus depend on cholinergic muscarinic receptors (Ji et al. 2001). The present results suggest that PPT and/or LDT inputs to IC underlie this cholinergic effect.

Recent studies have highlighted cells in the IC that appear specialized to respond to novel stimuli (Pérez-González et al. 2005). These cells fail to respond to repetition of a stimulus, but regain responsiveness if the stimulus is changed slightly. The authors discuss possible mechanisms for the adaptation, including intrinsic properties of IC neurons, feedback loops within the IC or descending inputs from cerebral cortex. We hypothesize that cholinergic inputs from LDT and/or PPT may be sufficient. Koyama et al. (1994) describe putative cholinergic neurons in the LDT that respond phasically to auditory and/or somatosensory stimulation and that show strong adaptation. The authors suggest that "...a group of the brainstem cholinergic neurons may function to produce a rapid elevation of excitability in response to novel, unfamiliar stimuli..." (Koyama et al. 1994, p. 1030). Such effects recall the conclusions of earlier studies in which ACh was suggested to enhance IC responses to specific stimuli and to set the level of neuronal activity in the IC (Farley et al, 1983; Habbicht and Vater, 1996). Interestingly, the novelty neurons are concentrated in the dorsal and external cortex of the IC and are rare in the central nucleus. If cholinergic inputs underlie the novelty response, it implies different functions for the cholinergic inputs to the cortical areas compared to the central nucleus. It would be interesting to determine whether the same cholinergic cells project to the central nucleus and the cortical areas of the IC.

The PPT and LDT have also been associated with the acoustic startle reflex, a motor response to an intense acoustic stimulus (Diederich and Koch, 2005; Yeomans et al. 2006). The cholinergic nuclei are not required for the startle itself, but are necessary for prepulse inhibition, whereby a non-startling stimulus can reduce the reaction to a subsequent stimulus (Bosch and Schmid, 2008). This inhibition is likely mediated by direct projections of PPT and LDT cholinergic cells to the caudal pontine reticular nucleus (Bosch and Schmid, 2008). It would

be interesting to determine whether these cholinergic cells are among those that project to the IC (i.e., do single cholinergic cells project to both the caudal pontine reticular nucleus and one or both ICs?). Whether or not there are collaterals, it seems likely that some of the PPT/LDT cholinergic projections to the IC are activated by stimuli that also cause startle. Prepulse inhibition has been suggested as a mechanism whereby startle is inhibited to allow for continued processing of sensory stimuli (reviewed by Fendt et al. 2001). Future studies of cholinergic effects in the IC may take advantage of startle and prepulse inhibition paradigms to examine possible gating of acoustic information flow.

Collateral projections—Branching projections from single cholinergic cells to multiple targets are common (e.g., Woolf and Butcher, 1986; Jourdain et al. 1989; Mena-Segovia et al. 2008). Woolf and Butcher (1986) suggest that the collateral projections contribute to coordinating sensory, motor, and limbic functions. Semba et al. (1990) discuss the cholinergic collateral projections in terms of simultaneously regulating thalamocortical rhythmicity and sensory transmission. We describe single cells in PPT and LDT that project to both ICs. If the cholinergic projection to IC is working in part to set the gain of the system (Habbicht and Vater, 1996), adjust the signal to noise ratio (Farley et al. 1983), or gate the transmission of auditory information (Goldstein-Daruech et al. 2002), collateral projections to both ICs could be useful in exerting these effects bilaterally. As discussed above, such effects are likely to be exerted in the context of arousal and control of behavioral state that has been associated with the midbrain tegmental cholinergic nuclei.

Acknowledgments

We gratefully acknowledge Arkadiusz Slusarczyk, Colleen Sowick and Megan Storey-Workley for their expert technical assistance. Thanks to Dr. Diana Peterson and Dr. Jeffrey Wenstrup for comments on an earlier draft of the manuscript. Supported by DC04391 and DC08463.

List of Abbreviations

ACh	acetylcholine
AF350	AlexaFluor 350
AF488	AlexaFluor 488
AF647	AlexaFluor 647
Aq	cerebral aqueduct
ChAT	choline acetyltransferase
ChAT+	choline acetyltransferase-immunopositive
cIC	contralateral inferior colliculus
cLDT	contralateral laterodorsal tegmental nucleus

cPPT	contralateral pedunculo pontine tegmental nucleus
DLL	dorsal nucleus of the lateral lemniscus
FG	FluoroGold
GABA	gamma-aminobutyric acid
IC	inferior colliculus
iIC	ipsilateral inferior colliculus
iLDT	ipsilateral laterodorsal tegmental nucleus
ILL	intermediate nucleus of the lateral lemniscus
iPPT	ipsilateral pedunculo pontine tegmental nucleus
LDT	laterodorsal tegmental nucleus
LL	lateral lemniscus
mlf	medial longitudinal fasciculus
PAG	periaqueductal gray
PnO	pontine reticular nucleus, oral part
PPT	pedunculo pontine tegmental nucleus
RB	red beads
scp	superior cerebellar peduncle
VLL	ventral nucleus of the lateral lemniscus

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Figure 1.

Examples of tracer injection sites in the inferior colliculus (IC). (A) Photomicrograph through the center of a FluoroGold injection site in the left IC. Transverse section; lateral is left and dorsal is up. The midline is near the right edge of the image. (B) Drawings of transverse sections through a FluoroGold injection site (solid black area) in the right IC. Crosshatching in section 1 indicates an area of tissue damaged during processing. Sections are spaced 300 μ m apart and are numbered from caudal to rostral. Entire sections were plotted; the drawings in the figure were truncated just ventral to the IC and, for sections 2–4, at the midline. Dorsal is up; lateral to the right. Scale bars = 1 mm. Aq – cerebral aqueduct; DLL – dorsal nucleus of the lateral

lemniscus; IC – inferior colliculus; PAG – periaqueductal gray; PPT – pedunculo pontine tegmental nucleus; scp – superior cerebellar peduncle.

Figure 2.

Cells in PPT (A) and LDT (B) that project to the ipsilateral IC. Upper panels show cells labeled with red beads (RB) or FluoroGold (FG) after injection of tracer in the ipsilateral IC. Lower panels show the same area viewed with a filter to visualize immunolabel for choline acetyltransferase (ChAT). Arrows: cells that are labeled with both retrograde tracer and ChAT immunolabel. Arrowheads: cells labeled with tracer only. Numerous cells (unmarked) contain ChAT immunolabel (green) but not FG or RB label. GP414 for A, GP421 for B. C, D) Cells in PPT that project to the contralateral IC. Arrows show double-labeled cells. GP421. E) Top panel: labeled cells in the lateral superior olive ipsilateral to a FG injection in the IC. Middle

panel: same area visualized for ChAT-immunolabel (AF647). Bottom panel: merged image showing there are no double-labeled cells. GP549. Scale bars = 20 μm for A, 10 μm for B–E.

Figure 3.

Plots of ChAT+ cells in the brainstem that project to the inferior colliculus. The borders of the PPT and LDT are not drawn; rather, the extent of the nuclei are indicated by the distribution of ChAT+ cells (green dots). The ChAT+ cells within the PAG delineate the LDT and those outside the PAG delineate the PPT. The superior cerebellar peduncle (scp) is shown in gray as it is a helpful landmark for finding PPT and LDT. An entire section (section 4) is shown for orientation. The labeled cells of interest were limited to a portion of the tegmentum (box); only this region is shown in the complete series of sections. The blue area in the IC indicates the FluoroGold injection site. The legend indicates the type of label; each symbol represents at least one labeled cell. Additional sections contained ChAT+ cells, but the cells were not labeled with tracer, and are not shown. Transverse sections, numbered from caudal to rostral. Sections are spaced 300 μ m apart. Scale bar = 1 mm. Aq – cerebral aqueduct; ChAT – choline acetyltransferase labeled cell; DLL – dorsal nucleus of the lateral lemniscus; IC – inferior colliculus; LDT – laterodorsal tegmental nucleus; LL – lateral lemniscus; PAG – periaqueductal gray; PnO – pontine reticular nucleus, oral part; PPT – pedunculopontine tegmental nucleus; scp – superior cerebellar peduncle.

Figure 4.

Contribution of ipsilateral and contralateral PPT and LDT to overall ChAT+ projection to IC. Tracer-labeled immunopositive cells were counted in every sixth section through each nucleus. Nine cases were used for calculations. Error bars indicate standard error of the mean.

Abbreviations: cLDT - contralateral laterodorsal tegmental nucleus; cPPT - contralateral pedunclopontine tegmental nucleus; iLDT - ipsilateral laterodorsal tegmental nucleus; iPPT - ipsilateral pedunclopontine tegmental nucleus.

Figure 5.

Photomicrographs of immunopositive cells that project to one or both inferior colliculi (IC). Red beads were injected in the left IC and FluoroGold was injected in the right IC (GP421). A, B) Labeled cells in the right (A) or left (B) pedunclopontine tegmental nucleus. Each panel shows a field illuminated to show red beads (RB, top panel), FluoroGold (FG, middle panel) or ChAT-immunoreactivity (ChAT) marked with AlexaFluor488 (green, bottom panel). Double arrows show cells that contained all three markers. Some cells contained the immunolabel and one tracer (single arrow: RB and ChAT; arrowhead - FG and ChAT). One cell (asterisk) contained only RB. C). A cell in the laterodorsal tegmental nucleus that contained RB, FG and ChAT (double arrow). Scale bars = 10 μ m.

Figure 6.

Plots of ChAT-immunoreactive cells in the brainstem that project to both inferior colliculi. The extent of the PPT and the LDT are indicated by the distribution of ChAT+ cells (green triangles). Conventions as in Figure 3. The blue area in the left IC indicates the FluoroGold injection site; the red area in the right IC indicates the red bead injection site. Many cells were labeled with just one tracer, or one tracer plus the immunolabel; these cells were plotted for analysis, but are omitted from the figure for clarity. Transverse sections, numbered from caudal to rostral. Sections are spaced 300 μ m apart. Scale bar = 1 mm. Aq – cerebral aqueduct; ChAT – choline acetyltransferase labeled cell; DLL – dorsal nucleus of the lateral lemniscus; IC – inferior colliculus; ILL – intermediate nucleus of the lateral lemniscus; LDT – laterodorsal tegmental nucleus; mlf – medial longitudinal fasciculus; PAG – periaqueductal gray; PnO – pontine nucleus, oral part; PPT – pedunculopontine tegmental nucleus; scp – superior cerebellar peduncle; VLL – ventral nucleus of the lateral lemniscus.

Figure 7.

Summary of collateral projections from cholinergic cells in the PPT (top panel) and the LDT (bottom panel) that project to both left and right IC. The numbers express the percentages of cells with collateral projections compared to the cells that project to the IC on one side or the other.

Table 1

Summary of injection sites and immunolabel.

Case	Tracer in left IC	Tracer in right IC	ChAT immunolabel
GP 379	red beads	green beads	AF350
GP 382	Fast Blue	Fluorescein dextran	AF647 [#]
GP 396	red beads	Fast Blue	AF647
GP 400	FluoroGold		AF647
GP 403		Fast Blue	AF647
GP 421	red beads *	FluoroGold *	AF488
GP 422	red beads *	-	AF488
GP 430	FluoroGold *	-	AF488
GP 462	-	FluoroGold	AF488
GP 468	FluoroGold	red beads *	AF488
GP 472	green beads	-	AF647
GP 482	red beads	-	AF647
GP 484	red beads	-	AF647
GP 547	FluoroGold	red beads	AF488
GP 549	FluoroGold *	red beads *	AF647
GP 550	FluoroGold	red beads	AF488
GP 560	Fast Blue *		AF488
GP 561	Fast Blue *		AF488

List of cases used in this study, the tracers injected into the inferior colliculus, and the fluorescent label used to visualize ChAT-immunoreactivity in each case. IC – inferior colliculus.

* cases that were used for quantitative summary.

[#] AF647 emits at near-infrared wavelengths; this label was visualized with a camera and the images were pseudo-colored magenta.

Table 2
Numerical summary of collateral projections to left and right IC.

Case	Nucleus	# cells to iIC	% double-tracer labeled	# cells to cIC	% double-tracer labeled
GP421	Left PPT	36	11%	18	22%
	Right PPT	58	7%	7	57%
GP549	Left PPT	81	10%	43	19%
	Right PPT	87	9%	37	22%
		mean = 9%		mean = 30%	
GP421	Left LDT	19	16%	9	33%
	Right LDT	14	14%	4	50%
GP549	Left LDT	27	15%	14	29%
	Right LDT	18	11%	10	20%
		mean = 14%		mean = 33%	

cells to iIC - the total number of tracer/ChAT+ cells that project to the ipsilateral inferior colliculus (iIC) in a series of every sixth section through the nucleus. % double labeled - percentage of cells listed in previous column that also project to the other IC. # cells to cIC - the total number of tracer/ChAT+ cells that project to the contralateral IC (cIC). LDT - laterodorsal tegmental nucleus; PPT - pedunculopontine tegmental nucleus.